UNIVERSIDADE FEDERAL DE OURO PRETO
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DEPARTAMENTO DE BIODIVERSIDADE, EVOLUÇÃO E MEIO AMBINETE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA DE BIOMAS TROPICAIS

Bruno Carlos Ramos

Evaluation of the toxicity of the pesticide acephate and his by-product methamidophos over the behaviour of zebrafish *Danio rerio* (C*ypriniformes, C*yprinidae)

Orientador(a): Eneida Maria Eskinazi Sant'Anna
Co-orientador: Cristiano Schetini de Azevedo

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MSc thesis presented to the Graduate program in Ecology of Tropical Biomes from the Exacts and Biological Sciences from the Federal University of Ouro Preto as a partial requirement for the acquisition of Master's degree.

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Dissertação apresentada ao Programa de Pós-graduação em Ecologia de Biomas Tropicais da Universidade Federal de Ouro Preto como requisito parcial para obtenção do título de Mestre em Ecologia de Biomas Tropicais

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Abstract

Worldwide, Brazil leads in the use of pesticides, and acephate is the fifth most used pesticide in the country. This component and its by-product methamidophos, may cause deleterious effects on the physiology and behaviour of various animals when in contact with water. Studies investigating the negative impacts of acephate in animals have used zebrafish larvae as a model species, but the effect on adult individuals remains unclear. Our main objective was to assess the behavioural changes in adult zebrafish (*Danio rerio*) exposed to acephate and methamidophos. To this end, 160 individuals of zebrafish were divided in three groups: acephate, methamidophos (exposed) and control groups (non-exposed), where subjects on the treatment groups were exposed to 7 µg/L of acephate and 7 µg/L methamidophos for 28 days. To compare the groups, behavioural assessments were carried out using scan sampling with instantaneous recordings of behaviour every 30 seconds, for ten minutes each day. Fish behaviour and fish location in the water column were compared between treatment and controls using Generalized linear Mixed Models (GLMMs). The results showed alteration in swimming patterns, an increase in aggression, an increase in abnormal behaviours and other stress-related behaviours, such as gasping for air, provoked by acephate and methamidophos. Similarly, fish in the treatment group preferred to swim near the surface of the water. These behavioural alterations caused by acephate and methamidophos may have serious consequences to wild fish communities, such as an increased risk of predation and may contribute to the extinction of local populations. These findings highlight the usefulness of behaviour as a tool to assess environmental impact caused by pesticides.
Keywords: pesticide, fish, behavioural alterations, water pollutant.

Resumo

Avaliação da toxicidade do pesticida acefato e seu sub-produto metamidofós sobre o comportamento do zebrafish *Danio reio* (Cypriniformes, Cyprinidae).

O Brasil é um dos lideres mundiais no uso de agrotóxicos, sendo o acefato o quinto agrotóxico mais utilizado no país. Este componente e seu subproduto metamidofós podem causar efeitos deletérios na fisiologia e no comportamento de vários animais quando em contato com a água. Estudos que investigaram os impactos negativos do acefato em animais têm usado larvas de peixe-zebra como espécie-modelo, mas o efeito em indivíduos adultos ainda não está claro. Nosso principal objetivo foi avaliar as mudanças comportamentais em peixes-zebra adultos (*Danio rerio*) expostos a acefato e metamidofós. Para tanto, 160 indivíduos de zebrafish foram divididos em três grupos: acefato, metamidofós (expostos) e controles (não expostos), onde os indivíduos dos grupos de tratamento foram expostos a 7 µg/L de acefato e 7 µg/L de metamidofós por 28 dias. Para comparar os grupos, foram realizadas avaliações comportamentais por meio do método scan sampling com registros instantâneos do comportamento a cada 30 segundos, durante dez minutos a cada dia. O comportamento dos peixes e a localização dos peixes na coluna d'água foram comparados entre os tratamentos e controles usando Modelos Mistos Lineares Generalizados (GLMMs). Os resultados mostraram alteração nos padrões de natação, aumento da agressividade, aumento de comportamentos anormais e outros comportamentos relacionados ao estresse, como pipping, provocada por acefato e metamidofós. Da mesma forma, os peixes do grupo de tratamento preferiram nadar perto da superfície da água. Estas alterações comportamentais causadas pelo acefato e metamidofós podem ter consequências graves para as comunidades de peixes selvagens, tais como um risco aumentado de predação e podem contribuir para a extinção das populações locais. Esses resultados destacam a utilidade do comportamento como ferramenta para avaliar o impacto ambiental causado por agrotóxicos.
Palavras-chave: pesticida, peixes, alterações comportamentais, poluentes hídricos.

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1. Introduction

Pesticides are substances whose products either physical, chemical, or biological, are used in agriculture to eliminate pests (i.e., usually insects) or diseases considered harmful to production in natural, urban and domestic environments (JABLONSKI, 2021). Despite being an efficient way to combat pests, its use may have harmful effects to the environment as several of the active principles used in pesticides composition have high toxicity and can contaminate the environment and natural resources, such as soil, water, fauna and flora (MATIAS et al., 2021; STEFFEN; STEFFEN; ANTONIOLLI, 2011). Furthermore, the use of pesticides can also be toxic to non-target organisms causing metabolic changes, genetic damage, teratology or even being lethal (SOLIMAN; MEHANA, 2015).

Brazil is one of the largest agricultural commodities producers in the world and is also considered the country with the highest consumption of pesticides. Including the commercialization of substances such as acephate, atrazine, and fipronil, which are banned in the USA and European countries (BRAGA et al., 2020; FRIEDRICH et al., 2021). Nevertheless, many of the detrimental effects of the usage of pesticides in the long term are still unknown (MARQUES; SILVA, 2021; MATIAS et al., 2021).

