Acute Toxicity, Behavioural and Morphological alterations in Indian Carp, *Labeo rohita* H., on exposure to Municipal Wastewater of Tung Dhab Drain, Punjab, India

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Abstract: ***Fingerlings of Labeo rohita were exposed to geometric concentrations (6.25 to 100%) of municipal wastewater of Tung Dhab Drain, Amritsar, India. 96h static non-renewal acute toxicity tests were conducted and simultaneously behavioural and morphological observations were noted. The 96h LC_{50} value along with 95% confidence limits were found to be 44.25% (38.47-50.92). The recorded values for chemical parameters such as TSS, BOD, NO_3-N, O&G and heavy metals like Cr (VI), Mn, Pb and As were far more than standard discharge limits of effluents into inland surface waters. Fish in toxic media exhibited increased air gulping, surfacing, abnormal schooling, irregular, erratic and hyperactive swimming movements, hyper excitability, loss of equilibrium and showed vertical position prior to death. Morphological deformities included dark body colouration, excessive mucus secretion, loosening and loss of scales, haemorrhages on skin and fins, lateral flexure in caudal region and altered posturing of pectoral fins.***

Keywords: *Labeo rohita,* municipal wastewater, acute toxicity, fish behaviour & morphology

1. Introduction

Toxicity refers to the potential of a substance to produce an adverse or harmful effect on a living organism. Acute toxicity tests determine whether some concentrations of test material or effluent will produce an adverse effect on a group of test organisms during a short-term exposure under controlled conditions [2]. Geometric increase in human population coupled with rapid urbanization, industrialization and agricultural development has resulted in high impact on both quality and quantity of water in India [5]. Anthropogenic activities lead to eutrophication and degrade water quality via oxygen depletion, elevated BOD & COD loads, changes in transparency, pH, phosphate and nitrate levels. Environmental pollutants are known to elicit adverse effects to aquatic organisms. Exploitation of ecosystem is exerting tremendous strain on aquatic communities including plankton, fishes and invertebrates [20]. In such scenario bioassays with fishes can be very useful for monitoring the quality of water polluted with complex mixtures of toxic substances. Growth retardation and a number of pathological effects have also been detected in polluted river waters using fish bioassays. Both non-specific effects (growth retardation, mortality, teratogenic effects and influence on embryo-larval development) and specific effects (enzyme activity, mutagenicity) can be screened through bioassay studies [19]. Behaviour allows an organism to adjust to external and internal stimuli in order to best meet the challenge of surviving in a changing environment. Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social and physiological aspects of the environment [10]. Behavioral and morphological abnormalities as a result of sublethal toxicity can reduce an aquatic organism's health and fitness. Changes in behavior and morphology are thus proven to be more sensitive diagnostic endpoints than the mortality [17]. Locomotor behavior is commonly affected by contaminants and the pattern of fish swimming is a highly organized species-specific response [9]. Behaviour links physiological function with ecological processes, hence behavioural indicators of toxicity appear ideal for assessing the effects of aquatic pollutants on fish populations. Importantly, studies are beginning to correlate physiological changes with behavioural disruption, thus providing ecological relevance to physiological measures of toxicity [18]. Through the present study, an attempt has been made to determine the physicochemical characteristics of municipal wastewater of Tung Dhab drain- an international water channel. A little work was documented on municipal wastewater toxicity to any fish, so the present study takes into account the effects of municipal wastewater to one of the economically important freshwater fish *L. rohita* by determining the acute toxicity, behavioural and morphological parameters during the period October and November, 2012.

2. Materials and Method

2.1 Test Specimens and Acclimatization

250 healthy and active specimens of fish *L. rohita* (Hamilton, 1822) (weight; 7.15±0.46, length; 5.4±0.39) were procured from Rajasansi Fish Farm Amritsar in the month of September, 2012 in aerated polythene bags. The aerated bag was put in the water filled tank (63x39x63cm) and fish slowly released to avoid mortality due to sudden temperature fluctuations. The entire fish stock was disinfected by dipping in potassium permanganate solution (2 mg/L) for four hours. Each tank was stocked with fish with a ratio of 1 g fish per litre water. Aerators were used both during acclimatization and experimental period so as to maintain the required level of dissolved oxygen. Fish were acclimatized for thirty days with a temperature 20-22^{o}C, pH 7.0-7.2 and dissolved oxygen 6-8 mg/l, 12-16 h photoperiod and were fed *ad-libitum* on commercial fish food. The fish species was

selected as test fish because of its economic importance, ready availability throughout the year, ease of maintenance, convenience of testing and relative sensitivity to pollutants.

