Bull. Environ. Contam. Toxicol. (2003) 70:753–760

DOI: 10.1007/s00128-003-0047-8

Bulletin of Environmental Contamination and Toxicology

Toxicity of the Azo Dye Methyl Red to the Organisms in Microcosms, with Special Reference to the Guppy (*Poecilia reticulata* Peters)

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Received: 9 November 2001/Accepted: 8 December 2002

Sanganer town, a suburb of Jaipur city (India) houses nearly 400 small-scale textile printing industries, which discharge about 10,000KL/day of wastewater loaded with azo dyes in the shallow pools and drains. Only about 1% of this is treated by physico-chemical and biological methods. The pollution of water bodies in Sanganer has led to the complete elimination of submerged macrophytes, zooplankton and fish, while phytoplankton occur in about 10% of the water bodies. The textile wastewaters containing azo dyes have been the subject of short-term ecotoxicological studies on algae, aquatic macrophytes, zooplankton, snails and fish by several workers (Sharma et al. 1999), while their long-term effects on fish and other organisms in the ecosystem are not known. The objectives of the present study were therefore, to examine: (i) acute toxicity of the simplest azo dye-methyl red, to Guppy fish (*Poecilia reticulata* Peters) and (ii) chronic toxicity of methyl red to Guppy fish and other organisms in the microcosms.

MATERIALS AND METHODS

Poecilia reticulata Peters (Guppy fish) - an ovoviviparous fish, were collected from a large concrete tank (6 m x 3.7 m x 2 m) in the University Botanical Garden, wherein their population has naturalised. These were acclimatised for 7 days in plastic tubs (20 L) filled with dechlorinated tap water prior to their use in the experiment. The submerged hydrophytes *Ceratophyllum* and *Hydrilla* supporting good growth of periphyton, were added in the tubs to oxygenate the water, while periphyton also provided food to fish.

A stock solution of methyl red was prepared by dissolving the weighed amount in 10-15 ml of ethanol, made up to 1000 ppm using the tap water. For determining the LC_{50} of methyl red, healthy mature fish of uniform size (length = 2.3±0.08 cm, width = 4.0±0.17 mm) were starved for 24 hr in dechlorinated tap water in 15 L plastic buckets. Different dilutions (5-50ppm) of methyl red, using tap water were prepared. These were added to separate 5 L plastic buckets (depth = 30 cm; diameter = 12.5 cm); while those containing tap water served as controls. Three replicates were made for each treatment. Five fish were carefully introduced into each bucket. The tap water/methyl red solution in the bucket was replaced every morning. Dead fish were removed immediately, and were counted everyday in each bucket. This study was terminated after 96 hr. In order to assess seasonal

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variation in mortality, separate studies were conducted during the rainy and winter seasons. All these studies were made without extra aeration, as textile wastewaters entering in the pools at Sanganer are low in oxygen content (0.8-2.0ppm). In another study, mortality of a mixed population of both sexes was assessed in both aerated (using aquarium pump) and non-aerated conditions in the spring season (March). The Probit analysis for calculating the LC_{50} values was made using the COMPAQ personal computer BASIC version 1.13.

During January (winter), the chronic toxicity of methyl red on fish and other organisms was examined at two sub-lethal concentrations (5ppm, 10ppm) in microcosms developed in six 15L plastic buckets buried 2/3 rd into the earthen floor of a green house. Each bucket had one outlet, 5cm below its top while its floor was laid with a 5cm thick layer of coarse sand. Each bucket was filled carefully with 10L of the Botanical garden tank water, causing minimum disturbance to the sand layer. After settlement of suspended particles therein, the floating impurities were removed with a sieve. Thereafter, 5-7 healthy branched shoots of *Ceratophyllum demersum* supporting good periphyton growth were added in each bucket to oxygenate the water. Every morning 7.5L of the tank water was introduced gradually in the bucket through a plastic pipe placed just above the sediment layer.