Among the different pesticides used in the Brazilian agriculture, acephate is the fifth most commonly used against pest insects/mites and well-known substance and has a high likelihood of being detected in environmental contamination after its use (ANVISA, 2015; FRIEDRICH et al., 2021; MATIAS et al., 2021). Acephate is part of the organophosphates group and belongs to systemic insecticides and acaricides of contact and ingestion class, whose half-life, when in soil, can vary from 1.5 to 13 days (HAN et al., 2009). Its primary function is the inhibition of acetylcholinesterase so that the accumulation of acetylcholine in the synaptic clefts changes the motor functions in insects and mites (COSTA-SILVA et al., 2015; LIU et al., 2018; VIRGENS; CASTRO; CRUZ, 2015).

Methamidophos is a by-product resulting from the contact of acephate with water, and despite being banned be the National Sanitary Agency in Brazil (ANVISA) since 2011 (ANVISA, 2023), is still among the most detected pesticides in water and soil samples in Brazil (KUMAR et al., 2015; MARQUES; SILVA, 2021). Methamidophos is considered a systemic insecticide-acaricide, acting on the oral apparatus of insects and mites, preventing the consumption of food through the inhibition of acetylcholinesterase in the cholinergic receptors of the peripheral and central nervous system (FARAG et al., 2012). This pesticide is classified as level 1 in terms of toxicity, being considered highly toxic when ingested, absorbed through the skin, or inhaled (ALBITER LÓPEZ et al., 2020).

Studies conducted in rats *Rattus norvegicus* (Berkenhout, 1769) demonstrated that methamidophos can also cause harmful effects on spermatogenesis, altering sperm motility and viability (VENTURA et al., 2016). In addition, the substance can inhibit acetylcholinesterase in synaptic clefts, causing changes in motor skills, reduced food consumption, which results in weight loss. Furthermore, it can cause cholinergic toxicity during pregnancy, resulting in foetus malformation (FARAG et al., 2012).

Another concerning feature of acephate and methamidophos is their tendency to accumulate in water bodies. Different from other pesticides, these substances cannot be appropriately removed through traditional treatments of effluents (COSTA, 2017; MENEZES et al., 2021). Both acephate and methamidophos are highly soluble in water, which may contaminate groundwater and soil, and are easily absorbed, potentially causing bioaccumulation (MOHAPATRA et al., 2011; LIN et al., 2020). In addition, these pesticides can contaminate aquatic ecosystems through rainfall, runoff or even atmospheric deposition (ARJMANDI; TAVAKOL; SHAYEGHI, 2010).

Several studies have evaluated the impacts of acephate on distinct organisms of the aquatic community, including the microcrustacean species *Daphnia magna* (Straus, 1820); which demonstrated that acephate can cause immobility by inhibition of acetylcholinesterase when present in concentrations as low as 100µM (PRINTES; CALLAGHAN, 2004). When exposed to acephate, tadpoles of the species *Bufo melanostictus* (Schneider, 1799) showed teratology (i.e. anatomical deformations), reduced food intake, skin discolouration, and skin detachment in concentrations higher than 0.1 µg/L (GHODAGERI; PANCHARATNA, 2011). Individuals of the Congolli fish *Pseudaphritis urvillii* (Valenciennes, 1832), when exposed to acephate at concentrations of 0.7 to 4.4mg/L and above 0.2mg/L, presented low concentrations of acetylcholinesterase in the brain and muscles, demonstrating that, even at low concentrations, possibly lethal effects can occur (DAVIES; COOK; GOENARSO, 1994; KUMAR et al., 2021). Similarly, methamidophos has shown to be lethal (LC50) in 25-51 mg/L exposure in rainbow trout *Oncorunchus mykiss* (Walbaum,1792), 46 mg/L exposure in guppies *Poecilia reticulata* (W. Peters,1859), and 100 mg/L exposure in carp *Cyprinus carpio* (Linnaeus, 1758) (ALBITER LÓPEZ et al., 2020).

Aquatic fauna can be exposed to acephate in various ways, including skin contact, direct absorption in a contaminated environment, by inhalation through the gills, ingestion of contaminated water or the ingestion of contaminated food or prey (SOLIMAN; MEHANA, 2015). Thus, this contaminant can be observed at all levels of the trophic chain, impacting producers to tertiary consumers, and may even affect humans. To identify those effects, organisms known as bioindicators are normally used (AMÉRICO et al., 2015; VAN DEN BRINK; MANN, 2011).

Bioindicators are organisms that exhibit noticeable responses to changes in the environment, making them an efficient tool to assessing environmental quality in a very representative and integrative way (SAHA et al., 2021). For pesticides, fish can be considered excellent bioindicators since the concentration of contaminants alters not only their physiology but also their behaviour, which enables early detection of contamination (UIEDA;BARRETTO, 1999; CARVALHO; ORSI et al., 2004; ERBE et al., 2011; PEREIRA NAVARRO LINS et al., 2017;). Additionally, freshwater fish make excellent models to studying the effects of contaminants, since their responses may mirror the potential consequence of bioaccumulation in vertebrates (PEREIRA NAVARRO LINS et al., 2017; PÉREZ-PARADA et al., 2018).

