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A comparative study on the toxic effects of textile dye wastewaters (untreated and treated) on mortality and RBC of a freshwater fish *Gambusia affinis* (Baird and Gerard)

Pratima Soni[1], Subhasini Sharma[2], Shweta Sharma[2], Suresh Kumar[1] and K. P. Sharma[1]
[1]Department of Botany, [2]Department of Zoology, University of Rajasthan, Jaipur-302 004, India

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Abstract: *Comparative toxicological studies of textile dye wastewater (untreated and treated) on a freshwater fish, Gambusia affinis, revealed a marked reduction in mortality and cytotoxic effects on RBCs, measured as reduction in their counts and percent changes in their shape (poikilocytosis) and variation in their size (anisocytosis)}, after subjecting them to both physicochemical and biological treatments. On comparing the data of mortality and the cytotoxic effects on RBCs, we found poikilocytosis is a better indicator for toxicity measurement of both untreated as well as treated wastewater, especially at their lowest concentrations where percent mortality was found to be either nil or lowerer than the percentage of poikilocytic RBCs. Although percent reduction in RBC counts and changes in their size (anisocytosis) indicated toxic effects of wastewaters, but* EC_{50} *values for RBC counts were usually higher than those for poikilocytosis and mortality, and non-calculable for anisocytosis suggesting their lesser sensitivity to pollutants. In view of these findings, we recommend monitoring of toxic effects of wastewaters during fish bioassay on both mortality and variation in RBC shape.*

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

Introduction

Fish are exposed to aquatic toxicants via the delicate respiratory surface of the gills, which comprise over half of the body surface area and make intimate contact with the surrounding water (Wendelaar, 1997). Hence, they are used as an excellent bioassay animal in the toxicological studies (Hollis *et al*., 1999; Kumar *et al*., 1999). Usually the low concentration of pollutant may not result in fish mortality, but it may still be toxic to them. Moss and Hathway (1964) reported that permeability of erythrocyte membrane to pollutants not only destroys them, but also alters their shape and size by effecting the structure and function of cell membrane (Udden, 2000; Suwalsky *et al.,* 2004). Thus, alongwith mortality, studies on erythrocyte morphology (shape and size) and their count may serve as important indices for monitoring pollutant toxicity. Keeping this in view, we examined toxic effects of dye wastewaters from the textile printing industries on a freshwater fish, *Gambusia affinis* (Baird and Gerard).

Materials and Methods

Textile dye wastewaters released at various steps of diazotization and silicate processes for dye fixing were collected separately into 35 litre plastic cans from textile printing industries at Sanganer, Jaipur. These were transported immediately to the laboratory and stored in a cold room. Toxicity of pass (a mixture of mineral acid and sodium nitrite used for fixing dyes on printed cloth in diazotization process) and wastewater of three washings (after fixing dyes on printed cloth) was examined. Their toxicity (pass and 1st wash wastewater) was also examined after neutralizing them with slaked lime (primary treatment). We analyzed the toxicity of only first wash wastewater of silicate process.

The toxicity of both untreated and treated textile dye wastewater from physicochemical and biological effluent treatment plants (ETP) in the Industrial area of Sanganer was assessed in order to quantify toxicity reduction. During physico chemical treatment, dyes and other impurities in the wastewater were removed by mixing $FeSO_4$, slaked lime and polyelectrolyte at the rate of 5kg, 3kg and 40g/5000 litre, respectively. In case of biological ETP, the wastewater neutralized (by slaked lime/acid) in the equalizer chambers, was microbially treated in a bioreactor and constructed wetland. The suspended impurities were then coagulated by alum treatment (100mg/l), which were finally removed by passing the effluent through a sand filter. The wastewaters (both untreated and treated) from ETPs (physicochemical and biological) were also collected and transported to the laboratory as described above.

In another set of study, treated wastewater from wetland was polished by adding slaked lime (pH = 11) to precipitate heavy metals if any, in the wetland outflow. Toxicity of this polished wastewater as such, and after adjusting its pH close to 7 by acid treatment was assessed.

Physicochemical characteristics of wastewaters were analyzed within 24hr of collection using standard methods (APHA, 1989). Toxicity of wastewaters was examined by fish bioassay performed on a freshwater fish, *Gambusia affinis*. The fish caught from the tanks were acclimatized for 15 days in a plastic trough (40 litre) containing good plankton population to serve as food and *Ceratophyllum* (submerged hydrophyte) to

Table – 1: EC_{50} values of wastewaters of diazotization and silicate (dyeing) process, ETPs (physicochemical and biological) and wetland outflow (unpolished and polished) for mortality, RBC count and poikilocytosis and percentage change in RBC diameter of exposed fish (*Gambusia affinis*) in comparison to control.