On day-9, ten mature healthy snails (*Lymnea luteola*) and 40 fish of uniform size (20 male: length = 2.2 ± 0.06 cm, width = 2.5 ± 0.14 mm; & 20 female: length = 3.3 ± 0.1 cm, width = 2.8 ± 0.13 mm) were introduced into each bucket. Among female fish, 10-15 were gravid. A plastic mosquito net was tied at the outlet of each bucket to prevent fish loss. Ten ml of fresh culture of *Spirulina* (OD >1 at 650 nm) was added daily as fish food in each bucket for a period of one week, and thereafter on alternate days throughout the study period.

After 7 days from fish transfer (i.e. on day-16), the buckets were divided into three groups, each comprising two buckets. In group-1, which served as the control, the replacement of bucket water with the tank water (7.5 L) continued until the end of this study. In Group-2 and 3, the water however, was replaced respectively with 5ppm and 10ppm methyl red solutions, prepared by diluting its stock solution (1000ppm) with the tank water. The methyl red solutions were then replaced daily until the end of the study (day 43). Thus, the fish and other organisms in the microcosms were exposed to methyl red for 28 days. This study was repeated in March (spring season) with three replicates each for the control and two methyl red treatments, but had to be terminated earlier (after 17^{th} day) on account of 100% fish mortality in the methyl red treatments. These two studies have been referred to as the first and second long-term study hereafter in the text. The occurrence of fish offspring was monitored carefully. Fish mortality (%) was noted in each bucket weekly, as described earlier.

To study external injuries and dye deposition in tissues; the operculum, mouth, gills and lateral line system of dead fish were examined under a binocular microscope during both short-and long-term studies. They were also dissected to assess potential morphological abnormalities of the internal organs. Using Wintrobe's method (Lee et al. 1994), RBC

counts were made at the end of short-term studies (96 hr), and at one-week intervals in the first long-term study. The dried and powdered fish were analysed for protein (Lowry et al. 1951) and total carbohydrate content (Roe 1955) at the end of first long-term study.

At the termination of the second long-term study, the density of snails (also in the first study) was recorded while both phytoplankton and zooplankton in the control and methyl red sets were identified by referring to standard keys (Pentecost 1984; Tonapi 1980) and also counted using a Sedgwick rafter. Since *Ceratophyllum* shoots growing in the methyl red treatments had comparatively longer internodes (20-30%, significant at 5%) and thin leaves in comparison to control shoots, adverse effects of dye on shoots were assessed. These were cut into ten 5cm long pieces, dried separately at 60°C in a hot air oven and weighed during both studies. The chlorophyll content of fresh shoots was also analysed according to the method described elsewhere (Sharma 1985). The physico-chemical characteristics of water (control set) and methyl red solutions were analysed at one occasion during both short-(at 4th day) and long-term studies (at 7th day), according to APHA (1989), while the dissolved oxygen content (morning) was determined using the azide modification of Winkler's method described in APHA (1989). Stastistical analysis of the data was carried out using SYSTAT computer program version 5.0.

RESULTS AND DISCUSSION

Except for oxygen and chemical oxygen demand (COD), physico-chemical characteristics of the control water and methyl red treatments were similar in both long-and short-term studies (Table 1). The COD values of controls and 5ppm methyl red solution were almost equal (25-34ppm) while they increased (60-170ppm) with the concentration of methyl red (10ppm-50ppm). After the first week of the second long-term study, a foul smell was observed in the 10ppm methyl red treatment.

Table 1. Physico-chemical characteristics (mean) of the medium in the control and methyl red treatments during the short (ST) and long-term (LT) studies.

	Temper-ature °C	pH	Conduc-tivity	Chlo-ride	Total hardness	Ca+2	Mg+2	Na+	K+
LT-1	*12-24	8.9-9.8	0.20-0.21	21-43	210-228	72-84	33-37	37-39	2
LT-2	*19-28	9.6-9.8	0.58-0.60	22-30	132-165	27-38	25-28	39-41	2
ST	**	7.8-8.2	0.50-0.54	24-28	184-230	50-64	32-41	40-43	2

Except for pH and conductivity (m mho/cm), all values are in ppm; * minimum & maximum temperature during the study period; ** varied seasonally therefore not included

The oxygen content of methyl red treatments were lower than the control, in both aerated and non-aerated conditions, decreasing with increasing concentration of methyl red, more so in the non-aerated condition (Table 2). During the long-term studies also, the oxygen content of methyl red treatments decreased markedly at both the 7th and 14th day of study;

1st study: 5ppm = 72%; 10ppm = 75% & 88%; 2nd study: 5ppm = 80% & 90%; 10ppm = > 95%, when compared with controls (1st study: 8.8-10.6ppm; 2nd study: 6.2-6.5ppm). The reduction in oxygen content in the methyl red treatments may partly be attributed to degradation of dye by the microbes.

