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Behavioural Responses and Mortality of Mozambique Tilapia *Oreochromis mossambicus* to Three Commonly Used Macadamia Plantation Pesticides

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Abstract: The use of pesticides in agricultural systems may have deleterious effects on surrounding environments. Aquatic systems are no exception and are increasingly polluted through the leaching of pesticides from agricultural activities. However, the pesticide pollution effects on key aquatic species have not been studied in many regions. In southern Africa, increasing pesticide use associated with macadamia tree *Macadamia integrifolia* farming presents a growing risk to surrounding aquatic ecosystems. This study assessed behavioural responses of an important and widely-distributed freshwater fish, Mozambique tilapia *Oreochromis mossambicus*, following exposure to three commonly used macadamia pesticides (i.e., Karate Zeon 10 CS, Mulan 20 SP, Pyrinex 250 CS) at different concentrations (0.7–200 µL, 0.3–1000 mg, and 0.7–8750 µL, respectively) over 24 h. Behavioural responses, i.e., swimming erratically, surfacing, vertical positioning, loss of equilibrium, being motionless and mortality were observed after pesticides exposure. Lethal dose 50 (LD50) values of Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS were 2.1 µL (per water litre dilution—WLD), 5.2 mg (WLD) and 21.5 µL (WLD), respectively. These concentrations are therefore expressed as a maximal threshold usage in the environment around macadamia farms and a minimum distance of the plantations to water systems should be considered. Further studies should examine effects on other fish species and aquatic invertebrates to inform on pesticide pollution threats and mitigation plans for the region.

Keywords: behaviour traits; environmental stressor; insecticide; neonicotinoids; organophosphate; pyrethroid

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1. Introduction

Freshwater ecosystems are among the most threatened by multiple anthropogenic stressors [1–3]. One of the major stressors for freshwater ecosystems is agricultural pesticides, which are introduced via point and non-point sources [4]. Pesticides are intended to protect agricultural production from pests; however, their residues reach far beyond their target areas via atmospheric, overland, sub-surface and groundwater routes [5]. Surface run-off and wastewater effluents from agriculture and urban/developed areas are among the most important entry pathways for pesticides into aquatic ecosystems, with well-documented adverse effects on these environments [6–8]. Pesticide contamination of surface waters affects aquatic invertebrate and vertebrate community structure and diversity [9]. Globally, pesticide use in agriculture has increased significantly to control problematic species, particularly in orchard plantations [10]. Within South Africa, macadamia nut plantations have been rapidly increasing in scale since the early 1990s, after the first introduction of *Macadamia integrifolia* seeds from Hawaii to South Africa more than 100 years ago [11]. The South African macadamia nut plantations are centred in the sub-tropical regions of the Mpumalanga Lowveld (i.e., Nelspruit area), Limpopo (i.e., Luvuvhu River Valley) and KwaZulu-Natal [11]. While relied upon for productivity, pesticide usage in these rapidly increasing macadamia nut plantations has the potential to pollute the aquatic ecosystems through several routes, including spray drift, drainage, surface run-off, and/or accidental spills [12–14]. Common and/or persistent pesticides are frequently detected as harmful pollutants in soil, surface water and groundwater because of their continuous application within agricultural landscapes [15–17].

Even though synthetic chemical production has increased rapidly in recent decades [14], the effect of these chemicals on non-target habitats and biota has been overlooked [18]. Fish play a keystone role in freshwater ecosystems through, for example, trophic interactions that structure food webs [19]. They are also very often important food sources for organisms further up the food web, including humans that readily exploit these food resources [20,21]. Fish can absorb pesticides through gills, skin, or alimentary ducts [22–24]. These organisms are potentially vulnerable to aquatic pollutants, such as pesticides used in macadamia plantations, which could severely compromise certain physiological and biochemical processes [24]. Several studies [25,26] have reported the mechanisms through which pesticides affect fish, with the goal of monitoring, controlling and possibly identifying concentrations that pose little or no effect on aquatic organisms. Pesticide exposure may affect fish abundance, causing significant mortalities and/or altering behaviours [27]. Although some fish metabolise and excrete certain pesticides, continuous dietary exposure may lead to bioaccumulation and impact their health or fecundity/offspring [28].

To our knowledge, no attempts have been made to document how pesticides used in the macadamia plantations in South Africa, such as Karate Zeon 10 CS, Mulan 20 SP, and Pyrinex 250 CS, affect fish. Macadamia plantations are often in close contact with the freshwater ecosystem and the use of these pesticides is an accepted management strategy for controlling pests in South Africa's macadamia plantations. As such, we aimed to assess the behavioural response of a common, often dominant, and economically/ecologically important fishery species, Mozambique tilapia *Oreochromis mossambicus* when exposed to Karate Zeon 10 CS, Mulan 20 SP, and Pyrinex 250 CS at various concentrations and any potential for recovery after exposure to pesticides. To this end, we used a median lethal dose (or LD50) approach, whereby for each test pesticide, the dose at which 50% of test animals die was determined. Once the LD50 of each pesticide was determined, sub-lethal concentration effects on behavioural responses of the fish were explored.

