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Effect of an Endocrine-disrupting Chemical Dimethyl Phthalate on *Poecilia sphenops*

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ABSTRACT

Dimethyl phthalate (DMP) has been recognized as a significant environmental contaminant due to wide applications in industry. It has been reported to be an endocrine disrupting chemical which promotes chromosomal injuries in human leucocytes, thereby causing abnormalities in reproductive system and interference with the development of animals and humans. In current investigation, a batch experiment was conducted under control conditions to assess the added effects of DMP on *Poecilia sphenops*. LC_{50} of DMP on *P. sphenops* was determined and observed to be 55.8 mg/L. Growth performance, macroscopic observation and mortality of fingerlings of *P. sphenops* in experimental tank containing 50 mg/L of DMP was monitored for 60 days. Mortality of the fish in the experimental tank was observed to be ~20% after 60 days of incubation. Significant changes in morphometric, behaviour and diminution in growth performance were observed. These results evidently indicate the sensitivity of *P. sphenops* to DMP.

INTRODUCTION

With the arrival of the technologically advanced century, the world is facing the threat from various problems such as the rapid growth of population, serious shortage of resources and the pollution by the discharge and dumping of waste containing high levels of toxic anthropogenic organic contaminates, which are difficult to degrade and have a strong tendency to concentrate in food chain. Understanding the mechanism of transport and transformation of these toxic chemicals is highly critical to manage their ecological effects. Dimethyl phthalate (DMP) is one the anthropogenic industrial chemicals, which is used in plastics, such as cellulose acetate and is also a component of paints, adhesives, printing inks and coatings (Prasad & Suresh 2015). Due to its wide applications in industry, DMP has been recognized as a significant environmental contaminant. DMP is relatively stable with a half-life of ~3.2 years under natural environmental conditions (Staples et al. 1997). DMP has been frequently reported in various environmental samples, including marine waters, freshwaters, sediments, soils, fatty food and cosmetics (Staples et al. 1997, Tan 1995). Presence of DMP in environmental compartments is possibly due to lack of chemical bonding between DMP and polymer matrixes. Therefore, it can be readily dispersed into the environment during their production, use and after their disposal. In aquatic environment, DMP interacts with particles due to their hydrophobic in nature. Microbial biodegradation plays a major role in the metabolism and degradation of DMP (Staples et al. 1997, Yuan et al. 2008). Several microorganisms have been isolated and characterized for their ability to degrade DMP under aerobic, anaerobic and facultative conditions (Gu et al. 2009, Kido et al. 2007, Li et al. 2005, Pranaw et al. 2014, Prasad 2016, Prasad & Suresh 2012a, 2012b, 2015, Surhio et al. 2014). Aerobic bacterial species are efficient biodegraders of DMP than other microorganisms.

DMP has been reported to be an endocrine disrupting chemical (EDC) which promotes chromosomal injuries in human leucocytes, thereby causing abnormalities in reproductive system and interference with the development of animals and humans (Yuan et al. 2008). Short to intermediate term exposure to DMP induced decrements in body weight gain, changes in haemoglobin, and increase in absolute and relative liver weight, which suggest that DMP is a sub-chronic toxicant to animals. DMP has been listed as a priority pollutant by the U.S. Environmental Protection Agency (Li et al. 2005). On the other hand DMP has not been designated as chronic hazard. To the best of our knowledge, acceptable daily intake values of DMP have not been calculated due to lack of comprehensive studies pertaining to particular organ systems or exposure duration.

