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Comparative Acute Toxicity Studies of Chlorpyrifos Technical Grade with its Emulsifiable Concentrate (20% EC) on *Labeo rohita*, a Freshwater Major Carp, and *Mystus vittatus*, a Freshwater Catfish

Rajib Majumder[1]

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Abstract

Chlorpyrifos is widely used across the world as an organophosphate insecticide and frequently contaminates freshwater bodies through runoff from agricultural fields. In the laboratory, static bioassays were undertaken to examine differences in acute toxicity caused by exposure to the technical grade (94% a.i.) and an emulsifiable concentrate (20% EC) of chlorpyrifos to two species of freshwater fish, *Labeo rohita* and *Mystus vittatus*. The recovery of actual chlorpyrifos concentrations varied from 83% (technical grade, T) to 89% (emulsifiable concentrate, F) after two hours in water. The susceptibilities of the two fish species to the two types of chlorpyrifos varied. The 96-h LC_{50} values for T and F chlorpyrifos in *L. rohita* were 68 and 36 µg/L, respectively, and 120 and 62 µg/L in *M. vittatus*, respectively. As the exposure period was extended, the LC_{50} values gradually decreased. LC_{50} values between the technical grade and formulation were compared following the criteria of Mayer et al. (1986), Schmuck et al. (1994), APHA (1995), and Demetrio et al. (2014). It was concluded from the study that the emulsifiable concentrate (20% EC) of chlorpyrifos was more toxic than technical-grade chlorpyrifos.

Keywords Organophosphates · Acute Toxicity · Fish · LC_{50}

Abbreviations

a.i	Active ingredient
T	Technical grade
F	Formulation
EC	Emulsifiable concentrate
h	Hour

Introduction

Intensive agricultural practices have had huge success all over the world for the last few decades, with huge inputs of chemical fertilisers and pesticides and the introduction of modern agro-equipment (Majumder 2023a). There is a worldwide, huge demand to increase food production for a growing human population. Application of pesticides to protect crops is a requisite for modern agriculture. Of the total pesticides applied to control pests, less than 0.1% ultimately reaches the target pest (Tudi et al. 2022). The majority of portions applied ultimately reach into the environment, causing contamination and adverse effects on non-target biota (Sarkar et al. 2021). Collaborative International Pesticides Analytical Council made a public list of 59 pesticide formulation types and their accompanying international classification system (CIPAC 2017). Pesticides are generally sold in Asia and the Pacific region in the form of dustable powder, wettable powder, granule, emulsifiable concentrate, suspension concentrates etc. (Hazra and Purkait 2019). One of the most popular and extensively used formulas is emulsifiable concentrate (EC), which is a transparent, homogeneous, oily liquid formulation comprising active ingredient, and other (or inert) ingredients or adjuvants or co-formulents dissolved in organic solvents. Surfactants, solvents, emulsifiers, propellants, antifoaming agents, carriers, diluents, stabilisers, dyes, preservatives, penetration agents, odor masking agents, etc. are the frequently used “inert ingredients” (Mesnage and Antoniou 2018). When diluted with water, emulsifiable concentrate

✉ Rajib Majumder
rajib.majumder2011@gmail.com

[1] Department of Zoology, Vivekananda Mahavidyalaya, Haripal, Hooghly 712405, West Bengal, India

produces an oil-in-water emulsion containing pesticides inside the oil droplets (Feng et al. 2018). Choosing to apply a pesticide formulation beyond its technical grade is done for a variety of reasons. It is simpler to work with and implement in the fields. It is less expensive than technical grade and offers consistent spreading, diluting capabilities, wide coverage, and stability in the field (Ohkouchi and Tsuji 2022). The active ingredient and the pesticide formulation have different levels of toxicity (Demetrio et al. 2014; Majumder and Kaviraj 2017, 2019; Nagy et al. 2020). The interaction between the active ingredient and other (or inert) ingredients in a commercial pesticide formulation must be revealed in order to fully comprehend the toxicological profile of the formulation (Nagy et al. 2020; Kalyabina et al. 2021). But, the company keeps 'other (or inert) ingredients' a trade secret and does not mention them in the literature supplied with commercial pesticide products (Cox and Surgeon 2006; Majumder and Kaviraj 2015). The term "inert" in pesticide products does not indicate that they are non-toxic (US EPA 2018), but rather that they have their own biological activity and pose a threat to the environment and human health (Kalyabina et al. 2021; Cox and Zeiss 2022). Co-formulants and added adjuvants influence toxicity of pesticide formulation (Adams et al. 2021). Demetrio et al. (2014) demonstrated that adjuvants contribute to the toxicity of commercial pesticide formulations. Therefore, toxicity data only on the active ingredient of the test pesticide on non-target organisms is not sufficient to predict the effect of pesticide formulations (Adams et al. 2021).

