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Acute toxicity of two organophosphorus pesticides to the early-life stage of flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758)

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The acute toxicity (96 h LC_{50}) of two organophosphorus pesticides (OPs), chlorpyrifos (CPF) and dimethoate (DMT) to the early-life stage (fry) of flathead grey mullet, *Mugil cephalus* (total length, 22±2 mm, wet weight, 285±4 mg) was evaluated. *M. cephalus* were exposed to concentrations of CPF (2, 4, 6, 8, and 10 µg/L) and DMT (300, 750, 1500, 3000 and 4500 µg/L) for 96 h. Values of 96 h LC_{50} indicated that CPF (3.45 µg/L; 95 % confidence limits, lower = 2.77 and upper = 4.06 µg/L) was found to be highly toxic to *M. cephalus* fry compared to DMT (854.10 µg/L; 95 % confidence limits, lower = 558.23 and upper = 1176.77 µg/L). Data obtained during the present study would be helpful in monitoring OP pollution in coastal waters with *M. cephalus* as key bioindicator species.

[**Keywords**: Bioindicator, Chlorpyrifos, Dimethoate, LC_{50}, Mortality, Pollution]

Introduction

Pesticides have an important role in boosting agricultural production by protecting crops from pests and vector-borne diseases. By virtue of their strong insecticidal ability and wide range of applications, organophosphorus pesticides (OPs) occupy the top segment of commonly applied pesticides/insecticides to the agricultural and horticulture crops such as cotton, rice, pasture, vegetables, etc.[1,2]. The toxicity mechanism of OPs in vertebrate and invertebrates is by inhibiting the activity levels of acetylcholinesterase — an important enzyme involved in transmitting nerve impulses and thus blocking the cleavage of acetylcholine[3,4]. Although obvious merits such as rapid biodegradation and low persistence nature in the aquatic environment outweigh in favour of OPs, the concern however, has been their effects on non-target organisms[1,2].

Pesticides may reach the estuarine, coastal and marine ecosystems *via* multiple anthropogenic routes such as industrial or domestic sewage, leaching from landfills, storm runoff, shipping and harbour activities, etc. The primary route of pesticide pollution in the aquatic ecosystems is through rainfall-runoff and spray drift[5]. Furthermore, the coastal marine environment has also been impacted by OPs pollution from different activities such as transportation of these compounds *via* sea surface, indiscriminate use on agricultural and horticultural crops near to seashore, direct release of agricultural wastewaters containing high concentrations, etc.[6]. According to Bhardwaj & Sharma[7], only 0.1 % of pesticide targets the pest, whilst the remainder gets permeated to the environment.

In view of the toxic limit for aquatic life and the lethal concentration and confidence limits, the results of acute toxicity tests are meaningful[8]. The concentration that kills 50 % of the test population within a given period is called the lethal concentration (LC_{50} values). It is an essential tool to measure the standard toxicity dose. The use of fish as biological indicators for assessing the effects of pollutants and their monitoring in aquatic environments has been widely recognized[2-5,9-12].

The flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758) is a benthopelagic catadromous, euryhaline and eurythermal fish known to inhabit in a wide-ranging environment (marine, brackishwater and freshwater) contributing a subsistence fishery in tropical, sub-tropical and temperate regions[13,14]. Due to its unique attributes, such as acceptable commercial value and adaptability to variable environmental conditions (salinity and temperature), renders *M. cephalus* as a suitable candidate species for aquaculture. Hence, *M. cephalus* is extensively cultured in estuaries and ponds along the Indian coast and is much relished for its good flavour and flesh quality[13,14].

Due to its extensive distribution, occupying diverse habitats and detritus-mud feeding habit often make *M. cephalus* a good bioindicator species for assessing the pollution levels[15-18]. Few toxicological studies with adult stages of *M. cephalus* as test species to different xenobiotic compounds have been carried out earlier[3,15-17]. Furthermore, due to obvious variations in the magnitude of sensitivity amongst the numerous test criteria and test species, it is pertinent to detect adverse effects of contaminants to early-life stages of organisms[3]. Compared to vast published literature assessing the acute toxicity of pesticides to adult fish species[2,3,9-12,19-21], studies on early-life stages of fish — generally regarded as the most sensitive to toxicants are limited[22,23]. With this background, the present study investigated the acute toxicity of two widely used OPs, chlorpyrifos (CPF) and dimethoate (DMT) to fry stages of *M. cephalus*. In view of the economic importance of *M. cephalus* and as a biomarker for the monitoring of the health of coastal habitats[24], the results of the present study would help to monitor pesticide pollution, particularly by OPs.