Wastewater		EC_{50} (%)			Mean diameter (% change)
		Mortality	RBC count	Poikilocytosis	
Diazotization process					
Pass	Untreated	0.7	1.58	0.396	+5-8
	Treated	3.7	10.40	3.86	-2-13
1st Wash	Untreated	9.6	9.41	8.99	-3-21
	Treated	12.5	21.88	50	Nil -+9
2nd Wash	Untreated	12.1	NA	23.14	+2-8
3rd Wash	Untreated	14.9	NA	NC	+7-15
Silicate process					
1st Wash	Untreated	5.8	9.48	5.40	+3-26
Effluent treatment plants (ETPs)					
1. Physicochemical					
	Inflow	18.8	NA	12.11	+2-12
	Effluent	47.5	NA	24.23	+5-7
2. Biological					
	Inflow	18.5	24.53	58.06	+8-26
Bioreactor outflow		30	18.02	5.0	+9-25
Wetland outflow	Surface sample	31.95	47.83	112.78	+15-32
	Bottom sample	33.31	NC	13.74	+17-30
Wetland outflow					
I. Unpolished		76.7	NA	4.8	-8-13
	Lime (pH 11)	45	NA	275	-7-13
II. Polished	Lime (pH =7)	93.8	NA	138.2	-7-11
	Alum	72.7	NA	17.9	-7-21

NA = Not available; NC = Not calculable

oxygenate water. Thereafter, 60 healthy mature fish of uniform size (Length: 2.3 $\pm$ 0.08cm, Width: 4.0 $\pm$ 0.17cm) starved for 24 hr in tap water were exposed to six different concentrations (increasing arithmetically) of textile wastewater (untreated/treated) for determining acute toxicity (96 hr). Dead fish were removed immediately. Fish autopsy was done after 96 hr of exposure for RBC counts (Dacie and Lewis, 1982) and blood smear preparation (Lee *et al.*, 1993). The percentage of morphologically abnormal RBC was calculated by observing approximately 100 RBCs in 10 microscopic fields (10x X 100x) using an oil immersion. Morphometric measurements of RBCs were made with an occulometer, standardized with micrometer scale as parallel magnification. The sum of 25 observations each of length and width of RBCs was divided by 50 to calculate their mean diameter. EC_{50} values for mortality, reduction in RBC counts and poikiolocytosis were calculated using Microsoft Basic Program, whereas correlation (r) was calculated using computer program (Systat Version-5).

Results and Discussion

In comparison to control fish, percentage mortality and abnormalities in RBC shape (poikilocytosis) increased with concentration of both untreated as well as treated textile wastewater, whereas an opposite trend was noticed for RBC counts. Similar relationship between wastewater concentration and percent change in RBC size (anisocytosis) of wastewater exposed fish was not evident. As a result, EC_{50} values of dye wastewaters were calculable only for mortality, RBC counts and poikilocytosis.

Diazotization process: EC_{50} values of wastewater released at different steps of diazotization process for mortality, RBC count and poikilocytosis were in the following order (Table 1):

3rd Wash > 2nd Wash> 1st Wash > Pass

EC_{50} values for mortality, reduction in RBC counts and their poikilocytosis had a significant negative correlation with conductivity and COD values of these wastewaters, while the correlation was positive in case of pH; suggesting that reduction in toxicity was on account of decrease in pollutant loads and increase in pH values due to dilution in the subsequent washing steps (Table 2, 3). Thus, all the three indices used during the present study had a definite relationship with wastewater characteristics. With the exception of 1st wash showing reduction in RBC size (microcytic condition), increase in RBC size (macrocytic condition) was observed in pass, 2nd and 3rd wash (Table 1).

The neutralization of pass and 1st wash with slaked lime decreased their toxicity to fish, as evident by an increase

Table – 2: Physicochemical characteristics of wastewaters of diazotization and silicate (dyeing) process and ETPs (physico chemical and biological).