Acute exposure of DMP via inhalation in humans and

animals results in irritation of the eyes, nose and throat (New Jersy Department of Health 1986, U.S. Department of Health and Human Services 1993a). However, oral and dermal acute exposures of DMP in rats have shown moderate toxicity (U.S. Department of Health and Human Services 1993b). On the other hand, oral LD_{50} values of DMP for rats, rabbits, guinea pigs, chicks and mice have been reported to be of 8,200 mg/kg, 5,200 mg/kg, 2,900 mg/kg, 10,100 mg/kg, and 8,600 mg/kg, respectively (Draize & Alvarez 1948). Dermal LD_{50} of DMP was reported to be > 11,000 mg/kg, > 4,800 mg/kg and 38,000 mg/kg for rabbits, guinea pigs and rats, respectively. However, no significant dermal irritation following dermal exposure has been reported. Oral chronic exposures of DMP in experimental terrestrial animals have shown an inconsequential effect on growth and development (U.S. Department of Health and Human Services 1993a, U.S. Environmental Protection Agency 1987). To the best of our knowledge, information on the chronic effects of DMP in humans and aquatic animals are scarcely available.

In current investigations, effect of DMP on growth and development of *Poecilia sphenops* was conducted in batch mode under control conditions. *P. sphenops* commonly known as "Black Molly" is an omnivorous ornamental fish. It has been reported to be highly adaptable and extensively thriving in many different environmental and ecological conditions as a model organism for the studies of ecology, evolution and behaviour, due to its viviparous nature and easy availability (Altaff et al. 2015, Sumithra et al. 2014).

MATERIALS AND METHODS

Source of chemicals: More than 99% pure analytical grade DMP was purchased from LobaChemie, India. All other chemicals were of high purity (>99%). Commercial pellet feed was purchased from Aini Pellet feed for ornamental fishes, China. All experiments were performed in duplicate unless otherwise specified.

Source of fish and thriving conditions: Mixed population of *P. sphenops* was procured and transported in 5 L of plastic bags filled with 3 L of water from Sagar Aquarium, Vadodara, India to a holding facility at the Environmental Monitoring Laboratory, Department of Environmental Studies, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India. The procured fishes were further cultivated in 20 L fish culturing experimental tank containing 10 L of water under control conditions with commercial pellet feed for further studies. The experimental tanks were located in an outdoor area, covered with a lid to prevent the entry of dust and rain water, and the fish were fed once a day. Dose of pellet feed was 10 mg/fish/day. Aeration was provided to all the experimental tanks with the help of aerators. During the experiment, the water quality parameters were analysed periodically to monitor the health of the fishes. The mean values for temperature, dissolved oxygen, pH and hardness were 22 ± 4°C, 4.85 ± 0.5 mg/L, 7.65 ± 0.2 and 248 ± 5 mg/L, respectively. During the experiments, heterotrophic bacterial cell counts in water were also measured using the plate count method. Serial dilution plating was performed on nutrient agar. The plates were incubated at 30°C and the colony forming units (CFU) were noted.

Characterization of fish: Morphometric characteristics of fish such as length, width and height of fishes were calculated with the help of thread, scale and Vernier Calliper measurement method. Fig. 1 shows the standard length, body width, caudal penduncle length, caudal penduncle depth, head length, head width, head depth, snout length (pre-orbital distance), eye diameter, postorbital distance, inter-orbital distance, pre-dorsal distance, post-dorsal distance, pre-pelvic distance, post-pelvic distance, pre-anal distance, post-anal distance, dorsal fin base, dorsal fin length, anal fin length, anal fin base, pectoral fin length, pelvic fin length, pectoral ventral distance, mouth width and pre-maxilla length of molly fish.

At the end of each experiment, morphological characteristics, survival rate and specific growth rate of the fish were measured and subjected to statistical analysis. Survival rate, mortality rate and specific growth rate were calculated according to equations 1, 2 and 3, respectively.

$$S = \frac{N_f}{N_i} \times 100 \quad ...(1)$$

In equation 1, S is the survival rate in percentage, N_f is the final number of fishes and N_i is the initial number of fish.

$$M = \frac{N_i - N_f}{N_i} \times 100 \quad ...(2)$$

In equation 2, M is the mortality rate in percentage, N_i is the initial number of fishes and N_f is the final number of fish.

$$\mu = \frac{W_f - W_i}{W_i \times t} \times 100 \quad ...(3)$$

In equation 3, μ is the specific growth rate in percentage per day, W_f is the final body weight of fish in mg, W_i is the initial body weight of fish in mg and t is the total days of incubation under experimental conditions.