When exposed to toxic agents, freshwater fish may present easy to identify behavioural changes, such as the ones observed in a study using the pesticide carbendazim, where locomotion and foraging behaviours were reduced (ANDRADE et al., 2016). In additional studies, other behavioural variations were observed, such as the increase in the occurrence of behaviours that are classified as abnormal were observed such as swimming in circles, swimming in the same trajectory (e.g. front to back), swimming near the bottom of the tank, hitting the head on the glass of the aquarium or opening and closing the mouth frequently (ZABEGALOV et al., 2019). All these behavioural modifications are usually observed in fish under stress or caused by the influence of chemicals in the water, such as the pesticides acephate and methamidophos (KALUEFF et al., 2013).

Freshwater fish also change the way they use the water column when under stress, which is usually accompanied by the presence of specific stress-related behaviours (KALUEFF et al., 2013). For example, behaviours such as piping (fish gulping air from the surface of the aquarium) cause an increase in the use of water surface, while lethargy causes an increase in the use of the aquarium bottom (KALUEFF et al., 2013). The same may be observed with aggressiveness. Despite varying with fish species, the presence of acephate or methamidophos in the water would probably change aggressiveness due to increased lethargy and motor changes caused by the accumulation of acetylcholinesterase in the synaptic cleft, leading to a decrease in aggression (FILBY et al., 2010; OLIVEIRA, 2013).

Zebrafish *Danio rerio* (Hamilton 1822) is a teleost freshwater fish widely used as a biological model, mainly in genetic, medical and pharmacological studies (CHOI et al., 2021; TEAME et al., 2019). Additionally, the zebrafish is a small freshwater fish species, considered one of the most popular in aquarium trade worldwide (RIBAS; PIFERRER, 2014). Likewise, it is used as a model in ecotoxicology studies, which evaluates the effects of contaminants (such as pesticides and heavy metals), not only on the morphology but also on the behaviour of animal species (FITZGERALD et al., 2021). The species presents genetic similarities with humans, has a small size and rapid development, it is relatively low cost to maintain and presents good resistance to changes in the environment, which makes it a suitable model for the laboratory environment (LAWRENCE, 2007; TEAME et al., 2019).

The choice of an experimental model for a study not only relies on the survival of the model species but also on the level of resistance of the animal to a contaminant and the easiness of maintenance in the laboratory. Therefore, the zebrafish is a promising candidate for studying the impact of pesticide concentration on aquatic matrices (FITZGERALD et al., 2021).

Considering the negative effects that the pesticides acephate and its by-product methamidophos may have on aquatic communities, this study aimed to investigate their effects on the behaviour of zebrafish *Danio rerio*. Initial investigations (LIU et al., 2018) with the pesticides have indicated that zebrafish larvae were severely impacted by these pesticides, but studies related on adult zebrafish individuals are non-existent. To this end, we intend to investigate the effects of these pesticides on zebrafish behaviour testing the following hypotheses: (I) pesticides will influence the behaviour of zebrafish, as a response to the neurological actions caused by contact with pesticides. We predict that fish will become more lethargic, less aggressive and will exhibit more abnormal behaviours and piping; (II) the position of the fish in the water column will be affected by the presence of pesticides in the water. We predicted that fish will more frequently stay next to the water surface due to physiological effects caused by both pesticides and (III) the difference in behavioural effects will be noticeable between methamidophos and acephate treatments; we predict that due to the higher level of toxicity, acephate will cause more severe behavioural changes, unlike methamidophos, that have a milder level of toxicity and should cause less severe behavioural effects.

2. Materials and Methods

3.1 Study site and ethical statement

The study was conducted at the Toxicology Laboratory of the School of Pharmacy (EFAR) at the Federal University of Ouro Preto (UFOP), Minas Gerais, Brazil. This project was revised and approved by the Ethics Committee on the use of animals of the Federal University of Ouro Preto (CEUA) protocol nº 7061120319.

3.2 Fish Management routines

All fish used in the study were purchased from specialized stores. One Hundred and sixty adult fish were randomly distributed in eight tanks containing 4L of water with 20 individuals per tank. The tanks were submitted to cycles of 14 hours of illumination (light) for every 10 hours of darkness. The water used in the tanks were locally sourced tap water treated with a chlorine neutralizer for commercial use, following the instructions provided by the manufacturer. In order to keep the concentration of pesticides constant during the experiment, partial water changes were performed every two days, in which two litres of water were removed from the tanks and then replaced with treated water. The correction of pesticide concentration was also performed at each partial water change using dilutions from stock solutions for both pesticides.

Mechanical aeration was employed with the use of "hang-on" filters to maintain oxygen levels within the acceptable range; this parameter was measured weekly with the use of specialized tests for commercial tanks. The temperature was kept constant at 26ºC $\pm$ 2ºC with the aid of a heater and measured daily using an aquarium thermometer.

The fish were fed once a day with, Alcon commercial food®, composed of fish derivates, vitamins and minerals, except for the euthanasia days, where individuals did not feed. Euthanasia trough liquid nitrogen immersion occurred at the end of the 28 days of exposure.

3.3 Experimental protocol

Zebrafish exposure to acephate and methamidophos followed the recommendations of OCED protocol No. 305, “Guidelines of Testing of Chemicals – Bioaccumulation in Fish: Aqueous and Dietary Exposure”. The concentration of acephate and methamidophos were standardized according to the maximum dosage allowed by ANVISA, which is 7 µg/L for its use in water (ANVISA, 2023).

The zebrafish individuals were placed in eight tanks containing 4L of water (labelled according to treatment; Fig. 1), two tanks were set as control group, and other six were designated for the treatment groups (three for acephate and three for methamidophos), where the fish underwent an acclimatization period of two weeks, without the presence of chemicals. From a stock solution of 1mg/L of acephate and 1mg/L of methamidophos with acephate, since the Brazilian resolution RDC nº 1 January 14th from 2011 does not allow the use of methamidophos by itself, several dilutions of acephate and methamidophos at a concentration of 7µg/L were obtained to be added to the tanks. The exposure to the pesticides was performed for 28 days.