Material and Methods

Chemicals

The commercial grade of OPs, chlorpyrifos (CPF) (diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl) oxy-λ^5-phosphane) and dimethoate (DMT) (O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] dithiophosphate) trade-named, 'PYRIBAN' (AIMCO Pesticides Ltd., Ratnagiri, India) and 'TAFGOR' (Rallis India Ltd., Akola, India), respectively procured from the local market were used. Commercial grades of CPF (20 % EC) and DMT (30 % EC), respectively, were diluted to prepare the stock solutions of 2 mg/L and 3 mg/L with MilliQ water.

Collection and acclimatization of *M. cephalus* fry

The fry stages of *M. cephalus* (total length, 20±3 mm; weight, 265±4 mg) were collected from the brackishwater ponds connected to the river Mandovi at Old Goa, Goa (India). Immediately after being caught, the fry were transferred to 100 L tanks fitted with an aeration system and transported to the Aquaculture Laboratory of the CSIR-National Institute of Oceanography, Goa (India). Prior to initiating acute toxicity experiments, *M. cephalus* fry were acclimated in a rectangular 2000 litre Fibre Reinforced Plastic (FRP) tank under a photoperiod of 16:8 h light:dark period for one week. The seawater drawn from Dona Paula Bay, Goa (Lat. 15°45' N; Long. 73°80' E) was first passed through a rapid sand filter followed by a set of cartridge filters (20 to 1 μm) and finally disinfected by ultraviolet radiation. Due to the absence of major industries and large-scale agriculture/horticulture practices in the vicinity of seawater intake point, the natural concentrations of OPs in the seawater and sediment were undetectable.

During the acclimation, experimental animals were fed with freshly hatched nauplii of brine shrimp (*Artemia salina*) *ad libitum* daily. Optimum levels of dissolved oxygen (DO) were maintained in the acclimation tank with continuous aeration (Hi-Blow 150 air-pump, Japan). During acclimatization, the measured water quality parameters such as temperature (28.4±0.2 °C), DO (6.5±0.6 mg/L), salinity (15±0.5 ppt), pH (7.8±0.2), NO_2–N (< 0.05 mg/L), NO_3–N (0.85±0.5 mg/L) and NH_3–NH_4 (0 mg/L) fell within the optimum range for the rearing of *M. cephalus*[25]. The water in the rearing tank was replenished every day to prevent the build-up of metabolites (excreta, uneaten feed etc.). The feeding was suspended 24 h prior to the initiation of the acute toxicity test. Experimental animals did not show any symptoms of stress or unusual behaviour during the acclimation and the mortality rate fell < 5 %. The dead fry noticed if any are removed immediately from the acclimation tank.

Acute toxicity experiments

Acute toxicity experiments were carried out in accordance with the standard procedures[8,26]. For assessing acute toxicity (96 h LC_{50}), healthy and actively moving fry of *M. cephalus* (total length, 22±2 mm; wet weight, 285±4 mg) were selected from the acclimation tanks and exposed to definitive test concentrations of CPF (2, 4, 6, 8 and 10 μg/L) and DMT (300, 750, 1500, 3000 and 4500 μg/L). These concentrations were selected after range-finding acute toxicity tests. All experiments were conducted under aqueous semi-static renewal conditions and each test concentration was prepared in triplicate. A negative control (without pesticide) was included to ensure the reliability of the experimental procedure. No feeding was done during the acute toxicity test. All the experiments were performed in triplicate per pesticide.

Acute toxicity tests, separately (CPF and DMT) were performed in the glass jars (2-litre capacity) containing 1 L seawater with a stocking density of 10 fry/L. According to the time schedule of 24, 48, 72 and 96 h, mortalities in each test container were noted during the 96 h exposure (HoE). Behavioural

alterations during the first 6 HoE and then at the end of 24, 48, 72 and 96 HoE were also observed. The absence of opercular movement and or no response to external stimuli were considered as indications of death. The dead fry were removed immediately to avoid any type of bacterial contamination and the survivors were counted. In order to maintain the uniform stocking density, dead fry if any, were replaced with similar sized fry reared separately in the same medium which have been exposed to the same HoE.