Table 2. Mean values of dissolved oxygen content (mg/L) in the controls and methyl red treatments after 24 hr and 96 hr of exposure of fish in the short-term studies.

	Control	Methyl red treatments					
		5ppm	10ppm	20ppm	30ppm	40ppm	50ppm
Non-aerated condition	5.9 (6.0)	5.0 (5.1)	4.6 (4.9)	4.6 (4.0)	4.9 (4.9)	2.6 NA[1]	2.0 NA[2]
Aerated condition	7.1 (7.1)	6.6 (6.5)	6.6 (6.6)	5.9 (5.9)	6.1 (4.5)	4.9 (6.1)	4.9 NA

Data in parenthesis are for oxygen content after 96 hr; NA: Data are not available due to fish death after 48 hr (2) and 72 hr (1) exposure

Ceratophyllum shoots in the controls were highly branched and bright green, while they were fragile and pale green in the methyl red treatments. In comparison to the controls, dry weights of *Ceratophyllum* shoots decreased in the methyl red treatments, more particularly during the second study (Table 3) while reduction in their chlorophyll content (30-35%) was almost similar in both studies. During the second study, marked reductions in both species diversity and counts of phytoplankton, periphyton and zooplankton (except counts) were observed in the methyl red treatments, especially at 10ppm (Table 3). Interestingly, *Paramecium* counts increased markedly (almost 9000 fold) in the methyl red treatments indicating its higher tolerance. The increase in *Paramecium* counts may either be due to reduction in the food intake of its predator/s or even their death in the methyl red treatments. Thus, methyl red was more toxic to the producers, adversely affecting the energy flow in the microcosm which may finally lead to death of its biotic components; a condition often found in the dye wastewater pools at Sanganer.

Table 3. Dry weights of *Ceratophyllum* shoots (mg) and species diversity and counts (L^{-1}) of phytoplankton, periphyton (cm^{-1} along leaf margin) and zooplankton in the controls and methyl red treatments at the termination of the second long term study.

Parameter	Control	5ppm	10ppm	Total no.of species
Ceratophyllum: I study	98±36	90±45	43±32*	-
II study	56±10	46±6*	16.7±10**	-
Phytoplankton	31×10^8 (15±2)	11×10^7** (6±1)**	41×10^6** (5±2)**	18
Periphyton	405±51 (4)	171±22** (3)	Nil	4
Zooplankton	397±275 (14)	389±26^{ns} (5±3)**	597±104^{ns} (4±2)**	14

Significant at *5% & at **1%; ns - not significant; Data in parenthesis are for number of species

The snails survived in the methyl red treatments until the 3rd week in the first study while 60% of them were dead on 17th day of the second long-term study. All these were however found dead at the bucket floor at the end of first study. In contrast, the snails bred in the control sets as evidenced by the occurrence of young ones (7-15) at the end of first study. The gravid fish of the controls released their young (5-8/set) during the first week of the long-term studies, but not until after the second week in 5ppm methyl red (1-3/set). Young were not released at 10ppm, and were found in the dissected dead fish. Young survived and were present throughout the study period in the control sets, but they died at the age of 3-5 days in the 5ppm solution. In the short-term studies, young (1-12) released at higher concentrations (>30 ppm) were found dead during observations made in the morning, noon and evening of the study period. Thus methyl red was highly toxic to the fry.

The LC_{50} value for the mixed population of both the sexes was higher in the aerated sets in comparison to non-aerated sets (Table 4, significant at 5%), suggesting high mortality of fish in the water bodies with low dissolved oxygen content receiving untreated textile wastewaters. LC_{50} values for both male and female fish were examined separately under the non-aerated condition, once in August (rainy season) and once in December (winter), to assess seasonal variation. During the rainy season (water temperature : min. = 26°C; max. = 30°C), LC_{50} values of male and female fish were similar, while during winter (water temperature : min. = 12°C; max. = 19°C) the value for male (25.7ppm) was higher than female (16.6ppm), but insignificant statistically (Table 4). It is thus evident that season had little effect on the mortality of male and female fish, suggesting Guppy to be a hardy fish having wide ecological amplitude.