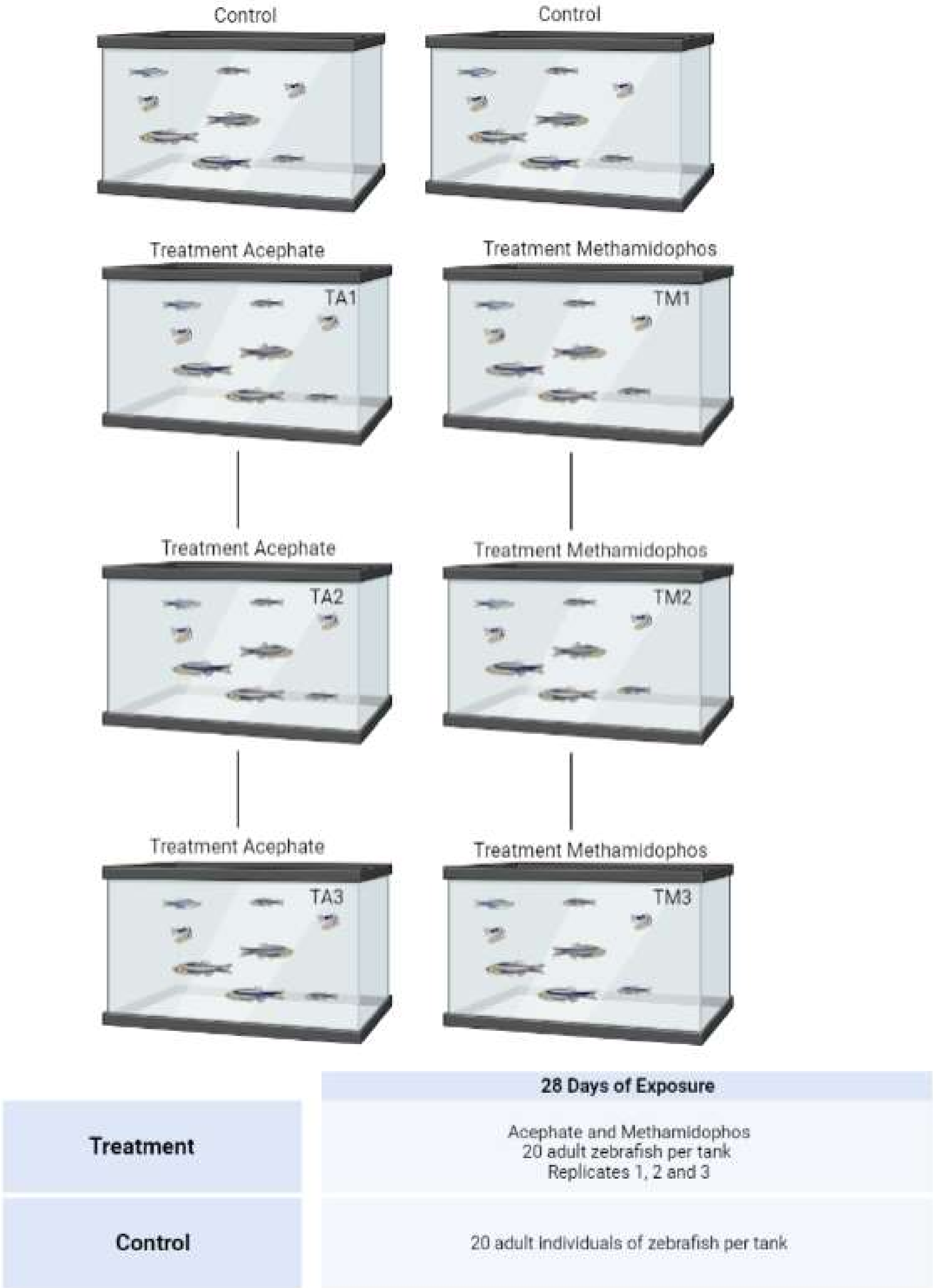

Figure 1: Representation of adult zebrafish exposed to acephate and methamidophos for 28 days as part of an experimental design (TA – Acephate treatment , TM – Methamidophos treatment).

3.4 Behaviour assessment

To assess the behaviour of zebrafish, an ethogram was elaborated based on published behavioural studies (KALUEFF et al., 2013; ORGER; DE POLAVIEJA, 2017) and 20h of preliminary *ad libitum* observations (Table 1).

Table 1: Ethogram used in the zebrafish (*Danio rerio*) study during exposure of acephate and methamidophos, created from a review of scientific bibliography and 20h of preliminary *ad libitum* observations (KALUEFF et al., 2013; ORGER; DE POLAVIEJA, 2017).

Behaviour – Acronym	Description
Active – AT	Includes the behaviours: a) Flutter: wave propagation through the body; b) reproductive behaviours such as cohort (pectoral fins display – fish opens to the maximum range, and swim side by side in circles, may display mouth-mouth touch and follow each other) and oviposition; c) Foraging: the act of looking for food in the aquarium; d) jumping out of the tank.
Aggression – AG	Include the behaviours: biting, attacking, chasing, and flicking (fish swim towards each other, touch mouth-to-mouth, and swim to the opposite side).
Eating – CO	Consumption of food provided
Cartesian Diving – CAR	Fish alternate between sinking and emerging vertically in the water column without moving their fins.
Abnormal Behaviour – ABN	Fish opening its mouth for too long or too often (grape and chatter, banging its head against the glass, shakes (weaver) or swims from side to side in the tank, in a circle or forming an 8.
Swimming abnormally – NA	Fish swim sideways, backwards, upside down, spiralling (corkscrew swimming) oy convulsing.
Piping – GAR	Fish gulping air from the surface of the aquarium.
Fleeing – FUG	Fish exhibit a rapid movement in the opposite direction of another fish or stimulus.
Freezing – FRE	Fish completely interrupts its movements, except for the eyes and operculum, while at the bottom of the tank.
Inactive – IN	Fish remains still but with eyes and pectoral fins movements.
Swimming slowly – ND	Slow swim where only the pectoral fins move (also known as creeping).
Swimming fast – NR	Dart: Rapid movement in one direction with the use of tail fin; Dash: Series of accelerations in dart style.