2. Materials and Methods

2.1. Pesticide Background

The three pesticides used in the experiment were Karate Zeon 10 CS (capsule suspension; Adama, Kempton Park, South Africa), Mulan 20 SP (soluble powder; Adama, Kempton Park, South Africa), and Pyrinex 250 CS (capsule suspension; Syngenta, Midrand, South Africa). Karate Zeon contains lambda-cyhalothrin (pyrethroid) as an active ingredient and consist of 100 g/L, whereas Mulan 20 AS contains acetamiprid (neonicotinoid) as an active ingredient and consists of 200 g/kg and Pyrinex contains chlorpyrifos (organophosphate) as an active ingredient and consists of 250 g/L. Pyrinex 250 CS and Karate Zeon 10 CS are capsule-suspension contact insecticides for the control of macadamia insects, such as thrips *Scirtothrips aurantii*, cotton aphid *Aphis gossypii*, and stink bugs *Bathycoelia distincta* and *Nezara viridula*. Mulan 20 SP, however, is a water-soluble powder systemic contact. These pesticides are broad-scale insecticides for the control of pests on macadamia, canola, citrus, tomatoes, wheat, barley, oats, cotton and rooibos tea, etc. The spray mixture (or dilution) volumes of 200 mL/100 L water, 5 mL/100 L water, and 40 g/100 L water for

Karate Zeon 10 CS, Mulan 20 SP, and Pyrinex 10 CS, respectively, are highlighted according to the manufacturer's recommendations. These pesticides can be applied by any medium or high-volume applicator, aerially or on the ground, provided that the applicator is correctly calibrated and fitted with an efficient agitation mechanism. These pesticides are diluted with Hydrobuff (Nutrico, South Africa) to maintain pH level and Wetta (Nutrico, South Africa) to act as a surfactant. For this study, these pesticides were selected because they are the most commonly used products by macadamia farmers in the Luvuvhu River Valley area for pest control [29,30].

2.2. Fish Sampling

Freshwater Mozambique tilapia *Oreochromis mossambicus* were caught from a small reservoir in Duthuni Village (coordinates: −22.967657, 30.397369), Limpopo Province of South Africa, using a 20 m seine net (depth 1.5 m, mesh size 0.5 cm) in October 2020 and 2021. All fish were transported in eighteen 20 L white polyethylene buckets (15–20 individuals per bucket) to the Department of Ecology and Resource Management Laboratory, University of Venda. We mixed 20 L of filtered reservoir water (through a 63 µm mesh) and 200 L of borehole water (1:10 ratio) which were also collected for the experiments. All the fish were size-matched with regard to total length (TL) to reduce the influence of size-related differences in behaviour when exposed to pesticides (TL, mean ± SD: 40.5 ± 2.3 mm). The Mozambique tilapia were housed in 20 L buckets with source water and continuous aeration at 23.2 ± 0.3 °C, i.e., ambient temperatures, and fed once during the 48 h acclimation period prior to experimentation, following procedures in Mbedzi et al. [31].

2.3. Experimental Design

The Organization for Economic Co-operation and Development (OECD) [32] guidelines for acute toxicity studies were followed for the experimental procedures. The experiment was conducted during October 2020 and 2021. Each pesticide (3) had four replicates, and a total of 216 fish were used for the entire experiment, excluding controls (9 treatments × 3 groups × 4 replicates × 2 fish per replicate; see Table 1). For each replicate, two individual fish of similar size/age (SL ± SD: 32.4 ± 2.1 mm) were placed randomly in an experimental container to avoid confounds (3 L glass containers with 2.9 L filtered reservoir and borehole water (ratio 1:10)) using a square 10.16 cm wide and long aquarium net made of fine mesh. Fish were juveniles and characterised by a silvery body with a straight forehead profile, with 6–7 vertical bars, three spots along flanks and 12–15 dorsal fin rays. After adding fish in each treatment, the fish were allowed to acclimate for 4 h prior to chemical addition. Further, a pesticide-free treatment was employed to serve as a control ($n = 36$) for each pesticide treatment with two fish of similar size. Once the chemicals were added and the experiment started, aeration was stopped, and no further aeration was provided for the duration of the experiment (24 h). Treatments K7 (100 µL), M8 (500 mg) and P8 (4500 µL) were field concentrations that the farmers use within the Luvuvhu River Valley area (Table 1). Concentrations for K1 (0.7 µL), M1 (0.3 mg) and P4 (33.3 µL) (Table 1) were decided based on background values in aquatic environments found in the literature: 0.11–0.14 µg L^{-1} [33], 0.2–5 µg L^{-1} [34] and 0.5 µg L^{-1} [35], for Karate Zeon 10 CS, Mulan 20 SP, Pyrinex 250 CS, respectively.