Chlorpyrifos is a broad-spectrum organophosphate that is widely used as an insecticide, acaricide, pesticide, and termiticide (Singh and Garg 2023). It is a moderately toxic (class II) insecticide (WHO 2019). Chlorpyrifos works inside an animal's body by blocking acetylcholinesterase, which causes acetylcholine buildup in the synaptic cleft, resulting in hyperexcitability and neurotoxicity (Philippe et al. 2022; Zhuo et al. 2024). Both surface and ground water samples contain chlorpyrifos residues (Sishu et al. 2022). It is highly toxic to non-target aquatic organisms, including fish (Majumder 2022; Zhuo et al. 2024). Chlorpyrifos can cause histopathological lesions (Majumder 2023b), oxidative stress (Zhao et al. 2021), endocrine abnormalities (Mansour et al. 2022) and growth retardation (Majumder and Kaviraj 2019) in fish. Chlorpyrifos is available on the market in a variety of commercial formulations with different ingredients. The majority of commercial formulations comprise 10–50% active ingredients and the remainders are inert ingredients. It ranks second in India in terms of sales (Stalin et al. 2019). In this context, comparing the toxicity of technical grade and a commercial formulation of chlorpyrifos to non-target organisms may become pertinent in designing pesticides and making pest management programmes more effective.

Fish are continually exposed to pesticides, heavy metals, and a number of other potentially hazardous chemicals as aquatic animal (Chaudhary et al. 2023). Some of these chemicals are biomagnified in the aquatic food web, posing health risks to consumers (Ray and Shaju 2023). Therefore, fish can be an ideal candidate for toxicological study due to their sensitivity to pollutants (Hong and Zha 2019; Zhuo et al. 2024). Among them, *Labeo rohita* is a fast-growing freshwater major carp that is widely employed in polyculture and has a high market demand as a tasty food fish. It is a good indicator for evaluating water quality and the presence of pesticides, and heavy metal pollution in the aquatic environment (Majumder et al. 2018; Chaudhary et al. 2023). On the other hand, *Mystus vittatus* is a representative of freshwater catfish and common test organism also in toxicological study because of their commercial importance and ability to withstand stress (Ali et al. 2017; Majumder et al. 2018). It is difficult to predict pesticide risk for non-target organisms if the toxicity of both technical-grade or active ingredient and commercial-formulated pesticides is not tested separately in a proper way. As inert ingredients are trade secrets, estimating their individual toxicity gets hampered, though their role in pesticide formulation is a matter of research interest. The objective of the present study was to evaluate differences in acute toxicity of technical-grade chlorpyrifos (94% a.i.) to a commercially available chlorpyrifos formulation (20% EC) to two species of freshwater fish: a carp (*Labeo roh*ita) and a catfish (*Mystus vittatus*). Additionally, attempts were made to report lethal values of chlorpyrifos for these two species of fish based on actual concentration of chlorpyrifos using chromatographic method. The obtained acute toxicity data from both technical grade and commercial formulations of chlorpyrifos for these species will shed light on the the possible eco-toxicological risk of agricultural runoff containing such pesticides to aquatic ecosystems.