Abnormal Body Position – ABP	Droopy tail: Contortion of the fish`s body in an abnormal position, normally occurring over a long period (a motor phenotype different from the standard horizontal position of the fish body).
Not Visible – NV	Fish not visible to the observer.
Other behaviours – OUT	Behaviours not described in this ethogram

Fish behaviour was recorded using two cameras (Canon SX50 and Nikon D5600) mounted over a tripod facing the tanks. Daily observations were conducted precisely at 08:40 am each day, for 10 minutes, over the period of 28 days. Fish were recorded 30 minutes after feeding to avoid oversampling of this behaviour. Similarly, to avoid observer bias, the researcher only remained in the room to turn the equipment on and returned after 10 minutes to turn it off, approximately 1 minute of buffer time were also used.

Behaviour data from all tanks were recorded using scan sampling with instantaneous recordings of behaviour every 30 second (ALTMANN, 1974; MARTIN; BATESON, 2021). Additionally, the position of the fish in the water column (bottom, middle or surface) was also recorded every 30 seconds.

3.5 Statistical analysis

The behavioural data were compiled in Excel spreadsheets and investigated using descriptive statistics and results are reported as either percentages or means with standard deviation. Data were investigated for normality using the Anderson–Darling test and found to be non-parametric.

Generalized mixed linear models (GLMMs) were constructed to assess whether pesticide exposure modifies zebrafish behaviour. Pesticide concentration of 7µg/L and time of exposure to pesticides of the control and treatment groups were considered explanatory variables, while fish behaviour and position in the water column (bottom, middle and surface) were considered response variables. As random variable we used the day of exposure. An alpha of 95% ($p < 0.05$) was considered for statistical analysis.

All analysis were conducted using the software Rstudio (GLMMs, glmer for non-normal data with lme4 package); graphics were constructed with ggplot and ggplot2 (Bates et al.; 2015). Whenever necessary, ANOVA tests, with Tukey's post-hoc, were performed to test the variance between the experimental models. The Poisson distribution was used for all models, indicated by residue variance tests.

3. Results

3.1 Overall activity profiles of fish

Descriptive statistics were calculated for behavioural metrics and water column use from 4,480 minutes of video, for all treatments (TA: Acephate treatment, TM: Methamidophos treatment, and C: Control), and results are summarised in Tables 2 and 3.

Overall, in the control group, ND was observed more often (36.4%), followed by NR (16.81%), AG and FUG (12.51% and 12.54%), respectively. AT comprised 5.35% of the behavioural observations and other remaining behaviours were recorded less than 1% of the time (Table 2).

For the TA, the most observed behaviour was ND (26.53%), followed by AG (15.34%) and FUG (15.34%). The least displayed behaviours were IN and OUT that together contributed to less than 1% of behavioural observations (0.35% and 0.01%, respectively). The fish in this treatment also presented a high display of the abnormal behaviour GAR (14.88%) (Table 2).

Similarly, ND was the most recorded behaviour (30.91%) in the TM, and the fish in this treatment also had a higher recording of the GAR abnormal behaviour (11.23% of total exposure time). In addition, the behaviours that were less recorded in the group were AT (2.63%), ABP (0.98%), IN (0.27%), and NA (0.01%). Moreover, behaviours such as CO, OUT, and FREE were not displayed in this group (Table 2).

For the position of the fish in the water column; in the control group the fish stayed longer in the middle and bottom sectors of the tanks (36.70% and 36.72% respectively), compared to the surface (26.25%). On the contrary, in the TA treatment, they spent more time in the surface (44.03% of observations), than in the middle (28.65%) and the bottom (27.32%) of the water column (Table 3). The same change was observed for the TM group, with fish recorded using the surface in more than half of the observations (50.60%), when compared to the other areas (middle: 27.15% and bottom: 22.25%) (Table 3).

Table 2: Mean and standard deviation for each displayed behaviour by zebrafish *Danio rerio* exposed to the pesticides methamidophos (TM), acephate (TA), and control group (C) for 28 days.

Behaviour	TM (±SD)	TA (±SD)	C(±SD)
IN	1.07 ±3.02	1.38±3.33	0.03±0.18
CO	0.00 ±0.00	0.00±0.00	0.00±0.00
ND	122.68±29.71	106.10±52.16	144.44±57.47
NR	59.81±29.71	59.51±28.09	67.17±33.72
NA	0.03±0.18	0.06±0.31	0.00±0.00
AG	44.69±23.11	61.36±23.16	50.02±19.65
ABN	0.02±0.21	0.09±0.35	0.00±0.00
AT	10.44±12.39	9.73±11.58	20.97±18.84
FUG	44.66±23.01	61.34±23	50.11±19.47
NV	65.00±30.11	55.93±24.79	66.06±28.42
OUT	0.01±0.11	0.04±0.32	0.03±0.25
ABP	3.89±18.29	1.32±8.65	0.06±0.50
GAR	44.59±42.07	43.06±45.40	0.60±2.25

Table 3: Usage of water column by zebrafish *Danio rerio* exposed to the pesticides methamidophos (TM), acephate (TA), and control group (C) for 28 days.