Statistical analysis

The median lethal concentrations (LC_{50} values) of CPF and DMT for different HoE (24, 48, 72 and 96 h) and their 95 % confidence limits (CL) were computed with a computer-based programme using the method of Log-Probit transformation for time and dose-mortality curves suggested by Finney's method[27]. One-way analysis of variance (ANOVA) in conjunction with Dunnett's test was used to verify the LC_{50} values between different HoE[28]. Statistical significance was adjudged at 5 % of probability (P = 0.05). A computer-based GraphPad PRISM 5.0 software (Graph Pad, San Diego, CA, USA) was used to perform all statistical analyses. The lowest observed effect concentration (LOEC) and no observable effect concentration (NOEC), respectively as the lowest concentration that had statistically significant mortality and the highest concentration that had no statistically significant mortality as in Pawar *et al*.[1] were determined.

Results

No fish died during the acclimation period before exposure and also in the control group during the experiments. The absence of mortalities in control tanks during acute toxicity tests implied that the test conditions were appropriate and the mortality recorded in the test tanks therefore could have been induced by pesticides. The percentages of mortality recorded in each test concentrations at different HoE are presented in Table 1. The mortalities in treatment tanks increased progressively with HoE and with the concomitant increase in the concentration of pesticides (Table 1; $P < 0.05$). During the 96 h acute exposure, fish exposed to higher concentrations (CPF > 6 µg/L and DMT > 750 µg/L) showed pronounced behavioural and morphological changes causing abnormal swimming such as loss of buoyancy, opercular and erratic movements, mucus secretion and discolouration of the skin.

The determined 96 h LC_{50} values and their 95 % confidence limits (CL) of CPF and DMT to *M. cephalus* fry were 3.45 µg/L (95 % CL, lower = 2.77 and upper = 4.06 µg/L) and 854 µg/L (95 % CL, lower = 558.23 and upper = 1176.77 µg/L), respectively (Table 2). The calculated LOEC and NOEC were 2 and 1 µg/L for CPF and 300 and 120 µg/L for DMT, respectively. The 96 h LC_{50} value was 245 times higher and showed a progressively decreasing trend in DMT compared to CPF. Furthermore, no significant variation ($P > 0.05$) in physico-chemical parameters of water between control and treatment tanks measured at 12 h intervals

Table 1 — Mortality rates (%) in the fry stage of *Mugil cephalus* for each tested concentration (µg/L) of chlorpyrifos (CPF) and dimethoate (DMT) at the end of different hours of exposure. Mortality data of three replicates (mean ± SD)

CPF					DMT				
Conc. (µg/L)	24 h	48 h	72 h	96 h	Conc. (µg/L)	24 h	48 h	72 h	96 h
0.00	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.00	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2.0	13.33±1.5	20.0±0.0	23.33±3.2	30.0±0.0	300	10.0±0.0	16.66±2.4	20.0±0.0	30.0±0.0
4.0	30.0±0.0	43.33±2.2	53.33±2.0	56.66±4.2	750	20.0±0.0	36.66±4.6	40.0±0.0	50.0±0.0
6.0	36.66±4.6	46.66±4.4	60.00±0.0	66.66±2.2	1500	33.33±2.2	40.0±0.0	46.66±4.0	60.0±0.0
8.0	43.33±2.2	56.66±4.2	66.66±4.40	80.0±0.0	3000	46.66±4.0	50.0±0.0	60.0±0.0	70.0±0.0
10.0	53.33±2.0	70.0±0.0	76.66±4.0	90.0±0.0	4500	56.66±4.2	60.0±0.0	70.0±0.0	80.0±0.0

Table 2 — The 96 h LC_{50} values (µg/L) with 95 % confidence limits (in parentheses) for the fry-stage of *Mugil cephalus* to chlorpyrifos (CPF) and dimethoate (DMT) at different periods of exposure. Different superscript letters in the same column significantly differ ($P < 0.05$)

Exposure period	CPF (µg/L)	DMT (µg/L)
24 h	9.39 (7.35–14.78)[a]	3397.74 (2466.54–5565.10)[a]
48 h	5.79 (4.74–7.22)[b]	2564.09 (1756.66–4604.50)[b]
72 h	4.32 (3.46–5.18)[c]	1600.61 (1156.25–2286.59)[c]
96 h	3.45 (2.77–4.06)[d]	854.10 (558.23–1176.77)[d]

throughout the acute toxicity experiment was discernible (Table 3).