Experimental design and conditions: Our experimental design consisted of a total of 40 fingerlings of *P. sphenops* (averaging 92 ± 7 mg in weight and 11.5 ± 4 mm in length), divided into two groups of 20. One group was exposed to

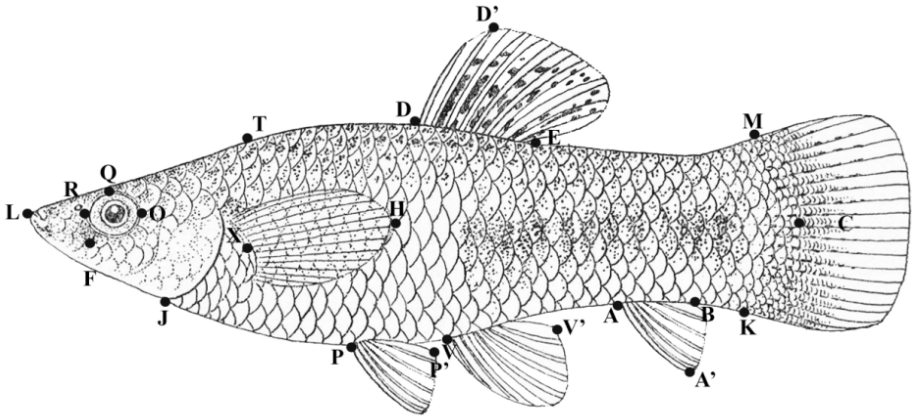

Fig. 1: Schematic diagram of molly fish showing morphometric characteristics. LC: Standard length, D↓: Body width, BC: Caudal penduncle length, B↑: Caudal penduncle depth, LH: Head length, XX': Head width, TJ: Head depth, LR: Snout length (pre-orbital distance), RO: Eye diameter, OH: Postorbital distance, QQ': Inter-orbital distance, DL: Pre-dorsal distance, EM: Post-dorsal distance, LV: Pre-pelvic distance, VK: Post-pelvic distance, LA: Pre-anal distance, BK: Post-anal distance, DE: Dorsal fin base, DD': Dorsal fin length, AA': Anal fin length, AB: Anal fin base, PP': Pectoral fin length, VV': Pelvic fin length, PV: Pectoral ventral distance, FF': Mouth width, LF: Pre-maxilla length

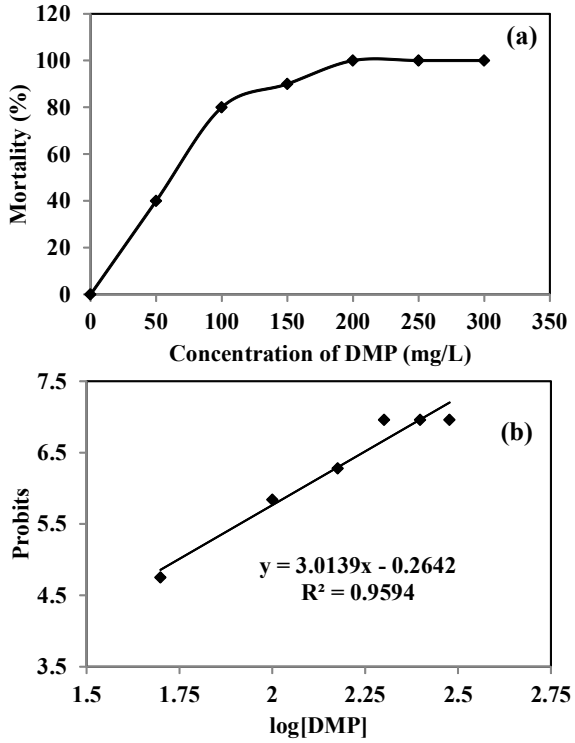

Fig. 2:(a) Mortality of *P. Sphenops* exposed to different concentrations of DMP and (b) probits at different concentrations of DMP for calculation of LC_{50}.