Group	Time spent Surface (%)	Time spent Middle (%)	Time spent Bottom (%)
TM	50.60%	27.16%	22.25%
TA	44.03%	28.65%	27.32%
C	26.57%	36.70%	36.72%

Behavioural category “other behaviours" and the behaviour "eating" were excluded from further analysis as they were registered with a low frequency (<0.01%). For all the other behaviours, changes in behaviour between control and treatment were found to be significant as reported in the GLMM models (Table 4).

Table 4: Generalized Linear Mixed Models (GLMMs) results describing the variation on behaviours presented by the individuals of zebrafish *Danio rerio*, according to condition (control x treatment), exposure, and number of individuals distributed between surface, middle and bottom of the water column.

Dependent Variable	DF	AIC	Distribution	Deviance	p-Value
Inactive – IN	3	975.1	Poisson	971.2	<9.395e-07
Swimming slowly – ND	5	6036.2	Poisson	6022.2	<2.2e - 16
Swimming fast – NR	3	3927.6	Poisson	3917.6	<2.2e - 16
Abnormal behaviour - ABN	3	90.2	Poisson	80.2	0.008322
Aggression – AG	5	3847.2	Poisson	3833.2	<2.2e - 16
Freezing – FRE	3	64.7	Poisson	54.7	<2.2e - 16
Active – AT	5	2440.8	Poisson	2426.8	<2.2e - 16
Fleeing - FUG	5	3810.6	Poisson	3796.6	<2.2e - 16
Abnormal Body Position – ABP	3	3010.4	Poisson	3000.4	4.986e-16
Piping – GAR	3	6408	Poisson	6398	<2.2e - 16
Other behaviours – OUT	3	71.1	Poisson	61.1	0.04455
Swimming abnormally -NA	3	84.2	Poisson	74.198	0.09165
Bottom	5	4730.5	Poisson	4716.5	3.436e-16
Middle	5	3462	Poisson	3448	7.424e-13
Surface	5	5688.1	Poisson	5674.1	<2.2e - 16

Display of “inactive” behaviour was observed to be higher on the acephate treatment (TA) when compared to the control (C) and methamidophos (TM) groups and it was the least observed behaviour in the control (C) group (Figure 2).

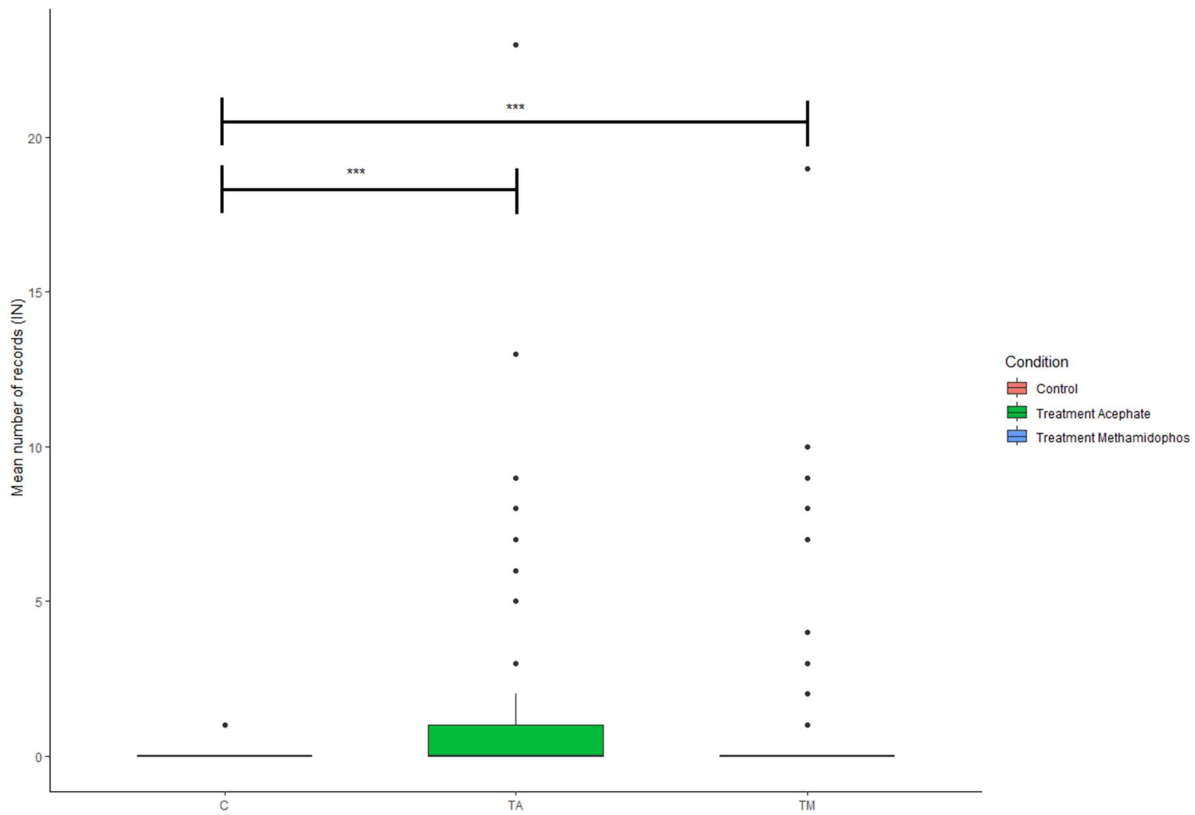

Figure 2: Mean record of “Inactive” behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (acephate treatment - TA x methamidophos treatment - TM x control – C) during the exposure period of 28 days. Tick line represent median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

The "swimming slowly" behaviour was widely exhibited during the experiment (Figure 3). However, in the control group, this behaviour was more frequently recorded when compared to the other groups. When compared the two treatments (TA and TM), it is possible to observe this behaviour was more often seen in the TM treatment (Figure 3).