	Wastewater	pH	EC (mS)	COD (mg/l)
Diazotization process				
Pass	Untreated	1.4	43.0	3061.0
	Treated	7.9	16.7	2676.0
1st Wash	Untreated	1.9	12.3	900.7
	Treated	7.6	7.8	806.7
2nd Wash	Untreated	2.9	4.0	544.9
3rd Wash	Untreated	7.2	2.6	291.4
Silicate process				
1st Wash	Untreated	11.6	15.2	459.2
Effluent treatment plants (ETPs)				
1. Physicochemical	Inflow	8.7	4.5	900.0
	Effluent	10.5	4.5	520.0
	Inflow	6.6	2.3	256.7
2. Biological	Bioreactor outflow	6.7	2.9	350.2
	Wetland outflow			
	I. Surface sample	6.9	4.4	73.1
	II. Bottom sample	7.2	4.4	74.8

Table – 3: Correlation (r) between physico chemical characteristics and EC_{50} values of wastewaters (untreated and treated) of diazotization process and ETPs (physico chemical and biological) for mortality (96hr), RBC count and poikilocytosis in fish (*Gambusia affinis*).

Wastewater	Characteristics	EC_{50}		
		Mortality	RBC count	Poikilocytosis
Diazotization process	pH	0.331	0.632	0.463
	EC (mS)	-0.905	-0.650	-0.630
	COD (mg/l)	-0.982	-0.667	-0.699
ETPs	pH	0.569	0.856	-0.317
(Physico chemical and	EC (mS)	0.473	0.882	0.011
biological)	COD (mg/l)	-0.211	-0.992	-0.501

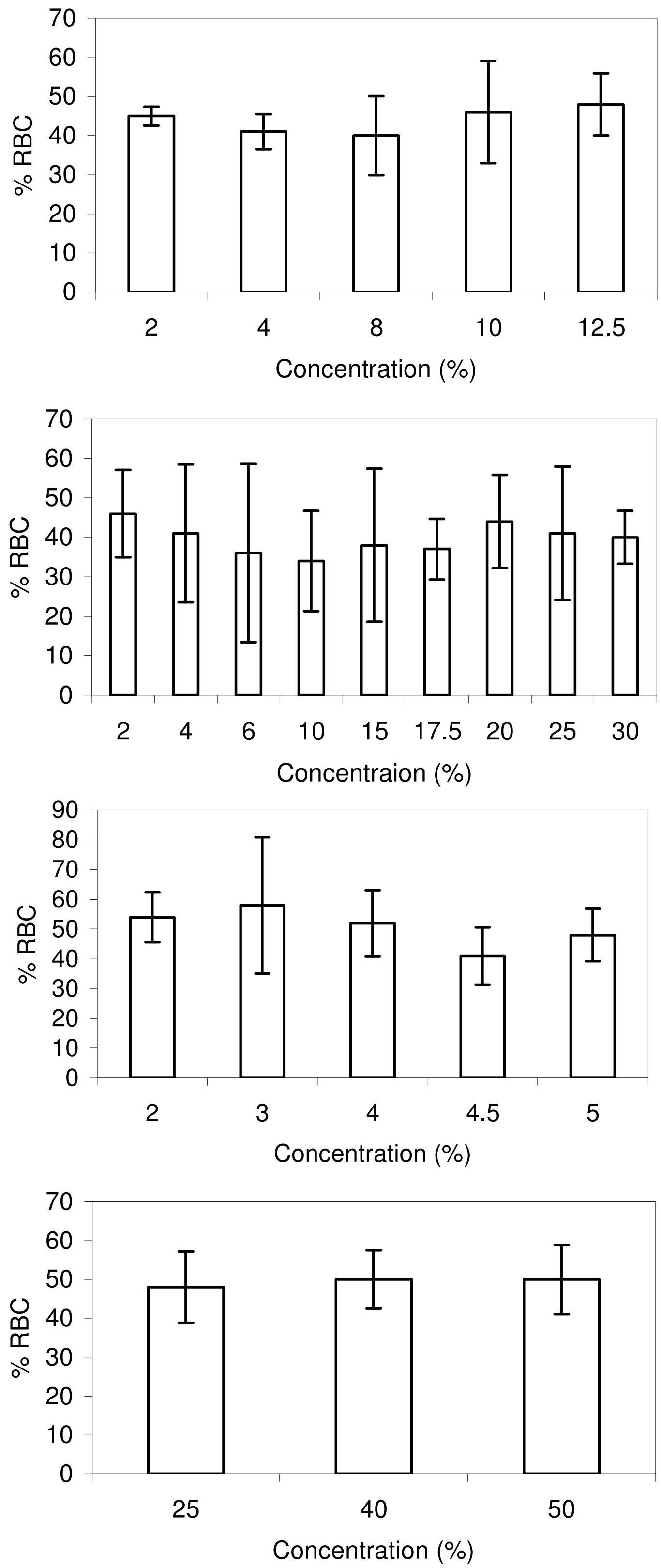
in EC_{50} values of all the three parameters, especially RBC count and poikilocytosis. This suggests marked reduction in cytotoxic effects of wastewater that may be ascribed to precipitation of dyes and heavy metals, especially Cu, present in them. Based on EC_{50} values, the parameters found most sensitive for examining toxicity were in the following order:

Pass
Untreated: Poikilocytosis > Mortality > RBC count
Treated: Mortality > Poikilocytosis > RBC count
1st wash
Untreated: Poikilocytosis > RBC count > Mortality
Treated: Mortality > RBC count > Poikilocytosis
2nd wash
Untreated: Mortality > Poikilocytosis
3rd wash
Untreated: Mortality

Thus, poikilocytosis was found to be the most sensitive parameter for measuring toxicity of untreated pass and 1st wash, whereas mortality for 2nd and 3rd wash, as also noted for treated pass and 1st wash.

It is important to note that percentage of morphologically abnormal RBCs (poikilocytosis) in 2nd and 3rd wash as well as in treated pass and 1st wash didn't increase significantly with concentration of wastewater and a very high percentage of them (45-50%) was observed even at very low concentrations (Fig. 1a-d). Thus, poikilocytosis and mortality were the most sensitive parameters for toxicity assessment.

As described earlier, we exposed fish at six different concentrations of a wastewater increasing in arithmetic progression for assessing its LC_{50} value. A comparison between percent mortality, reduction in RBC counts and percentage of poikilocytic RBCs at the lowest and maximum (at which fish survived) concentrations of the six test solutions used for the experiment revealed interesting facts. Percentage of poikilocytosis was maximum at the lowest concentration of wastewaters, whereas reduction in RBC counts at their maximum concentration, indicating higher sensitivity of RBCs towards pollutants (Table 4). It is evident from the data that poikilocytosis can detect toxicity of wastewater better at its lowest concentration when mortality is either low or even nil.



1a

1b

1c

1d

Fig. 1: Percentage of poikilocytic RBCs at different concentrations of wastewaters. Fig. 1a: 2nd wash (untreated); Fig. 1b: 3rd wash (untreated); Fig. 1c: Pass (treated) and Fig. 1d : 1st wash (treated).

Table – 4: Mortality, RBC count and poikilocytosis in fish (*Gambusia affinis*) at the lowest and maximum concentration of dye wastewater of diazotization, silicate (dye) process, ETPs (physico chemical and biological) and wetland outflow (unpolished and polished).

Wastewater				Mortality (%)	RBC count (% reduction)	Poikilocytosis (%)
Diazotization process						
Pass :	Untreated :		L (1.0%)	45	30	53
			M (2.25%)	71	85	63
	Treated :		L (2.0%)	38	42	54
			M (5.0%)	43	50	48
1st Wash :	Untreated :		L (2.0%)	Nil	26	43
			M (12.5%)	43	59	49
	Treated :		L (25%)	29	52	48
			M (50%)	43	68	50
2nd Wash :	Untreated :		L (2.0%)	Nil	NA	45
			M (12.5%)	57	NA	48
3rd Wash :	Untreated :		L (2.0%)	14	NA	46
			M (30.0%)	71	NA	40
Silicate process						
1st Wash :	Untreated :		L (0.5%)	Nil	28	40
			M (6.0%)	40	41	56
Physico chemical	Inflow :		L (10.0%)	Nil	NA	48
			M (22.5%)	80	NA	64
	Effluent :		L (25.0%)	Nil	NA	46
			M (45.0%)	60	NA	57
Effluent Treatment Plants (ETPs)						
Biological	Inflow :		L (5.0%)	Nil	10	56
			M (25.0%)	50	52	49
	Bioreactor outflow:		L (15.0%)	20	29	66
			M (20.0%)	40	79	NA
	Wetland outflow					
	Surface sample :		L (15.0%)	20	20	63
			M (25.0%)	40	29	61
	Bottom sample :		L (15.0%)	Nil	22	52
			M (25.0%)	20	20	54
Wetland Outflow;	Unpolished		L(25%)	Nil	NA	54
			M (75%)	40	NA	61
	Polished	Lime (pH 11) :	L (25%)	Nil	NA	60
			M (50%)	80	NA	59
		Lime (pH 7) :	L (25.0%)	Nil	NA	61
			M (100%)	80	NA	52
		Alum ;	L (25.0%)	Nil	NA	52
			M (75.0%)	60	NA	58