Materials and Methods

Collection and Acclimatisation of Test Animals

Fingerlings of *Labeo rohita* (mean length 5.51 ± 0.29 cm, mean weight 2.11 ± 0.26 g) and *Mystus vittatus* (length 5.88 ± 0.34 cm, weight 3.45 ± 0.32 g) were collected from a fish farm located at Naihati, West Bengal, India. Before being used in bioassays, the fingerlings were brought to the laboratory in well-aerated polythene packets with water. They were then unpacked and bathed in 3% sodium chloride (NaCl) solution for 15 min to eliminate ectoparasites, and then stocked in outdoor cement tanks to acclimatise

Table 1 Physicochemical parameters of the control and test mediums used in the acute toxicity study

Parameters	Value
Temperature	29 ± 1 °C
pH	7.3 ± 0.1
Free CO_2	3.59 ± 0.35 mg·L^{-1}
Dissolved oxygen	7.2 ± 0.2 mg·L^{-1}
Total alkalinity	129.21 ± 3.21mg·L^{-1} as $CaCO_3$
Total hardness	137.24 ± 6.48mg·L^{-1} as $CaCO_3$

for a week. Each cement tank (diameter 90 cm and depth 75 cm) had a 3-cm layer of soil at the bottom and was filled with 300 L of deep tubewell water stored in an overhead tank. Thirty fingerlings were stocked in each cement tank. Throughout the acclimatisation phase, the fish were fed a balanced diet with 30% crude protein. The water tanks were kept aerated by using air pumps. To ensure appropriate water quality, water was partially exchanged (30–35%) on alternate days.

Test Chemicals

The study used two test chemicals: Technical grade chlorpyrifos (94% active ingredient) obtained from Krishi Rasayan Group of Companies, Kolkata-700020 (India) marked as T; and an emulsifiable concentrate (20% EC) of chlorpyrifos under the brand name Dursban® obtained from Dow Agro Sciences India Pvt. Ltd., Mumbai-400079 (India) marked as F.

Experimental Design

In the laboratory, each glass aquarium (15 L) was filled with 10 L of deep tube-well water (Table 1) and stocked with five acclimatised fingerlings to conduct static bioassays in accordance with the standard methodology (APHA 1995). Appropriate amount of the Technical grade (T) or formulation (F) chlorpyrifos was dissolved in 10 ml of acetone or water to prepare a stock solution containing 100 mg/L of the pesticide. Necessary dilutions were made before actual treatment. Because of its low solubility, the active ingredient of chlorpyrifos was mixed with acetone before being given to the test medium. Several test concentrations of chlorpyrifos (both T and F) were prepared from stock solution for use in bioassays, each with three replicates (Table 2). Two separate bioassays were conducted for two of the fish species: one using the technical grade chlorpyrifos and the other using the formulation (emulsifiable concentrate). There was a control group with an equal number of replicates in each category. All treatments, including control, were made in three replicates. Negative controls were run both with and without a solvent. 0.1 ml/L of acetone was added to the test water as an acetone control since the maximum amount of acetone contained in the highest concentration of this category of chlorpyrifos tested was below 0.1 ml/L. In order to avoid the test chemical from being impacted by the excretory products of the test organisms, food was not supplied throughout the bioassay.

Analytical Methods

After 2 h of treatment, 250 ml of water samples were collected from each aquarium to measure chlorpyrifos concentrations in water using chromatographic method. A Gas Chromatograph (Agilent 6890 N) in conjunction with a 7683 B series auto injector fitted with an Electron Capture Detector (ECD). Concentration of chlorpyrifos was quantified from the calibration curve prepared from standard chlorpyrifos concentrations, using ChemStation software. Table 3 summarises instrumental parameters of GC-ECD used for residue analysis of chlorpyrifos.

Statistical Analyses

The lethal concentration (LC_{50}) of chlorpyrifos at which 50% test organisms died and their 95% confidence limits were estimated for 24, 48, 72, and 96-h from the mortality data using US EPA-Probit analysis version 1.5 statistical software based on probit analysis method (Finney 1971). LC_{50} values of the technical grade and formulation of chlorpyrifos were compared according to the criteria of Mayer and Ellersieck (1986), Schmuck et al. (1994), APHA (1995) and Demetrio et al. (2014). The quotient (LC_{50} of X / LC_{50} of Y) is used to evaluate and compare acute toxicity data for

Table 2 Actual concentrations of chlorpyrfos (μg/L) used to determine LC_{50} values for different test organism (T: technical grade; F: Formulation)