After the additions of the pesticide solutions to the experimental containers, the behaviour of the fish was observed constantly for the first 1.5 h of the experiment and then observed again at 3 h, 6 h and 24 h for each pesticide treatment exposure by personnel. At 3 h, 6 h and 24 h, the constant observation time was 30 min. In an event where personnel were absent, behaviours of the fish were video recorded and estimated using a stopwatch. The time to the onset of certain behavioural endpoints associated with each pesticide exposure was assessed during the study. These behaviour endpoints were the time from initiation of pesticide exposure (t_0) to when the first fish (a) started swimming erratically (thereafter referred to as swimming), (b) were seen positioned vertically in the water column, (c) exhibited motionless behaviour, (d) attempted to breach the surface to breathe air, (e) was first seen exhibiting loss of equilibrium, and (f) showed mortality, defined as the cessation or absence of movement after repeated tactile stimulation/prodding for a period of 2 min [36]. These were carried out for each replicate, treatment and concentration. Any fish surviving to the end of the 24 h exposure period was moved to a water recovery tank to determine if a moribund state resulted in death or if recovery was possible, being subsequently inspected after 24 h.

Table 1. Pesticide treatments and volumes per litre—thereafter referred to as concentrations, were applied to 2.9 L water with Mozambique tilapia, *Oreochromis mossambicus* to assess behavioural responses and mortality.

Treatment	Group	Concentrations
K1	Karate Zeon 10 CS	0.7 µL
K2	Karate Zeon 10 CS	3.3 µL
K3	Karate Zeon 10 CS	6.7 µL
K4	Karate Zeon 10 CS	10 µL
K5	Karate Zeon 10 CS	16.7 µL
K6	Karate Zeon 10 CS	80 µL
K7	Karate Zeon 10 CS	100 µL
K8	Karate Zeon 10 CS	150 µL
K9	Karate Zeon 10 CS	200 µL
M1	Mulan 20 SP	0.3 mg
M2	Mulan 20 SP	0.5 mg
M3	Mulan 20 SP	1.7 mg
M4	Mulan 20 SP	3.3 mg
M5	Mulan 20 SP	6.7 mg
M6	Mulan 20 SP	100 mg
M7	Mulan 20 SP	300 mg
M8	Mulan 20 SP	500 mg
M9	Mulan 20 SP	1000 mg
P1	Pyrinex 250 CS	0.7 µL
P2	Pyrinex 250 CS	3.3 µL
P3	Pyrinex 250 CS	16.7 µL
P4	Pyrinex 250 CS	33.3 µL
P5	Pyrinex 250 CS	66.7 µL
P6	Pyrinex 250 CS	1250 µL
P7	Pyrinex 250 CS	2250 µL
P8	Pyrinex 250 CS	4500 µL
P9	Pyrinex 250 CS	8750 µL
C	Control	No chemicals

3. Data Analyses

Prior to analysis, all data were tested for normality (Shapiro–Wilk test, $p > 0.05$) and homogeneity of variances (Levene's test, $p > 0.05$) and were found to be normal with homogenous variances. To assess the behavioural endpoints and mortality among the pesticide treatments, we used one-way ANOVA. To assess the onset of behavioural changes within each pesticide group (i.e., Karate Zeon 10 CS, Mulan 20 SP, Pyrinex 250 CS) as a grouping variable for all replicates, we used pesticide treatments (i.e., K1–K9, M1–M9, P1–P9) as explanatory variables and each onset behaviour as response variables. Individuals were included as fixed factor to account for onset of fish behaviours. To assess lethal dose 50 of each pesticide group we used probit analysis (95% confidence limits for concentration). Chi-square goodness-of-fit tests were also performed to check if the probit models fit the data adequately. Tukey post hoc tests were used to evaluate multiple comparisons among pesticides concentrations where effects were significant. In all analyses, significance was inferred at $p < 0.05$. All statistical analysis were performed using SPSS version 16.0.0.0 [37].