L= Lowest concentration ; M = Maximum concentration

Toxicity of wastewater of silicate process: In case of silicate wastewater, EC_{50} values for poikilocytosis and mortality were almost similar and were significantly lower than that for RBC counts (Table 1). Macrocytic condition was observed in RBC similar to diazotization process. Contrary to wastewater of diazotization process, values for poikilocytosis were higher than mortality at both lowest and maximum concentrations of test solutions, indicating poikilocytosis to be a better indicator of toxicity measurement of silicate wastewater (Table 4).

Toxicity of wastewaters of ETPs: Toxicity of wastewaters decreased markedly after both physico chemical and biological treatments as evident by EC_{50} values for untreated and treated wastewaters (Table 1). EC_{50} values had significant negative correlation with COD values of these wastewaters, suggesting that reduction in toxicity was on account of decrease in pollutant load after treatments (Table 2,3). Based on EC_{50} values, the parameters found to be most sensitive for examining toxicity of these wastewaters were as follows:

ETP (physico chemical)
Untreated: Poikilocytosis > Mortality
Treated: Poikilocytosis > Mortality
ETP (biological)
Inflow (primary treatment): Mortality > RBC count > Poikilocytosis
Microbial treatment
Bioreactor: Poikilocytosis > RBC count > Mortality
Wetland (surface sample): Mortality > RBC count > Poikilocytosis
Wetland (bottom sample) : Poikilocytosis > Mortality

Thus, poikilocytosis was the most sensitive parameter for examining toxicity of untreated as well as physico chemically treated wastewater. In case of biologically treated wastewater however, both mortality and poikilocytosis were the sensitive parameters. Macrocytic condition was found in RBC of fish exposed to both untreated and treated wastewater of ETPs. A comparison of mortality, RBC count and poikilocytosis at the lowest concentration of test solutions revealed poikilocytosis to be the most sensitive index (Table 4). The trend for maximum concentration was different. Percent mortality was greater than poikilocytosis, in case of untreated as well as physico chemically treated wastewater, whereas in biologically treating ETP, the percentage mortality was equal to poikilocytosis for inflow while it was lower than poikilocytosis for wetland outflows. It is thus evident that poikilocytosis is a sensitive parameter for examining toxicity of even treated wastewater, especially at its lowest concentration.

Toxicity of polished wetland outflow: Maximum value of EC_{50} for mortality was observed when pH of lime treated (pH 11) wetland outflow was decreased close to pH 7 (Table 1), whereas an opposite trend was found for poikilocytosis. Thus, higher pH value found more toxic to fish was less cytotoxic to RBC. Venkata Subbaiah *et al*. (2003) reported stress in fish, following their exposure to sublethal acidic (pH 5.0 ± 0.1) and alkaline (pH 9.0 ± 0.1) medium, which has been attributed to reduction in oxygen consumption by them. Thus, higher fish mortality during the present investigation (at pH 11) seems to be on account of oxygen stress. Alum treatment of wetland outflow had little effect on fish mortality, but proved to be more toxic to RBC in comparison to lime treatment, as evident by lower EC_{50} value of poikilocytosis. Thus, for lime polished outflow, mortality was the most sensitive parameter whereas poikilocytosis for alum treated outflow.

Poikilocytosis was found to be the most sensitive parameter for examining toxicity at the lowest concentration of both unpolished and polished wetland outflow, whereas mortality at the maximum concentration (Table 4).

Present study has thus established poikilocytosis as the most sensitive parameter for monitoring toxicity of textile dye wastewaters, especially at their lowest concentrations, at which either mortality was nil or very low. In view of these findings, we recommend monitoring of both fish mortality and poikilocytosis in their RBCs for toxicity measurement of wastewaters, especially at their non-lethal concentrations.

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Correspondence to:
Dr. Subhasini Sharma, Associate Professor
C-141 A, Mahaveer Marg
Malviya Nagar, Jaipur - 302017 (Rajasthan), India
E-mail: subhasini_Sharma@yahoo.co.in
Tel.: 0141-2521502