Discussion

Many researchers have reported high concentrations of pesticides in agricultural wastewaters and their toxicity to aquatic organisms, especially in fish species[10]. In general, pesticide contamination in the aquatic environment occurs *via* rainfall-runoff[4]. During the present study, the 96 h LC_{50} values of two OPs to fry of *M. cephalus* as assessed by static bioassay tests displayed a varying degree of sensitivity (CPF, 3.45 µg/L; DMT, 854.10 µg/L). Furthermore, the acute toxicity indices are known to be affected by the concentration and exposure time and the relationship between concentration and exposure time was highly significant ($P < 0.05$).

The acute toxicity values revealed that CPF (3.45 µg/L) was relatively more toxic to fry stages of *M. cephalus* compared to DMT (854.10 µg/L). The median lethal concentrations of CPF to different fish species have been widely determined previously[17,19,29]. Marigoudar *et al.*[3], reported LC_{50} values of 1.13 µg/L and 3.20 µg/L to fingerlings of *M. cephalus* and *Chanos chanos*, respectively. On the other hand, Schimmel *et al.*[17], observed that LC_{50} values of CPF to be 5.4, 4.1 and 1.7 µg/L to *M. cephalus*, longnose killifish (*Fundulus similis*) and Atlantic silverside (*Menidia menidia*), respectively. Tilak *et al.*[29] has reported 96 h LC_{50} values of technical grade CPF to three Indian major carp species (*Gibelion catla*, 350 µg/L; *Labeo rohita*, 470 µg/L; and *Cirrhinus mrigala*, 650 µg/L). Rao *et al.*[2] reported LC_{50} value of CPF to be 25.97±0.01 µg/L for fish *Oreochromis mossambicus*. According to Pathiratne & Athauda[11], the LC_{50} value of CPF to fingerlings of *Oreochromis niloticus* was observed to be 117 µg/L. Acute toxicity (96 h LC_{50} values) of CPF to freshwater catfish, *Heteropneustes fossilis* during different reproductive phases were reported as 0.174 mM/L (resting phase), 0.123 mM/L (preparatory phase) and 0.026 mM/L (pre-spawning phase)[19].

Compared to the extensive literature in assessing the toxicity of CPF in diverse fish species, relatively few studies on the acute ecotoxicity of DMT have been carried (Table 4). A LC_{50} value of 14840 µg/L to

Table 3 — Water quality parameters (mean ± SD) in treatment and control tanks stocked with the fry stages of *Mugil cephalus* during acute exposure experiment chlorpyrifos (CPF) and dimethoate (DMT). Superscripts sharing similar letter in the same row are not significantly different ($P > 0.05$)

Parameter	Control	CPF	DMT
Temperature (°C)	28.4±0.20^a	28.0±0.22^a	28.6±0.24^a
Salinity (ppt)	15.0±0.5^b	15.0±0.4^b	15.0±0.4^b
Dissolved Oxygen (mg/L)	6.5±0.6^c	6.2±0.4^c	6.1±0.4^c
pH	7.8±0.2^d	7.4±0.4^d	7.3±0.2^d

Table 4 — Comparison of LC_{50} values for chloropyrifos (CPF) and dimethoate (DMT) obtained in the present study with those reported for other teleosts

		CPF	
Common name	Scientific name	96 h LC_{50} values (µg/L)	References
Mozambique tilapia	*Oreochromis niloticus*	25.97	Rao *et al.*[2]
Milk fish	*Chanos chanos*	3.20	Marigoudar *et al.*[3]
Grey mullet	*Mugil cephalus*	1.13; 5.4	Marigoudar *et al.*[3]; Schimmel *et al.*[17]
Longnose killifish	*Fundulus similis*	4.1	Schimmel *et al.*[17]
Atlantic silverside	*Menidia menidia*	1.7	Schimmel *et al.*[17]
Catla	*Gibelion catla*	350; 1660	Tilak et al.[29], Hossain *et al.*[40]
Rohu	*Labeo rohita*	470; 2350	Tilak et al.[29], Hossain *et al.*[40]
Mrigal	*Cirrhinus mrigala*	650; 2350	Tilak et al.[29], Hossain *et al.*[40]
Chola barb	*Puntius chola*	219	Verma & Saxena[41]
Common carp	*Cyprinus carpio*	8; 149	De Mel & Pathiratne[42], Li *et al.*[43]
		DMT	
Jarbua terapon	*Terapon jarbua*	700	Lingaraja & Venugopalan[31]
Stinging catfish	*Heteropneustes fossilis*	2980	Pandey *et al.*[20]
Common carp	*Cyprinus carpio*	1600	Singh *et al.*[30]
Striped Gourami	*Trichogaster fasciata*	21650; 9300	Singh *et al.*[30]; Shukla[37]
Guppy	*Poecilla reticulata*	19000	Gupta *et al.*[38]
Blue gill	*Lepomis macrochirus*	6000; 22400	CCME[39]