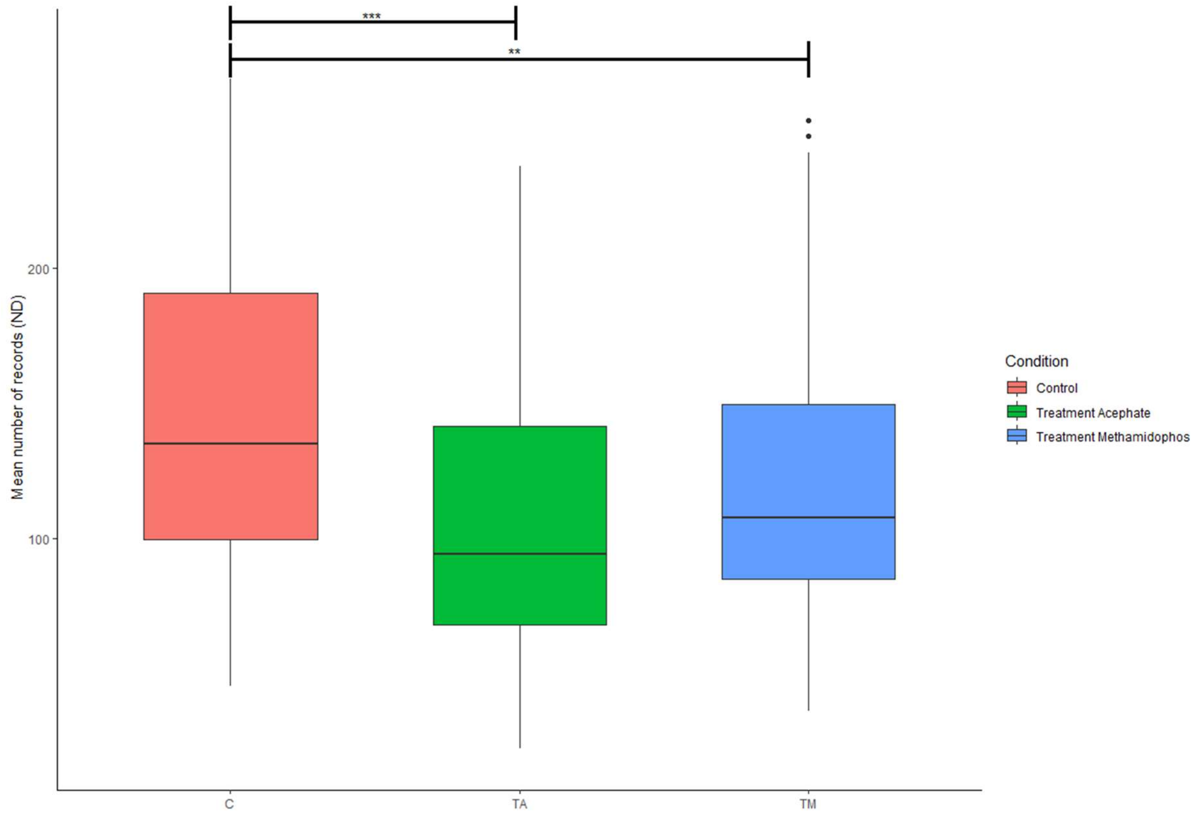

Figure 3: Mean record of “swimming slowly” behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (Acephate treatment - TA x Methamidophos treatment - TM x Control – C) during the exposure period of 28 days. Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

“Swimming fast” behaviour was also displayed in both treatments and control group. Nevertheless, the control group obtained the highest number of records for this behaviour when compared to TM and TA groups (Figure 4).