4. Results

Mozambique tilapia *Oreochromis mossambicus* in the control treatments did not die or exhibit any abnormal behaviour. When *O. mossambicus* were exposed to Karate Zeon 10 CS, Mulan 20 SP, and Pyrinex 250 CS, abnormal behaviour such as fast swimming, surface breaking (i.e., air breathing), vertical positioning, being motionless, loss of equilibrium and mortality were observed. The average time to onset of behavioural symptoms associated with pesticide toxicity exhibited a dose-dependent response, with slower vertical positioning, motionless, loss of equilibrium and mortality responses generally exhibited at low concentrations and faster swimming and surfacing at high concentrations of each treatment (Figure 1).

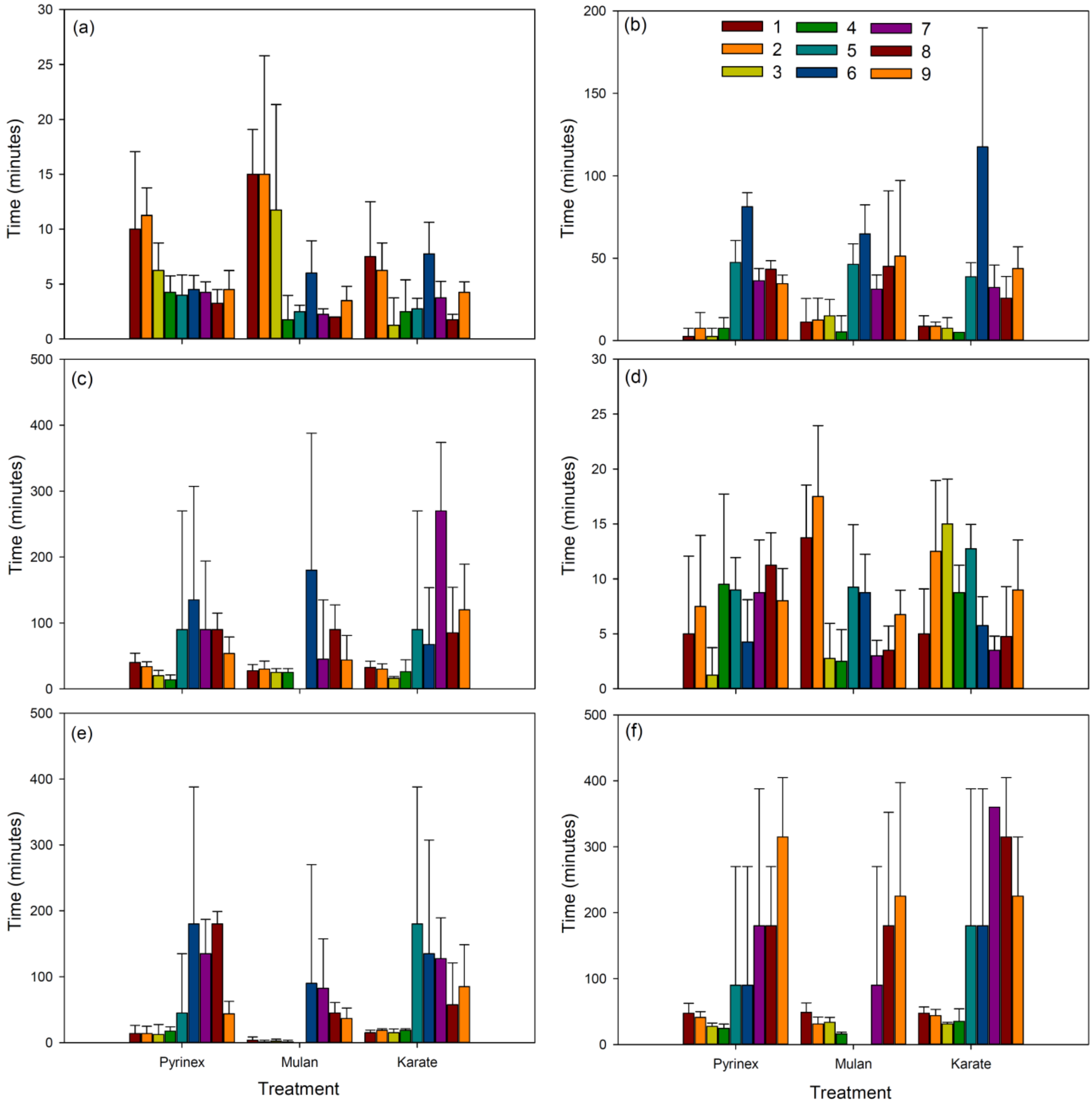

Figure 1. The mean time (±standard deviation) to onset of behaviour effects associated with (**a**) swimming erratically, (**b**) vertical, (**c**) motionless, (**d**) surface, (**e**) loss of equilibrium and (**f**) mortality under various chemical treatments. The numbers represent various concentrations across Karate Zeon 10 CS, Mulan 20 SP, Pyrinex 250 CS, see Table 1 and note the differences in time scales.