2.2 Collection and Physicochemical Analysis of Municipal wastewater

The Municipal wastewater for the present study was collected (between 0500-0700 hours) from Tung Dhab Drain (31^{o}67ˈ612ˈˈN and 74^{o}74ˈ280ˈˈE) near village Mahal, Amritsar, India. Tung Dhab drain is 20 km long, has a catchment area of 80.63 sq.miles, capacity of 31 cusecs, bed width of 45 ft (at outfall) and 4ft (at starting point). The drain receives effluents from industries & mills, agricultural runoff and also receives domestic sewage of the Amritsar city. The drain originates from village Talwandi Bharath (Gurdaspur) and joins with Hudiara drain near village Boparai Khurd (Amritsar) and further enters into the river Ravi. Wastewater was collected in polypropylene cans of 80 L capacity and transported to laboratory within fifteen minutes from the source. For physicochemical analysis, grab samples were also collected in well labelled plastic bottles, chilled immediately following collection and stored at 4^{o}C until used. The wastewater was collected every two weeks (October and November, 2012) both for bioassay and physicochemical studies. A portable water analyzer kit (WTW Multy 340i/ SET) was used for measuring four parameters i.e. pH, temperature, dissolved oxygen (DO) and electrical conductivity (EC) at the sampling site itself. Electrodes in the kit were calibrated prior to every sampling event by following the instructions supplied with the equipment. TURBIQUANT 1100 IR was used for measuring turbidity. Biological oxygen demand (BOD) was calculated using Oxitop measuring system for five days at 20^{o}C in a thermostat (TS 606-G/2-i). Acidity (AD), alkalinity (AK), total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), total hardness (TH), calcium (Ca), magnesium (Mg) and oil & grease (O&G) were calculated using standard methods recommended by APHA/AWWA/WEF [3].

Chemical oxygen demand (COD), ammonium as N (NH_4-N), nitrates as N (NO_3-N), total phosphates (∑P), nitrogen (N), potassium (K), chlorides (Cl^-), sulphates (SO_4^{2-}), iron (Fe), copper (Cu), cadmium (Cd), chromium (Cr VI), lead (Pb), manganese (Mn), nickel (Ni), arsenic (As) and zinc (Zn) analysis was done using Merck testing kits and their concentrations were measured using the UV/VIS spectrophotometer (Spectroquant® Pharo 300). Quality Assurance/Quality control methods were performed by collecting duplicate wastewater samples in case of titrations, whereas test reagents and standard solutions were used for testing samples using Merck testing kits. Detection limits for various kits and instrumental procedures are given in Table 1. For physicochemical parameters of diluent water and wastewater, mean± S.E. values were obtained using Minitab (version 14) statistical software.

2.3 Acute Toxicity Bioassays, Behavioural and Morphological Studies

Acute, static, non-renewal tests were conducted with five wastewater concentrations (100, 50, 25, 12.5 and 6.25%) and a control was simultaneously run using tap water in accordance with standard methods given in the manual of APHA/AWWA/WEF [3]. The experiments were conducted in triplicates (n=10, total 180 fish). Acute toxicity tests were carried out for a period of 96 h and feeding was suspended a day before the test. The wastewater was thoroughly mixed before preparing concentrations to ensure proper mixing and to avoid stratification of toxicant. The wastewater concentrations of the quantity 70 L (v/v) were prepared using tap water for dilutions and fish were released slowly and randomly to toxicant containing tanks (100 L capacity, 42x57x42). The mean ± S.E. values of various physicochemical parameters of tap water used for dilutions, like temperature (23±0.93), pH (7.1±0.06) and DO (6.9±0.35) are summarized in Table 1. A control tank was maintained simultaneously containing only tap water. The mortality rate was determined at the end of 24, 48, 72 and 96 hours and dead fish were removed immediately as and when observed. Fish were declared dead when there were no gill movements and there was no reaction upon touching caudal peduncle. The control and the treatments were keenly observed for behavioural and morphological characters daily for 30 min every 1, 6, 24, 48, 72 and 96h for each concentration [17]. Behavioural observations were recorded for air gulps, startle response, mode of swimming, schooling, equilibrium and general activity of fish during the experiment. Data was also collected for morphological studies that included the effects on fish colouration, fish scales, presence/absence of hemorrhages and any other abnormality in structure such as abnormal lateral flexure and posturing of pectoral fins. Operational definitions of behavioural and morphological responses were considered according to methods given by Rice *et al* [17]. The 96h LC_{50} values along with 95% confidence limits were calculated and regression equation was prepared by EPA computer probit analysis program (version 1.5) as proposed by Finney [8].