the experimental water containing 50 mg/L of DMP, and the second group was exposed to the experimental water without DMP, as control. DMP was first dissolved in a half litre of water taken from the experimental tank, and then this concentrated DMP solution was mixed with the remaining water (9.5 L) in the tanks. The fish were fed with commercial pellet feed once a day. Dose of pellet feed was 10 mg/fish/day. Aeration was provided to all the experimental tanks with the help of aerators. The tank was kept in such a manner that maximized natural conditions in the laboratory. The experimental tanks were maintained under control conditions for 60 days to observe change in behaviour and death of the fish. In order to study the anatomical and morphometric characteristics, fish were sacrificed after 60 days of incubation.

Determination of lethal concentration of DMP: Toxicity tests were performed in accordance with the standard methods given in APHA (APHA 2012). The batch experiments were conducted in 20 L fish tank containing 10 L of water to assess the lethal concentration (LC_{50}) values of DMP on *P. sphenops*. The fish culturing conditions and tank maintenance were same as discussed earlier, except the feed was omitted. The initial concentrations of DMP used were 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L and 300 mg/L. One additional experimental tank was maintained without any concentration of DMP as control. Aeration was provided to all the tanks with the help of aerators. Ten juvenile fish of *P. sphenops* in each experimental tank were introduced. Behavioural changes and mortalities were recorded at 24, 48, 72 and 96 hours of exposure, and dead fish were removed immediately from the experimental tank. LC_{50} was calculated by probit analysis Finney method (Singh & Manjeet 2015) on the basis of mortality of *P. sphenops* in each experimental tank.

RESULTS AND DISCUSSION

Effect of varying initial concentrations of DMP on *P.*

Table 1: Toxicity level of DMP on experimental organisms.

Tested organisms	Life stage	Study duration	Toxic dose (mg/L)	Acute toxicity rating	References
Alburnusalburnus (Bleak)	80 mm	96 h	100-115	Slightly Toxic	Linden et al. 1979
Lepomismacrochirus (Bluegill)	-	24 h	350	Not Acutely Toxic	Buccafusco et al. 1981
Lepomismacrochirus (Bluegill)	-	96 h	50	Slightly Toxic	Buccafusco et al. 1981
Pimephalespromelas (Fathead minnow)	18 mm, 80 mg	96 h	121	Not Acutely Toxic	Geiger et al. 1984
Gymnodinium breve (Dinoflagellate)	1000-5000 Cells/mL	96 h	125 & 185	Not Acutely Toxic	Wilson et al. 1978
Americamysisbahia (Opossum shrimp)	-	96 h	74	Slightly Toxic	EPA 1978
Daphnia magna (Water flea)	<24 h	24 h	150	Not Acutely Toxic	LeBlanc 1980
Daphnia magna (Water flea)	<24 h	48 h	33	Slightly Toxic	LeBlanc 1980
Nitocraspinipes (Harpacticoid copepod)	0.6-0.8 mm	96 h	62	Slightly Toxic	Linden et al. 1979
Poeciliasphenops (Black molly)	11.5 ± 4 mm 92 ± 7 mg	96 h	25-300	Slightly Toxic	Current study

Table 2: Growth performances of *P. sphenops* in experimental tank containing DMP and fed with pelletized feed for 60 days feeding trials.