Table 4. LC_{50} values of male (M), female (F) and mixed population of both the sexes (M & F) of Guppy fish during different seasons.

Season	Sex	LC_{50}	95% confidence limits	
			Lower	Upper
Spring	M & F [1]	27.2	24.7	33.9
	-do-[2]	33.4	30.8	39.7
Rainy	M[1]	24.0	20.1	29.6
	F[1]	23.5	20.5	30.0
Winter	M[1]	25.7	25.1	34.8
	F[1]	16.6	11.2	22.9

1- non-aerated sets; 2- aerated sets

During the first long-term study, fish mortality was low in both the control (8%) and 5ppm methyl red treatment (6%) during the first week, but almost four-fold (30%) higher at 10ppm. Thereafter, no fish mortality occurred in the control sets, increased gradually at 5ppm (14th d = 15%; 21st d = 23%; 28th d = 26%), and rapidly at 10ppm (14th d = 44%; 21st d = 55%; 28th d = 62%) during the first study. During the second long-term study, fish mortality was similar to the first study until the second week. All fish were however, found dead on the 17th day in the methyl red treatments. Mortality showed a significant negative correlation with oxygen content of the methyl red treatments during both short-term (non-aerated sets: r >0.80; aerated sets: r = 0.64) and long-term (r = 0.50-0.75) studies, indicating that oxygen stress is one of the important factors in expressing methyl red toxicity.

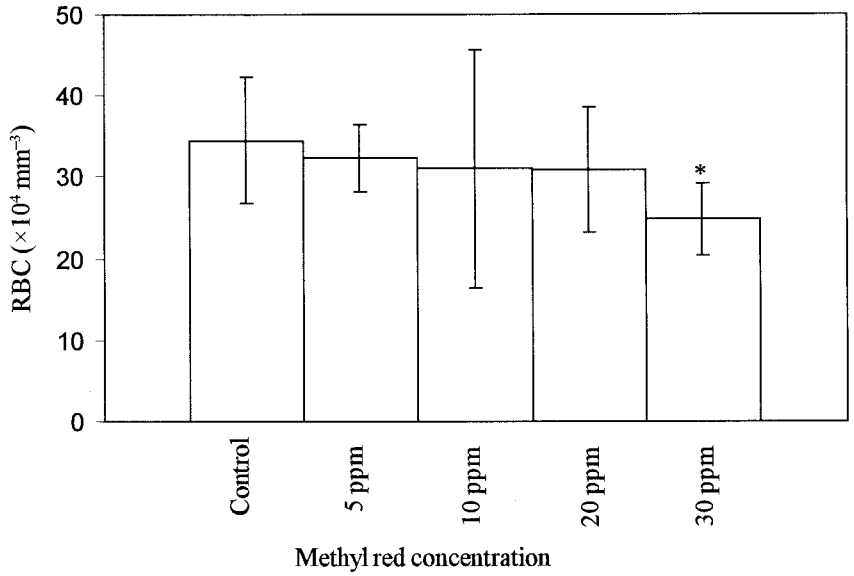

Figure 1. RBC counts of fish after 96 hr of exposure to methyl red treatments. Error bars represent the standard deviations. * - significant at 5%.

An examination of dead fish under a binocular microscope revealed marked deposition of dye on the gills and lateral line system during both short-(only at >20ppm) and long-term studies. This deposition of dye may adversely affect respiration and body balance. Unlike control fish having an off-white brain, blackish brown spots were seen over the brain of methyl red exposed fish in the long-term studies. The effect of this abnormality on the fish requires further investigation.