Fish behaviour was affected by pesticide treatment concentrations and time to onset behaviour (Table 2, Figure 1). Immediately after exposure to Karate Zeon 10 CS, Mulan 20 SP, and Pyrinex 250 CS separately, the fish started to swim actively and surfacing prior to vertical positioning, being motionless, losing equilibrium, and then dying off; however, these varied in pesticide concentrations and time to onset. When *O. mossambicus* was exposed to Karate 10 CS, the onset to swimming, vertical, motionless, and mortality differed significantly ($p < 0.05$) among concentrations; however, the loss of equilibrium behaviour did not differ significantly ($p > 0.05$) among concentrations (Table 2, Figure 1). When exposed to Mulan 20 SP, the onset to swimming and surface behaviours differed significantly ($p < 0.05$) among concentrations, however, the vertical, motionless, loss of equilibrium and mortality behaviours indicated no significances ($p > 0.05$) among concentrations (Table 2, Figure 1). Lastly, in fish exposed to Pyrinex 250 CS, the swimming, vertical and loss of equilibrium behaviours all differed significantly ($p < 0.05$) among concentrations, with motionless, surfacing and mortality behaviours showing no significances ($p > 0.05$) among concentrations (Table 2, Figure 1). Tukey's post hoc analysis highlighted significant differences among pesticide concentrations associated with onset behaviours, and are presented in Tables S1–S3, for Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS, respectively.

Table 2. Onset behaviours as response variables associated with Mozambique tilapia *Oreochromis mossambicus*, identifying multiple comparisons between concentrations within each pesticide (i.e., Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS) as explanatory variables. Bold values indicate significant differences at $p < 0.05$.

Behaviour	Karate Zeon 10 CS	Mulan 20 SP	Pyrinex 250 CS
	p	*p*	*p*
Swimming	**0.013**	**0.004**	**<0.001**
Vertical	**<0.001**	0.225	**<0.001**
Motionless	**0.005**	0.161	0.691
Surface	**0.001**	**<0.001**	0.304
Loss of equilibrium	0.134	0.439	**0.007**
Mortality	**<0.001**	0.057	0.069

Mortality following exposure to Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS at 1.5 h, 3 h, 6 h and 24 h was variable. Across treatments, non-significant differences (F = 2.424, df = 2, p = 0.110) were observed in mortality rates within 24 h (Table 3). Across treatments, 100% mortality was observed after 1.5 h at high concentrations, i.e., treatment 6–9 (Table 3, Figure 1). Overall, high mortality rates were observed in Karate Zeon 10 CS followed by Mulan 20 SP and lastly Pyrinex 250 CS.

Table 3. Mortality of Mozambique tilapia *Oreochromis mossambicus* (%) following exposure to various concentrations of C—control, Karate Zeon 10 CS 250 CS (K1–K9), Mulan 20 SP (M1–M9) and Pyrinex 250 CS (P1–P9), with 8 fish each per treatment.

Treatment	Karate Zeon 10 CS				Mulan 20 SP				Pyrinex 250 CS			
	1.5 h (%)	**3 h (%)**	**6 h (%)**	**24 h (%)**	**1.5 h (%)**	**3 h (%)**	**6 h (%)**	**24 h (%)**	**1.5 h (%)**	**3 h (%)**	**6 h (%)**	**24 h (%)**
C	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	25	0	0	0	12.5	0	0	12.5	12.5
2	0	0	0	50	0	0	0	25	0	0	12.5	12.5
3	0	0	0	75	0	0	12.5	12.5	0	0	12.5	25
4	0	0	0	100	0	0	25	25	0	0	25	50
5	0	25	62.5	100	0	12.5	25	50	0	0	12.5	12.5
6	100	0	0	0	100	0	0	0	100	0	0	0
7	100	0	0	0	100	0	0	0	100	0	0	0
8	100	0	0	0	100	0	0	0	100	0	0	0
9	100	0	0	0	100	0	0	0	100	0	0	0

Recovery following 24 h exposure to Karate Zeon 10 CS, Mulan 20 SP and Karate 250 CS treatments was variable. Across treatments, non-significant differences (F = 1.480, *df* = 2, *p* = 0.248) were observed in survival rates after individuals were transferred from exposure treatments to clean water for certain treatments (i.e., K1–K3, M1–M5, P1–P5) (Table 4). Of the fish that were alive at the end of the 24 h treatment exposure, survivability ranged from 12.5–62.5%, 50–87.5%, 50–87.5%, at 24 h to 50–87.5%, 37.5–87.5%, 12.5–62.5 at 48 h in clean water, in Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS treatments, respectively (Tables 1 and 4). Fish recovery survival rate increased with decreasing toxicity of each pesticide.