3. Results and Discussion

3.1 Analytical results

Limit, range and mean± S.E. values were obtained from data for physicochemical parameters of diluent water and municipal wastewater, and are given in Table 1. The recorded values were totally different for dilution water and wastewater used for experimentation. The wastewater samples did not meet the discharge standards of effluents into inland surface waters whereas values were within limits for dilution water. The pH of sample water was slightly alkaline, dark grey in colour with a pungent smell. The recorded values for parameters such as TSS, BOD, NO_3-N, O&G and heavy metals like Cr (VI), Mn, Pb and As were much higher than the recommended discharge limits stated under Environment Protection Amendment Rules [7].

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Table 1: Physico-chemical parameters of municipal wastewater collected from Tung Dhab drain and the tap water (for dilutions)

Parameters	*Municipal wastewater*			*Tap water (used for dilutions and running control)*			*MPL EPAR -2012*	*D L*
	Limit	*Range*	*Mean ±S.E*	*Limit*	*Range*	*Mean ± SE*		
Temp	29.0-30.2	1.2	29.7±0.38	22.4-25.5	3.1	23.7±0.93	20-35	-
pH	7.02-7.08	0.06	7.04±0.02	7.00-7.20	0.2	7.13±0.06	5.5-9	-
DO	0.32-0.46	0.14	0.39±0.04	6.4-7.6	1.2	6.9±0.35	-	-
EC	958-961	3	959.3±0.88	469-498	29	483.3±8.37	-	-
TD	124.5-127	2.5	125.5±0.75	0.22-0.30	0.08	0.27±0.02	300	-
AD	80-84.37	4.37	82.3±1.26	29.2-34.2	5.07	31.9±1.49	-	-
AK	537-553	16	545.3±4.63	170-189	19	179.7±5.5	-	-
TH	265.8-283.6	17.8	276.2±5.39	128-139	11	133±3.21	-	-
Ca	76.2-95.23	19.03	83.1±6.08	43-52.1	9.1	47.7±2.63	100	-
Mg	33.2-46.2	13.02	39.78±3.76	24-30.3	5.91	27.6±1.74	100	-
TS	1274-1290	16	1283.3±4.8	420-450	30	436.7±8.82	-	-
TDS	853-868	15	861.7±4.48	290-310	20	300±5.77	-	-
TSS	408-422	14	416.0±4.21	73-87	14	79.7±4.06	100	-
Cl^-	71.9-82.8	10.9	77.03±3.16	17-17.9	0.85	17.3±0.28	1000	1- 250
SO_4^{2-}	55-67	12	60.3±3.53	2.87-3.24	0.37	3.04±0.11	1000	50-500
N	32-48	16	42.3±5.17	3.8-6.2	2.4	4.8±0.72	-	10-150
P	3.09-3.95	0.86	3.5±0.25	0.03-0.06	0.03	0.05±0.008	10	0.01-5.0
K	17.7-21.5	3.8	19.4±1.11	4.5-5.9	1.4	5.0±0.45	-	5.0-50
NH_4-N	17-19	2	18±0.58	2.2-2.8	0.6	2.5±0.18	50	2.0-150
NO_3-N	13.8-15.8	2	14.8±0.58	1.4-1.9	0.5	1.6±0.15	10	0.10-25
BOD	222.5-237.5	15	230.9±4.43	12-Aug	4	10±1.15	30	0-400
COD	276.5-288.7	12.2	282.4±3.53	45-48	3	46.6±0.88	250	25-1500
O&G	248.8-267.0	18.2	256.4±5.45	4.3-5.2	0.9	4.8±0.27	10	-
Cd	0.195-0.33	0.14	0.26±0.04	0.003-0.007	0.004	0.05±0.001	2	0.02-1.0
Cr (VI)	0.41-0.79	0.38	0.7±0.12	0.05-0.08	0.03	0.07±0.008	0.1	0.01-3.0
Mn	2.15-2.63	0.48	2.4±0.14	0.1-0.5	0.4	0.3±0.12	2	0.01-10.0
Ni	1.83-3.45	1.62	2.5±0.48	0.05-0.08	0.03	0.06±0.009	3	0.02-5.0
Pb	2.15-3.70	1.55	2.7±0.49	0.18-0.23	0.05	0.21±0.01	0.1	0.01-5.0
Cu	1.07-1.26	0.19	1.14±0.06	0.05-0.09	0.04	0.07±0.01	3	0.02-6.0
Fe	0.25-0.84	0.59	0.56±0.17	0.017-0.02	0.003	0.02±0.0008	3	0.01-5.0
As	0.1-0.5	0.4	0.2±0.13	0.05-0.10	0.05	0.07±0.01	0.2	0.005-0.5
Zn	2.0-4.0	2	3.0±0.57	0.0-0.0	0	0.0±0.0	5	0-250