In comparison to controls, RBC counts in the methyl red exposed fish decreased with an increase in concentration during the short-term studies, being significant at 5% probability only at 30ppm in the non-aerated condition (Fig. 1). In comparison to the non-aerated condition, reduction in RBC counts of the methyl red exposed fish was a little lower (<5%; insignificant) in the aerated condition. RBC counts also decreased with time at both 5ppm (39-58%) and 10ppm (40-68%) in the long-term study, being significant at 1% probability (Fig. 2). This may be due to defective absorption of iron and destructive or suppressive effects on erythropoiesis (Kurde 1992). The anaemic condition of fish was perhaps responsible for their dose dependent mortality in the methyl red treatments.

During the long-term study, the total carbohydrate content of fish exposed at 5ppm (1.13 $\pm$ 0.06%) was almost equal to control fish (1.20 $\pm$ 0.10%), whereas it declined a little at 10ppm (1.00 $\pm$ 0.20%; insignificant). Total protein content in the methyl red exposed fish (5ppm = 55 $\pm$ 3%; 10ppm = 54 $\pm$ 2%) however, decreased moderately (significant at 10%) in comparison to control fish (67 $\pm$ 3%). This decrease in carbohydrate and protein contents may have reflected the need to meet extra energy requirements due to pollutant stress (Rajamannar and Manohar 1998).

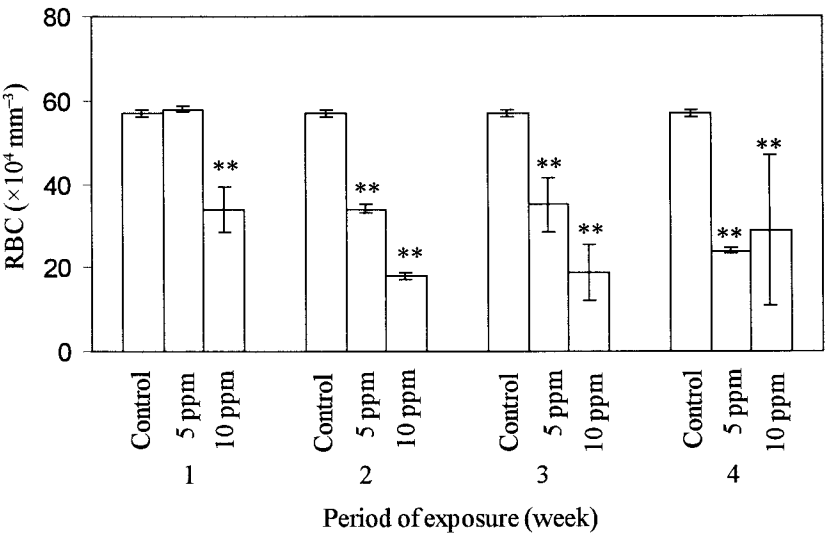

Figure 2. RBC counts of fish after 1-4 weeks of exposure at sublethal concentrations of methyl red. Error bars represent the standard deviations. **-significant at 1%.

The present study has revealed toxicity of methyl red dye to both flora and fauna in the microcosms, being relatively higher during spring in comparison to winter season. This may be attributed to an increase in oxygen stress caused by higher microbial activities as a result of increasing water temperature. The fish toxicity at 5ppm of methyl red was also higher in the short-term experimental study, as evident by a 6% decrease in RBC counts after 96hr exposure, which was nil even after 7 days of fish exposure in the microcosms (See; Figs. 1-2). The presence of additional factors governing methyl red concentration in the microcosms such as adsorption of the pollutant on the plants, animals, particulate matter and sediment may be the cause for the results obtained. The pollutant may also be modified chemically by the natural microbial community in the microcosms, which might results into antagonistic effects on their toxicity. As a result, unlike the short-term experimental study, both fish and other organisms in a microcosm were often exposed to a relatively lower concentration of the pollutant. Besides, the long-term ecotoxicological study provides an explanation for the overall impact of the pollutant on its biotic components, rather than on a single species. This may be helpful in understanding the response of the biotic community in an aquatic ecosystem receiving pollutant/s; which will guide its restoration.

Acknowledgements. We are thankful to the Department of Biotechnology, New Delhi (Grant no: 1758/BCE/12/123/99) for the financial support and the Co-ordinator SAP, Department of Zoology, Univ. of Rajasthan, Jaipur, for the laboratory facilities.

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