Test Organisms	Number of organisms used (*n*)	Chlorpyrifos	Actual concentrations (μg/L)
Labeo rohita	210	T	0.00, 16.60, 33.20, 49.80, 66.40, 83.00, 99.60, 116.20, 132.80, 149.40, 166.00, 182.60, 207.50, 232.40
	225	F	0.00, 4.42, 8.84, 17.68, 35.36, 53.04, 70.72, 88.40, 106.08, 123.76, 141.44, 159.12, 176.80, 194.48, 212.16
Mystus vittatus	225	T	0.00, 24.90, 41.50, 66.40, 91.30, 116.20, 132.80, 149.40, 174.30, 190.90, 207.50, 232.40, 249.00, 273.90, 290.50
	180	F	0.00, 8.84, 17.68, 35.36, 53.04, 70.72, 88.40, 106.08, 123.76, 141.44, 159.12, 176.80

Table 3 Instrumental parameters of GC-ECD used for residue analysis of chlorpyrifos

Model of GC-ECD	Agilent 6890 *N*
Column	wide bore HP-5, 30 m, 0.32 mm ID, 0.25 µm film thickness
Column flow rate	1 ml/min
Make up (N_2) gas	59 ml/min
Split ratio	20:1
Run time	10 min
Injection volume	1 µl
Retention time	3.12 ± 0.1 min
Oven temperature	Starting at 180^0 C for 1 min hold, then increased at the rate of 6^0 C per min to 220^0 C hold for 3 min.
Injector Temperature	275^0C
Detector Temperature	300^0C
Limit of Detection	5 µg/L
Limit of Quantification	150 µg/L

pesticides, both the technical grade (X) and formulation (Y). Mayer and Ellersieck (1986) assumed formulation as more toxic when quotient > 1, however Schmuck et al. (1994) recognised that there was a natural range of quotient between 0.5 and 2.0, and formulation was regarded more toxic when quotient > 2. Demetrio et al. (2014) advocated accepting the criterion as valid only if the concentration effect lines were parallel. According to the criterion of APHA (1995), LC_{50} values for two forms of a chemical can differ at each time point if the confidence limits of the LC_{50} do not overlap.

Results

The recovery percentage for technical grade (T) and emulsified concentrate (F) chlorpyrifos was 83±0.2% and 89±2%, respectively. There was a linear rise in the 2 h chlorpyrifos concentration with the increase in nominal concentration exposure. Figure 1 shows the actual recovery of chlorpyrifos in water following a 2 h of exposure to the nominal concentrations of technical grade (T) as well as formulation (F) of chlorpyrifos. The LC_{50} values (µg/L) and 95% confidence limits of technical grade (94% a.i) and emulsifiable concentrate (F) of chlorpyrifos (20% EC) for *L. rohita* and *M. vittatus* are given in Table 4. The variations in LC_{50} values of T and F chlorpyrifos with respect to exposure hour for *L. rohita* and *M. vittatus* indicated that the LC_{50} values decreased as the exposure duration increased. On the basis of the 96-h LC_{50} values for both species, emulsifiable concentrate (F) of chlorpyrifos 20% EC is 1.89 (in *L. rohita*) to 1.94 (in *M. vittatus*) times more toxic to the two fish species than technical grade (94% a.i) chlorpyrifos. Furthermore, *L. rohita* was more sensitive to chlorpyrifos (both T and F) compared to *M. vittatus*, which could be attributed to the *M. vittatus*' hardiness, accessory respiratory organ and better adaptability than *L. rohita*, indicating that the susceptibility of the test organisms varied with the chemical form of the pesticide. The quotient of LC_{50} values (LC_{50} T/LC_{50} F) varied in *L. rohita* from 1.89 to 2.08 and in *M. vittatus* from 1.89 to 1.94 (Table 4). Table 5 shows the regression parameters of the log concentration-probit mortality line for 96 h mortality data for two fish species. It was found that the slopes between T and F were determined to be more or less parallel (Fig. 2).

In general, the two test fish species showed signs of frequent surfacing, gulping air at the surface, an initial rise in opercular movement followed by a progressive decline as exposure length increased, abundant mucus production, loss of stability, and spiral swimming. Finally, the fish drop to the bottom and die, their jaws open wide There were signs of severe internal bleeding around the dead fish's pharynx.