Table 4. Mozambique tilapia *Oreochromis mossambicus* survival rates at 24 h after pesticide concentrations of C—control, Karate Zeon 10 CS 250 CS (K1–K9), Mulan 20 SP (M1–M9) and Pyrinex 250 CS (P1–P9) and recovery after a further 24 h (i.e., 48 h) following exposure to in clean water.

Treatment	Karate Zeon 10 CS		Mulan 20 SP		Pyrinex 250 CS	
	24 h (%)	48 h (%)	24 h (%)	48 h (%)	24 h (%)	48 h (%)
C	100	100	100	100	100	100
1	75	62.5	87.5	87.5	87.5	87.5
2	50	37.5	75	75	87.5	75
3	25	12.5	87.5	87.5	75	75
4	0	0	75	75	50	50
5	0	0	50	37.5	12.5	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0

The LD50 values obtained at 24 h exposures and the 95% confidence limits revealed that Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS showed high toxicity. The effects of each pesticide on *O. mossambicus* were strongly influenced by the level of concentration (Figure 1). No significant differences were observed in mortality across treatments ($p > 0.05$). Results from probit analyses showed 2.1 µL, 5.2 mg and 21.5 µL LD50 values of Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS, respectively (Table 5).

Table 5. Lower limit, upper limit, and lethal-dose 50 of Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS following exposure of Mozambique tilapia *Oreochromis mossambicus* at various concentrations.

Groups	*p*	Lower Limit	Upper Limit	LD50
Karate Zeon 10 CS	0.897	0.8 µL	3.7 µL	2.1 µL
Mulan 20 SP	0.701	2.4 mg	13.5 mg	5.2 mg
Pyrinex 250 CS	0.684	8.8 µL	54.1 µL	21.5 µL

Figure 2 presents the probit model. Log doses of each pesticide were plotted against probits, i.e., response of *O. mossambicus* when exposed to Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS at various concentrations, used to analyse LD50 (Table 1). Karate Zeon 10 CS and Mulan 20 SP were not distributed randomly compared to Pyrinex 250 CS.