Concentration of DMP (mg/L)	Initial length of fish (mm)	Final length of fish (mm)	Gain in length (mm)	Initial weight of fish (mg)	Final weight of fish (mg)	Gain in weight (mg)	Specific growth rate (%/day)	Survival rate (%)	Mortality rate (%)
0 (Control)	11.5±4	18.9±2	7.4	92.5±7	208.7±9	116.2	2.09	100	0
50	11.5±4	17.8±2	6.3	92.5±7	131.5±8	39.0	0.71	90	10
200	11.5±4	-	-	92.5±7	-	-	-	0	100

***sphenops* under starvation condition:** Increase in mortality of *P. sphenops* in experimental tank was observed with the increase in the concentrations of DMP (Fig. 2a). Death probit were computed and plotted against log of DMP concentrations to calculate lethal concentrations (Fig. 2b). The LC_{50} value of DMP for *P. sphenops* was estimated to be 55.8 mg/L. To the best of our knowledge, LC_{50} value of DMP for *P. sphenops* has not been reported in the literature. However, LC_{50} values of DMP for *Hyalella azteca*, *Chironomus-tentans* and *Lumbriculus variegatus* were reported to be 28.1, 68.2 and 246 mg/L, respectively (Singh & Manjeet 2015). The differences in the value of LC_{50} and overall toxic effect were often reported in the literature for different species and even for same species (Table 1). This is possible due to several factors, including differences in the test species, age, feeding habit, sex, composition of toxicant and also the experimental conditions under which the tests are performed.

Based on the current finding, it is evident that *P. sphenops* is highly sensitive towards lower phthalate esters like DMP. However, effect of higher phthalate esters such as diethyl phthalate, dibutyl phthalate, diethyl hexyl phthalate etc. on *P. sphenops* need to be investigated further.

Effect of DMP on *P. sphenops* under normal feeding condition: Growth performance and macroscopic observation of fingerlings of *P. sphenops* in experimental tank containing DMP and fed with commercial pellet feed was monitored for 60 days. *P. sphenops* exposed in experimental tank containing 200 mg/L of DMP showed very less movement of pectoral fin and tail fin as compared to control fish. Within 15 minutes of incubation >80% of the fish were rested on floor of the tank and rest were moving along with the flow of air in water. However, in case of control experiment, fish were active and showed continuous movement of pectoral fin and tail fin. After 1 hour of incubation >90% of fish were floating on the surface. They hardly showed any movement as they were simply floating and a few fry kept on drifting as dead. Within 18 hours of incubation around 50% fish were dead as they were not showing any movement of fin and gill and did not respond to touch. Remaining fish were breathing heavily and showing rapid movement of gills. More than 90% of the total fish died within the first 24 hours of incubation in 200 mg/L of DMP. This is possible due to high toxicity of DMP for young ones of *P. sphenops* or it may be possibly due to their high viscosity in nature which impedes swimming and movement of fish. However, in control experimental tank (without DMP) all the fish survived and showed continuous movement of pectoral and caudal fin during the entire incubation period. In order to scruti-

Table 3: Diminution of growth performance in presence of DMP.