Discussion

In the present study, 96-h LC_{50} values of chlorpyrifos for *Labeo rohita* and *Mystus vittatus* ranged from 68 µg/L to 120 µg/L in case of technical grade (94% a.i.) and 36 µg/L to 62 µg/L in case of emulsifiable concentrate (20% EC), respectively. These values are in line with what several other workers have reported. The 96-h LC_{50} value of 68 µg/L for chlorpyrifos (94% a.i.) in *Labeo rohita* (present study) is near to the 96-h LC_{50} value of 82 µg/L for chlorpyrifos (95% a.i.) in *Oreochromis mossambicus* (Kunjamma et al. 2008) but higher compared to 96-h LC_{50} value of chlorpyrifos technical grade (10.39 µg/L) for *Labeo rohita* (Ikram et al. 2023). However, much higher LC_{50} values have been reported for *Cnesterodon decemmaculatus* (105.3 µg/L, Paracampo et al. 2015), *Cyprinus carpio* (149 µg/L, Li et al. 2013), *Cyprinus carpio* (160 µg/L, Halappa and David 2009), adult *Oreochromis niloticus* (154 µg/L, Oruç 2010), *Poecilia reticulata* (176 µg/L, Sharbidre et al. 2011), *Danio rerio* (289 µg/L, Singh et al. 2017), *Cyprinus carpio* (318 µg/L, Ambareen and Venkateshwarlu 2022), and *Cirrhinus mrigala* (440 µg/L, Bhatnagar et al. 2016). The 96-h LC_{50} value of chlorpyrifos (94% a.i) for *Mystus vittatus*