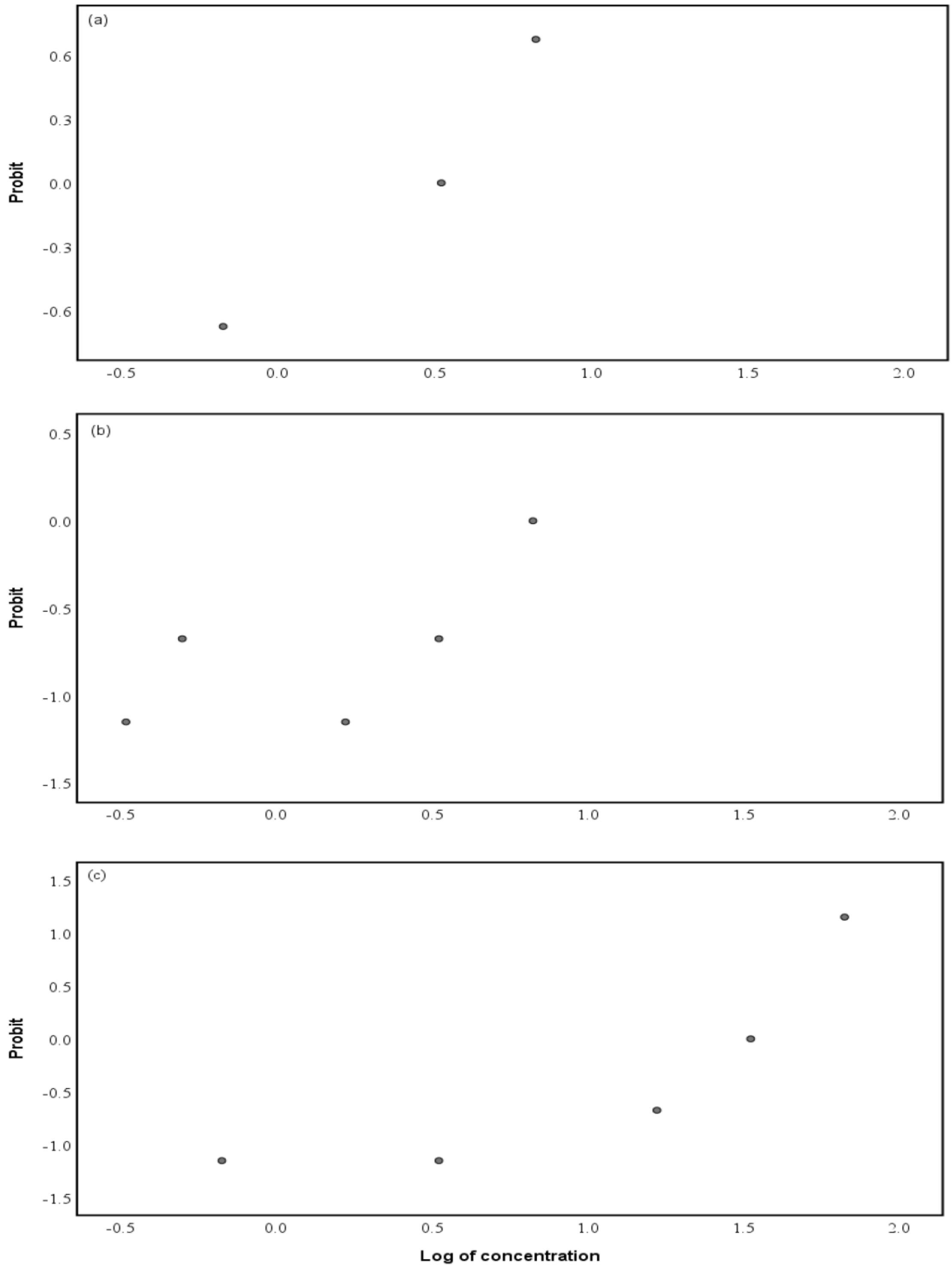

Figure 2. Output for the determination of the 24 h median lethal dose (LD50) of (**a**) Karate Zeon 10 CS (µL), (**b**) Mulan 20 SP (mg) and (**c**) Pyrinex 250 CS (µL) to Mozambique tilapia *Oreochromis mossambicus*. Probits also referred to as proportion killed/mortality, i.e., the response of *O. mossambicus* and log of concentration (toxicity of chemicals), i.e., dose of each pesticide.

5. Discussion

Alteration in behaviour patterns is one of the most sensitive indicators of environmental stress [38]. Behavioural information provides insights into external (morphological) and internal (physiological) adaptive changes of an organism due to the impacts of chemical pollutants [39]. In the present study, we assessed onset times for dysfunctional behaviours in Mozambique tilapia *O. mossambicus*, exposed to field and background concentrations of Karate Zeon 10 CS, Mulan20 SP and Pyrinex 250 CS, but found total mortality onsets within a critical 3 h observation period for field concentrations. These results serve to increase the breadth of knowledge regarding the impacts of macadamia pesticide applications, in particular on this fish species. While the effects of aquaculture chemicals on fish have generally been well-assessed, relatively few studies have examined the effects of agricultural pesticides such as Karate Zeon 10 CS [40], Mulan 20 SP [41] and Pyrinex 250 CS [42] on aquatic organisms, and, according to our knowledge, none on *O. mossambicus*.

Studies conducted by Boone and Semlitsch [43], Camargo and Alonso [44], and Majumder and Kavjraj [45] indicate that when fish or other animals are exposed to a pesticide at lethal or sub-lethal concentrations, a wide variety of behaviour changes occur, but higher concentrations of pesticides are more often associated with negative effects and more rapid onsets [46]. Nevertheless, chronic exposure might present additional adaptation to the chemical stress that, if successful, will permit the organisms to continue to function normally in their environment, or they might indicate partial failure of one or more physiological functions [47].

The present study indicated that the behavioural responses from exposure to Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS differed significantly, ranging from early onset behaviours, i.e., at high concentrations to delayed behaviours, i.e., at low concentrations (Figure 1). Some non-significant differences were observed across pesticide treatments, for instance, loss of equilibrium within Karate Zeon 10 CS; vertical, motionless, loss of equilibrium and mortality within Mulan 20 SP; and lastly, motionless, surface and mortality within Pyrinex 250 CS treatments. Nevertheless, significant differences were found among concentrations of each pesticide group. Mortality and recovery rates did, however, not differ across pesticide groups. These findings are similar to those of other research on the responses of *O. mossambicus* exposed to pyrethroids [48], neonicotinoids [49], and organophosphates [50]. Although we did not find any literature on the response of *O. mossambicus* to Karate Zeon 10 CS, Mulan 20 SP and/or Pyrinex 250 CS, other studies have revealed that when *O. mossambicus* is exposed to agricultural chemicals at different concentrations, they also exhibited different behavioural traits. The behavioural traits included differential swimming patterns, surfacing activity, gills operculum activity, mucous secretion, food intake and excretions from the fish, which occurred at various levels of pesticides [51–53]. For example, a study by Parithabhanu [54] showed behavioural traits of *O. mossambicus* such as restlessness, obscured swimming patterns, loss of equilibrium, and mortality, however, when treated to cypermethrin. Here, *O. mossambicus* was recorded to change its swimming pattern and surfacing activity after exposure in all treatments. The fish swam erratically with signs of suffocation at the water surface before becoming motionless, with some fish positioned upside-down prior to mortality. According to Kane et al. [55], the erratic swimming movements in the fishes might be due to the inhibition of AchE (acetylcholinesterase) by the toxic effect of pesticides. Increased surfacing activity after exposure suggests an elevated rate of metabolism and altered physiology of fish due to hypoxia. According to Saxena and Parashari [56], the surfacing activity compensates for oxygen deficiency from the medium and extracts more oxygen to meet the extra energy to cope with toxicity.