Note: Values represent mean ± SE; n=4. All the values except pH, temp (oC), TD (NTU) and EC (µS/cm) are reported in mg l-1, EPAR- Environment Protection Amendment Rules, MPL-Maximum Permissible Limits and DL- Detection Limits

3.2 96h LC_{50}

From the probit analysis, the 96h LC_{50} value (95% confidence limits) of municipal wastewater to the freshwater fish, *L. rohita* was found to be 44.25% (38.47-50.92) of Tung Dhab drain water (Table 2). The regression equation of the expected probit (Y) and log concentration (X) is Y = a+bx = -5.728+ 6.517X. The percent mortality increased with an increase in toxicant concentration and also duration of the exposure, hence a linear and positive relationship was obtained between log concentration and empirical probability. Mondal and Kaviraj [14] reported LC_{50} values of 37.55% and 57.54% to *L. rohita* and *Hypophthalmicthys molitrix* when exposed to different dilutions of jute-retting water (JRW). Mishra *et al* [13] observed 96h LC_{50} value of 25.5% to *L. rohita* when exposed to raw dairy effluent. Therefore, we concluded from our results that municipal wastewater was extremely toxic to fish as significant mortality was recorded during the experiments.

3.3 Behavioural Observations

The control fish showed active feeding, normal schooling behaviour, and well-synchronized body movements and was attentive to slight disturbance near the tank. In the present study, the behaviour of the control groups did not alter notably, hence taken as standards for the entire experimentation. Comparative behavioural responses given by experimental fish *L. rohita* after exposure to different wastewater concentrations noted at durations of 1, 6, 24, 48, 72 and 96h is given in the Table 3.

Table 2: Acute toxicity (96 h LC_{50}), regression equation and 95% confidence limits of municipal wastewater to *L. rohita*

Exposure(in hours)	*Regression equation in Log Y = a + bX*	*R^2*	*95%confidencelimits Lower*	*Upper*	*LC_{50} (%)*
96	-5.728+6.517X	0.928	38.47	50.92	44.25

Treated fish exhibited a number of altered behavioural responses on exposure to different wastewater concentrations and time durations. Increased air gulping and surfacing was recorded immediately after exposure and continued further although the intensity was not the same as in the beginning of experiment. This type of behavioural display directly points towards the avoidance behaviour of fish to the toxicant as previously mentioned by Kasherwani *et al* [11].

Table 3: Comparative and cumulative behavioural responses given by *L. rohita* after exposure to different concentrations of municipal wastewater noted for 1, 6, 24, 48, 72 and 96

Behavioural Changes	*Control*	*100%*	*50%*	*25%*	*12.50%*	*6.25%*
Jumping	-	++++	+++	+	+	-
Loss of balance	-	++++	++++	++	-	-
Restlessness	-	++++	+++	+	-	-
Schooling	-	++++	++++	++	-	-
Startle response	-	++++	++++	++	+	-
Un-coordinate Swimming	-	++++	++++	++	-	-

Note: Symbol (-) ---> Normal response, (+) ---> Abnormal response, (++) ---> Mild increase response, (+++) ---> Moderate increase response, (++++) ---> Maximum increase response

Surfacing phenomenon i.e., significant preference of upper layers in exposed group might be a demand of higher oxygen during the exposure periods [16, 6]. Fish showed violent, hyperactive, rapid movements initially and swam haph hazardly on slight disturbance. It showed jumping to escape from the toxicant. Fish in the higher concentrations remained in vertical position with mouth pointed towards the surface before death. Similar behavioural observations were made by Pathan *et al* [15], Adakole [1] and concluded that such responses could be due to respiratory impairment and nervous system failure caused by the toxicant.