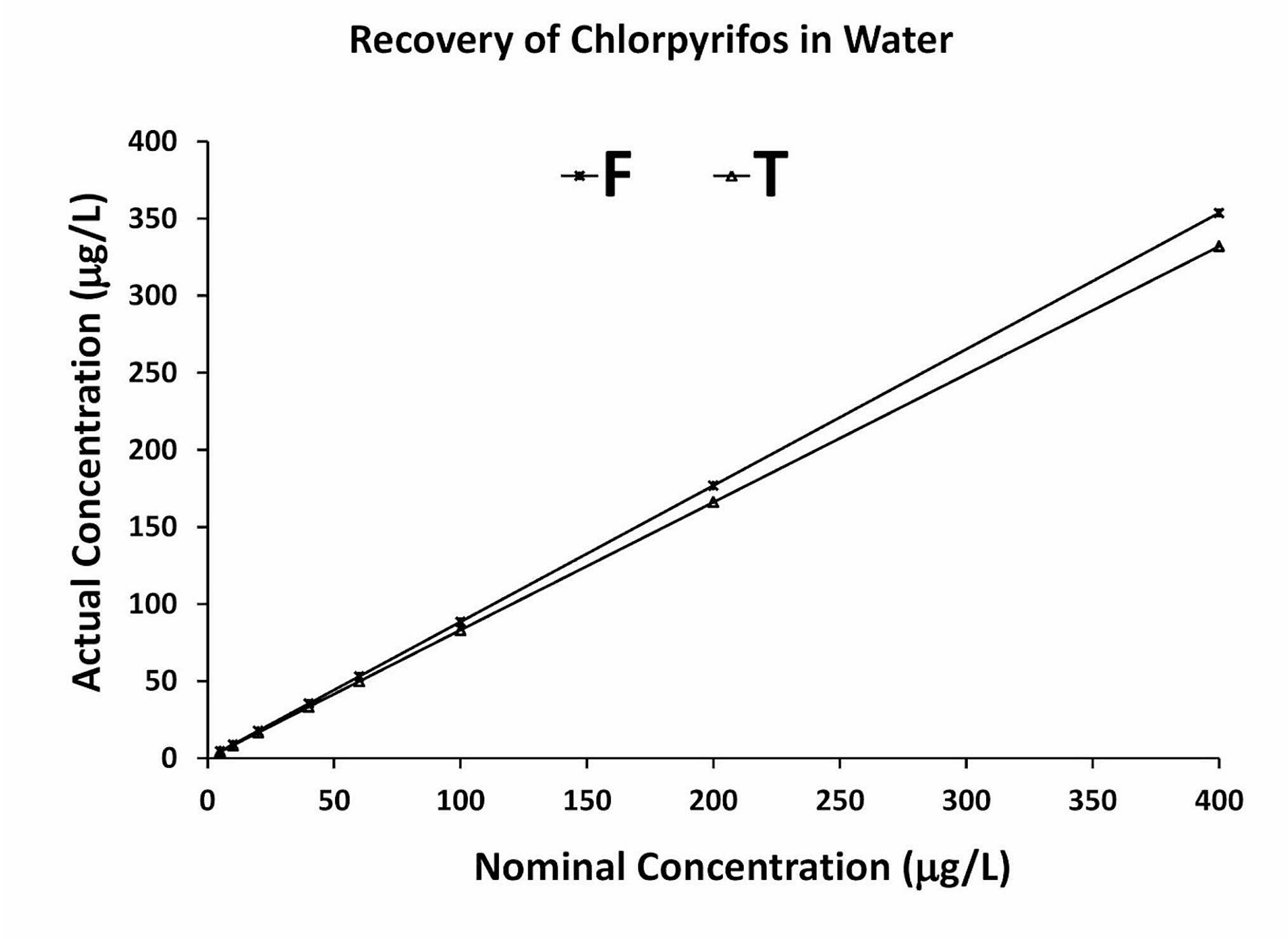

Fig. 1 Chlorpyrifos recovery in water after 2 h of exposure to technical grade (T) and emulsifiable concentrate (F) chlorpyrifos

Table 4 LC_{50} values (µg /L) with 95% confidence limit in parentheses of technical grade and formulation of chlorpyrifos to two fish species and quotient calculation

Hour of exposure	*Labeo rohita*	*Mystus vittatus*
24 h		
T	108 (94–122)	155 (136–176)
F	55 (41–71)	82 (68–96)
Quotient (LC_{50} T / LC_{50} F)	1.96	1.89
48 h		
T	89 (75–104)	147 (129–165)
F	47 (32–64)	77 (64–90)
Quotient (LC_{50} T / LC_{50} F)	1.89	1.91
72 h		
T	75 (61–91)	128 (108–151)
F	36 (25–50)	68 (53–84)
Quotient (LC_{50} T / LC_{50} F)	2.08	1.88
96 h		
T	68 (54–82)	120 (101–140)
F	36 (24–50)	62 (49–77)
Quotient (LC_{50} T / LC_{50} F)	1.89	1.94

Table 5 Regression parameters of log-probit lines for two fish species from 96-h toxicity data of technical grade (T) and emulsifiable concentrate (F) based on actual concentration of chlorpyrifos

Organism	Chemical form	Slope (b)	Intercept (a)	*n*	r^2
Labeo rohita	T	2.39	0.62	9	0.83
	F	1.36	2.83	9	0.90
Mystus vittatus	T	2.79	-0.79	11	0.86
	F	2.19	1.06	9	0.87

(120 µg/L) of the present study can be compared with these studies. The 96-h LC_{50} value of chlorpyrifos 20% EC for *L. rohita* (36 µg/L) and *M. vittatus* (62 µg/L) is close to the 96-h LC_{50} value of chlorpyrifos 20% EC reported by Majumder and Kaviraj (2019) for *O. niloticus* (42 µg/L) and by Hossain et al. (2022) for *O. niloticus* (46.80 µg/L). However, a much higher 96-h LC_{50} value was reported in case of: chlorpyrifos 40% EC for *L. rohita* (442.8 µg/L, Ismail et al. (2014); chlorpyrifos 50% EC for *Heteropneustes fossilis* (1900 µg/L, Tiwari et al. 2019). All of the variations in chlorpyrifos sensitivity among fish suggest that it is species-specific.