fingerlings of *O. niloticus* has been reported[11]. Singh *et al.*[30] reported the 96 h LC_{50} of DMT to fingerlings of *Cyprinus carpio* to be 1600 µg/L. According to Lingaraja & Venugopalan[31], the LC_{50} value of DMT to estuarine teleost (*Terapon jarbua)* was 700 µg/L. The median lethal concentrations (96 h LC_{50}) of DMT to Stinging catfish (*Heteropneustes fossilis*)[20] and Striped/Banded gourami (*Trichogaster fasciatus*)[21] have been reported to be 2.98 mg/L and 21.65 mg/L, respectively. From these limited studies, it appears that DMT is relatively less toxic compared to CPF.

Our results are in good consonance with the previous reports validating high toxicity of CPF and DMT to various fish species[2,11,17,29,30]. A wide range of responses by aquatic organisms following exposure to OPs depending on the compound, exposure time, water quality and species have been reported[2]. A significant variation in acute toxicity (LC_{50} values) of CPF and DMT to different fish species might be related to species-specific response, size of fish used for conducting bioassay, type of rearing water (freshwater or seawater/brackishwater) and interaction of pesticides with biotic/abiotic factors[11,29,32]. Relatively higher sensitivity of early life stages of fish and invertebrates to toxicants compared to adult organisms have been previously reported[1,23].

During the present study, LOEC and NOEC values determined to be 2 and 1 µg/L for CPF and 300 and 120 µg/L for DMT. Marigoudar *et al.*[3], reported the impact of sublethal concentration of CPF and determined mean values of NOEC and LOEC values to be 0.09 and 0.17 µg/L for *M. cephalus* and 0.16 and 0.32 µg/L for *Chanos chanos*, respectively. During the present study, 96 h LC_{50} values proved that CPF is highly sensitive to the fry of *M. cephalus* as compared to DMT. Relatively higher permeation by CPF into the lipid-rich tissues of an aquatic organism by virtue of its lipophilic nature leading to enhanced levels of bioaccumulation and severe damage or death of the organism has been reported[9,30,33]. However, further research to understand the processes by which different OPs affect the physiology and histology of different fish species is warranted.

The vulnerability of estuarine habitats and the associated fish assemblages potentially being impacted by a variety of anthropogenic inputs having a direct bearing on the food resources, distribution, diversity, breeding, abundance, growth, survival and behaviour of both resident and quasi-resident fish species has been highlighted[24]. The impact of pesticide toxicity to other aquatic organisms, including ichthyofauna which constitute the food chain in addition to agriculture crops has been documented[3,12,34]. *M. cephalus* is a key indicator of coastal environment pollution, which feeds primarily on soft mud and other detritus. Owing to its detritivorous feeding habit, the likelihood of uptake of xenobiotic compounds (organics, pesticides, and heavy metals) by *M. cephalus* is quite high if the feeding areas are contaminated by these pollutants that settle from the water column or become bound to sediment[35]. The greater vulnerability of *M. cephalus* to pesticide pollution compared to other filter-feeding fish species such as Tilapia, attributing to its detritivorous feeding behaviour, has been reported[36]. Consumption of such pesticide-contaminated fishes may adversely affect the health of human beings. Therefore, the results of this study are quite useful for monitoring the pollution of OPs, specifically CPF and DMT, with *M. cephalus* as key bioindicator species in the estuarine, coastal and marine environments.

In conclusion, our results demonstrated that CPF showed higher toxicity compared to DMT to the fry stages of *M. cephalus* under similar experimental conditions. In both tested OPs, the magnitude of mortality was found to be directly proportional to the test concentration and exposure time. Lastly, this study indicated that the fry stages of *M. cephalus* are highly sensitive to OPs and thus could meet the requirements of bioindicator species.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Conceptualization: ASD, APP and SVS; Software and formal analysis: ASD and APP; Supervision: RAS and APP; Writing original draft: ASD; and Writing – review and editing: RAS and SVS.

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