Characteristics	0 mg/L of DMP (Control)	50 mg/L of DMP	Diminution (%)
Standard length (mm)	18.9 ± 2.28	17.8 ± 2.20	5.93
Body width (mm)	6.48 ± 1.48	5.68 ± 1.28	12.35
Caudal penduncle length (mm)	4.36 ± 0.91	3.90 ± 0.57	10.55
Caudal penduncle depth (mm)	4.82 ± 1.12	4.28 ± 1.11	11.20
Head length (mm)	4.52 ± 1.11	4.06 ± 1.29	10.18
Head width (mm)	3.28 ± 0.53	2.90 ± 0.66	11.59
Head depth (mm)	4.00 ± 0.91	3.52 ± 0.95	12.00
Snout length (mm)	2.76 ± 1.02	2.32 ± 0.65	15.94
Eye diameter (mm)	0.80 ± 0.44	0.62 ± 0.26	22.50
Postorbital distance (mm)	2.54 ± 0.72	2.16 ± 0.76	14.96
Inter-orbital distance (mm)	3.76 ± 0.96	3.12 ± 0.93	17.02
Pre-dorsal distance (mm)	6.90 ± 0.73	6.48 ± 1.00	6.09
Post-dorsal distance (mm)	6.68 ± 0.72	6.30 ± 0.71	5.69
Pre-pelvic distance (mm)	8.56 ± 1.26	8.06 ± 0.96	5.84
Post-pelvic distance (mm)	12.7 ± 1.10	11.7 ± 1.10	7.57
Pre-anal distance (mm)	14.2 ± 0.71	13.8 ± 0.69	2.68
Post-anal distance (mm)	2.70 ± 0.57	2.36 ± 0.43	12.59
Dorsal fin base (mm)	3.34 ± 0.78	3.18 ± 0.59	4.79
Dorsal fin length (mm)	1.66 ± 0.32	1.50 ± 0.35	9.64
Anal fin length (mm)	0.90 ± 0.41	0.88 ± 0.34	2.22
Anal fin base (mm)	1.20 ± 0.34	1.08 ± 0.10	10.00
Pectoral fin length (mm)	2.90 ± 0.54	2.66 ± 0.53	8.28
Pelvic fin length (mm)	1.18 ± 0.21	1.20 ± 0.12	-1.69
Pectoral ventral distance (mm)	3.34 ± 0.44	3.06 ± 0.64	8.38
Mouth width (mm)	1.60 ± 0.22	1.50 ± 0.18	6.25
Pre-maxilla length (mm)	1.00 ± 0.00	1.00 ± 0.00	0.00

nize the effect of DMP on morphometric, gonadal and hepatic, the concentration of DMP was reduced from 200 mg/L to 50 mg/L and similar experiments were conducted.

In 50 mg/L of DMP, within 15 minutes of incubation, about 50% of fingerlings were settled on the floor of experimental tank. This is possibly due to increase in viscosity of water in the presence of DMP, which hinder the movement of caudal fin. This observation was in contrast with control fish which show active movement of caudal fin. After 2 days of incubation in the experimental tank, about 90% of fingerlings were acclimatized to 50 mg/L of DMP and showed regular behaviour as observed in control. However, they were not able to recognize the pellet feed when provided. In 60 days of incubation, two fingerlings were found dead in the experimental tank which contained 50 mg/L of DMP, one each after 3 and 8 days of incubation. However, no death was observed in the control experimental tank (without DMP). The death of fingerlings could be possibly due to change in physical conditions of water in the presence of DMP or could be due to toxic nature of DMP or due to lack of feed recognition. Detailed study on change in physical conditions of water in presence DMP could be crucial to justify the above observation.

Specific growth rate of *P. sphenops* in experimental tank containing 50 mg/L of DMP and 0 mg/L (control) was observed to be 0.71 % and 2.09 % per day, respectively (Table 2). However, specific growth rate of *P. sphenops* in experimental tank containing varying initial concentrations of DMP need to be investigated further to assess the maximum diminution in specific growth rate. Results presented in the Table 3 evidently suggested the diminution in the growth performance of *P. sphenops* in experimental tank containing 50 mg/L of DMP as compared to control experimental tank (without DMP).

Based on the forgoing discussion, it suggests that DMP has a significant detrimental effect on growth and development of *P. sphenops*. Detailed comprehensive studies on specific toxicity of DMP on internal organs and organ systems are highly crucial to understand the fate of DMP in the experimental animals.

CONCLUSION

In current investigations, lethal concentration (LC_{50}) of DMP on *P. Sphenops* was investigated and found to be 55.8 mg/L under experimental conditions. The change in growth performance, morphometric and macroscopic observations evidently indicate the toxicity of DMP to *P. Sphenops*. Specific growth rate of *P. sphenops* in experimental tank containing 50 mg/L of DMP was observed to be 0.71% per day. However, in case of control experiment about 2.09% per

day was observed. These observations clearly indicate that DMP has a significant detrimental effect on growth and development of *P. sphenops*. The effect of DMP on internal organ systems such as gonads, liver, brain, etc. need to be investigated further to understand the specific toxic effect.

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