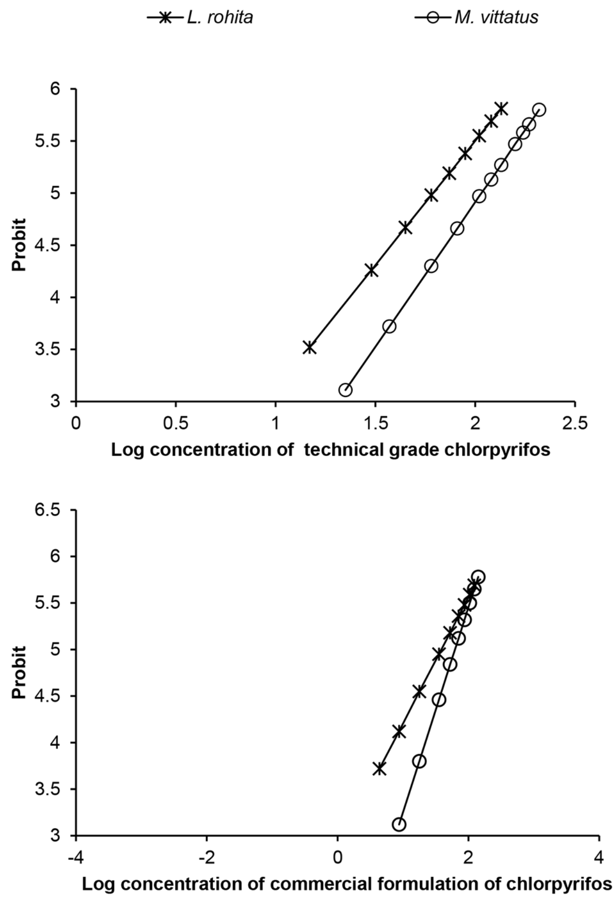

Fig. 2 Regression lines for log concentration versus probit mortality of *Labeo rohita* and *Mystus vittatus* following 96-h exposures to technical (T) and emulsifiable concentrate (F) of chlorpyrifos

In the current study, the quotient of 96-h LC_{50} values (LC_{50} T / LC_{50} F) for *Labeo rohita* and *Mystus vittatus* was between 1.89 and 1.94. When the current study's results were evaluated, it became clear that, according to Mayer and Ellersieck's (1986) criteria, the formulation was more toxic than the technical grade chlorpyrifos for the two fish species, and that, according to Schmuck et al. (1994), there was no difference in toxicity between T and F chlorpyrifos for *Labeo rohita* and *Mystus vittatus*. However, sensitivity comparisons based on quotient are not accepted universally. The slopes between T and F were found to be parallel for the two fish species, which satisfied the criteria of Demetrio et al. (2014). Since there is no overlap in the confidence limits of the LC_{50}, the LC_{50} values for T and F chlorpyrifos differ at each time point in the two test fish species, which meets the criteria of APHA (1995).

The present findings clearly indicate that commercial chlorpyrifos formulation (20% EC) is more toxic than the chlorpyrifos technical grade (94% a.i.) in both of the two fish species, which are in agreement with the earlier works of Tilak and Veeraiah (2001) on *Labeo rohita*, De Silva et al. (2010) on *Perionyx excavatus*, Demetrio et al. (2014) on *Daphnia magna*, and Majumder and Kaviraj (2019) on *Oreochromis niloticus*. The degree to which T and F chlorpyrifos differed in toxicity, however, varied depending on the species. Chlorpyrifos 20% EC, marketed as Dursban® by Dow AgroSciences India Pvt. Ltd., contains solvent naphtha (petroleum) and heavy aromatic as other (= inert) ingredients (MSDS 2014). The use of solvent naphtha (petroleum) in pesticide formulation and consequent additional toxicity of the formulated pesticide products compared to the active ingredient were reported in a few recent studies (Adams et al. 2021; Tu et al. 2023). Solvent Naphtha (P) HA has already been recognised as a neuro- and hepatotoxin (Haz-Map 2023). The synergistic interactions of the chlorpyrifos active ingredient with added inert ingredients make Dursban® (chlorpyrifos 20% EC) more toxic compared to the technical grade of chlorpyrifos (94% a.i).

Conclusions

This study concludes that both of the two fish species are highly susceptible to chlorpyrifos, with *Labeo rohita* being more susceptible than *Mystus vittatus*. The LC_{50} values have an inverse relationship with exposure time. The sensitivity of the organisms varies with the chemical form of the chlorpyrifos used. The synergistic interactions of the active ingredient chlorpyrifos with inert substances make commercial formulations or emulsifiable concentrate of chlorpyrifos (20% EC) more toxic than technical-grade chlorpyrifos. Therefore, in order to minimise the amount of pesticides in the environment and promote sustainable agriculture, attention should be focused on developing target-specific pesticide generation.

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Authors Contribution The author is solely responsible for conceptualisation, experiment designing, performing, formal analysis, writing-reviewing-editing and finalisation of the manuscript.

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Data Availability All the relevant data, including descriptive statistics and statistical calculations have been given in the supplementary file.

Declarations

Ethical Statement Animal care and handling was performed in accordance with the standard animal care protocols of the University of

Kalyani as well as all applicable national and international guidelines.

Conflict of Interest There is no conflict of interest.

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