At the start of experiment, fish occupied the water surface in the first three highest concentrations, mid to upstream column in fourth and confined to the bottom in case of lowest concentration and control. With an increase in exposure duration, the fish in different concentrations occupied the bottom of tank. As per mode of swimming, treated fish at the highest concentrations showed frequent sinking and rising, whirling, side swimming and hung vertically in tank prior to death. Fish at three lowest concentrations showed head up swimming and later on became stationary and rested on bottom. Fish in higher concentrations were hyperactive than the fish at lower concentrations. No schooling is observed at highest concentrations as the fish occupied greater area of tank than control. At lowest concentrations, fish occupied the area similar to control and showed schooling behavior. Similar conclusions regarding alerted schooling behavior were drawn by Bhat *et al* [4] to fish *L. rohita* when worked with bio-pesticide. Equilibrium was lost in higher treated groups as fish showed irregular swimming movements, back swimming and hanging vertically in tank. Similar observations were reported by Kasherwani *et al* [11],

Bhat *et al* [4] when worked with cadmium (*H. fossilis*) and bio- pesticide (*L. rohita*). Observations regarding startle response showed that the fish were hyper-excitable and darts away from stimuli faster than the control in highest concentrations as compared to lowest concentrations that showed less excitablility. Observations were more severe at the higher concentrations and showed there prevalence as the duration increased although the frequency was not the same as at the start of experiment.

3.4 Morphological Observations

Results indicated apparent changes in the external morphology of the test fish when exposed to different wastewater concentrations as compared to control fish (Table 4). Bulging of eyeballs, dark body colouration along with excessive mucus secretion was recorded in fish exposed to wastewater concentrations. Mucus secretion helps in reducing the contact with toxicant, hence minimizing the irritation by forming a barrier between fish body and exposed toxic media. Our results were in accordance to observations made by Dube and Hosetti [6], Patil and David [16] for *L. rohita* when exposed to different pollutants. Post-mortem examination of dead fish revealed clumping of gills and the effect was concentration dependent.

Table 4: Comparative morphological changes observed when *L. rohita* exposed to different municipal wastewater concentrations for 96 h

Morphological Changes	*Control*	*100%*	*50%*	*25%*	*12.50%*	*6.25%*
Abnormal lateral flexure	-	++++	++++	-	-	-
Clumping of gills	-	++++	++++	+++	++	+
Discoloration of skin	-	++++	++++	++++	+++	++
Lesions on skin	-	++++	+++	-	-	-
Mucus secretion	-	++++	++++	++	+	+
Posturing of pectoral fins	-	++++	+++	+	-	-
Sedimentation of pollutant on body	-	++++	++++	+++	-	-
Shedding of scale	-	++++	++++	++	-	-

Note: Symbol (-) ---> Normal response, (+) ---> Abnormal response, (++) ---> Mild increase response, (+++) ---> Moderate increase response, (++++) ---> Maximum increase response

The gill adhesion decreases the respiratory surface area and hence the metabolic rate of the fish as reported by Kasherwani *et al* [11]. Lateral flexure in caudal region and posturing of pectoral fins was observed in fish when exposed to higher wastewater concentrations. Intensity of effect increased with the increased concentration of wastewater. Lateral flexure in caudal region hinders the locomotion of the fish, hence its survival in the environment. Our results were in agreement with Pathan *et al* [15] and Adakole [1].

4. Conclusion

The present study indicated that various physicochemical parameters of municipal wastewater collected from Tung Dhab drain showed values that were above the given standard discharge limits set by environmental laws. It was clearly demonstrated from the LC_{50} value, behavioural and morphological manifestations that the waste was harmful to freshwater fish and its discharge into other water bodies will be hazardous to aquatic ecosystem.

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