Oreochromis mossambicus showed a low rate of mortality at a low concentration and high rate of mortality at high concentration across each pesticide. Using Finney's probit analysis as recommended by Miller and Tainter, [57], the LD50 values of *O. mossambicus* in this study were found to be 2.1 µL, 5.2 mg and 21.5 µL for Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS, respectively. This shows that selected pesticides are highly toxic to *O. mossambicus* at field concentration and can cause 50% mortality even at background aquatic environment concentrations. Chlorpyrifos is highly toxic to fish (LD50 for 24 h 5.38 µL/L) and affects their survival [58]. A study conducted by Sharma [59] concerning organophosphate insecticide found an LD50 value of 0.03 µL/L; however, at 96 h exposure. Sánchez-Bayo [60] found LD50 value of 0.01–0.5 mg/L looking at non-target fish organisms exposed to neonicotinoids insecticides.

Non-target organisms in different aquatic ecosystems contaminated with pesticides at different concentrations from various sources such as agricultural sectors may suffer deleterious or deadly consequences, resulting in an indirect disruption in food webs. However, the effects of pesticides at field concentrations on *O. mossambicus* cannot directly correspond to what happens in aquatic environments. Moreover, the distribution and stability of these pesticides in water systems and their effects on non-target organisms may vary with the size of water bodies and the amount of chemicals being applied. For example, fish predators such as birds may suffer severe effects from trophic transfer when they feed on fish exposed to pesticides. The overall behavioural responses of *O. mossambicus* when exposed to each pesticide at different concentrations suggest high sensitivity at the tested concentrations, but further studies should examine those under a greater degree of dilution to determine critical thresholds for mortality. Furthermore, the recommended field concentration of each pesticide is about 70–80-times the LD50 determined in this study.

In conclusion, the present study indicated that pesticides, i.e., Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS, which are commonly used by macadamia farmers in South Africa, affect behaviours of *O. mossambicus.* Several onset behaviours, i.e., response variables, were found to differ significantly among concentrations of each pesticide group, indicating the capacity of *O. mossambicus* to tolerate various concentrations differently. After assessing the effects of Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS on *O. mossambicus* and behavioural changes, the following main conclusions can be drawn: (*a*) the nature of behavioural responses of *O. mossambicus* differed when exposed to selected pesticides, but varied in terms of onset times and concentrations; (*b*) effects of Karate Zeon 20 CS, Mulan 20 SP and Pyrinex 250 CS at high concentrations, i.e., field concentrations caused high mortality rates compared to background concentrations; and (*c*) LD50 of each pesticide was determined and was well-within concentrations used in the present study. Regarding the increasing rate of macadamia plantation and the utilization of chemicals as pest controls, continuous exposure of non-target organisms will result in a high concomitant decline in aquatic populations. Further research is needed to assess other macadamia pesticides that are likely to pose less effects, even at a high concentration, to non-target animals and behavioural responses of aquatic macroinvertebrates. Such studies will improve our understanding of the interaction between toxicological and ecological mechanisms [61], helping to improve our understanding of the environmental impacts of chemicals and their risk assessment. The present study's findings can be used for effective management and to determine the safe level of Karate Zeon 10 CS, Mulan 20 SP and Pyrinex 250 CS disposal through agricultural run-off to natural water bodies to minimise its toxic effect on non-target organisms and aquatic ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14081257/s1, Table S1: Tukey's multiple comparisons results following one-way ANOVA for onset behaviour across Karate Zeon 10 CS concentrations; Table S2: Tukey's multiple comparisons results following one-way ANOVA for onset behaviour across Mulan 20 SP concentrations; Table S3: Tukey's multiple comparisons results following one-way ANOVA for onset behaviour across Pyrinex 250 CS concentrations.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Venda Research and Animal Ethics Committee (Ethical clearance number: FSEA/22/ERM/01/1103).

Data Availability Statement: The datasets generated and/or analysed during the current study are not publicly available as they are part of larger study that is currently on-going but are available from the corresponding author on reasonable request.

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