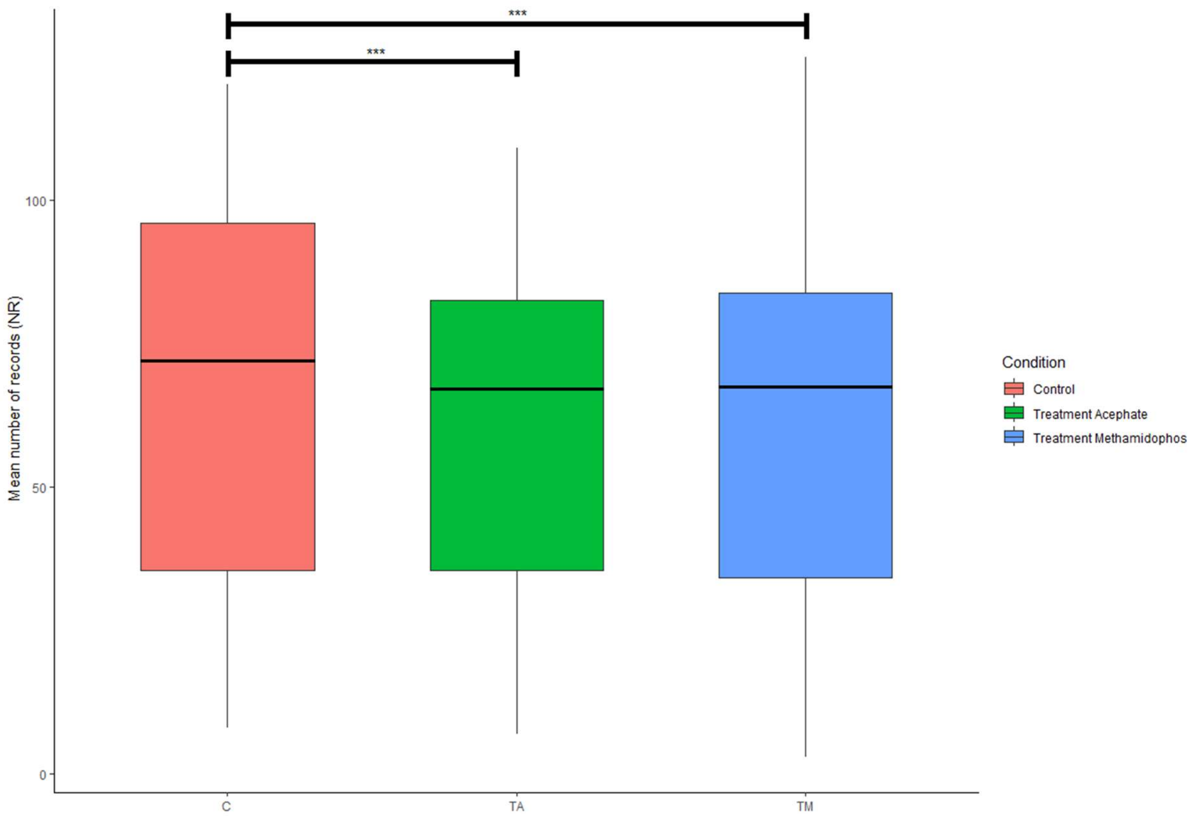

Figure 4: Mean record of "swimming fast" behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (Acephate treatment - TA x Methamidophos treatment - TM x Control – C) during the exposure period of 28 days. Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

Although not often recorded, the "abnormal behaviours – ABN" were more observed in the TA and TM groups respectively, but not recorded in the control group.

The "aggressive" behaviour was registered in all groups. However, the TA had the highest number of records compared to the other groups, followed by C and TM (Figure 5).

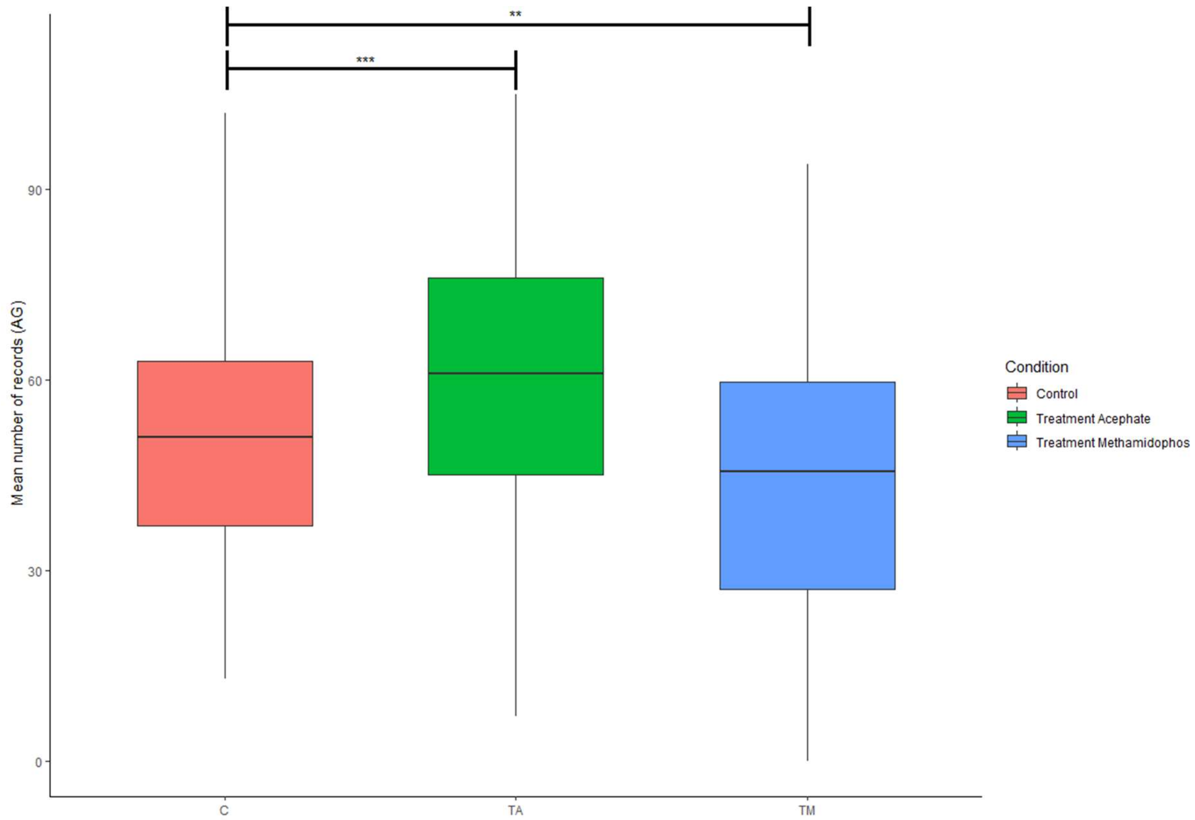

Figure 5: Mean record of “Aggressive” behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (Acephate treatment - TA x methamidophos treatment - TM x Control – C) during the exposure period of 28 days. Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

For the “active” behaviour, when comparing the TA and TM groups to the Control, was recorded more often in TM than in TA treatment. However, none of the other groups surpassed the number of records observed in the C group (Figure 6).

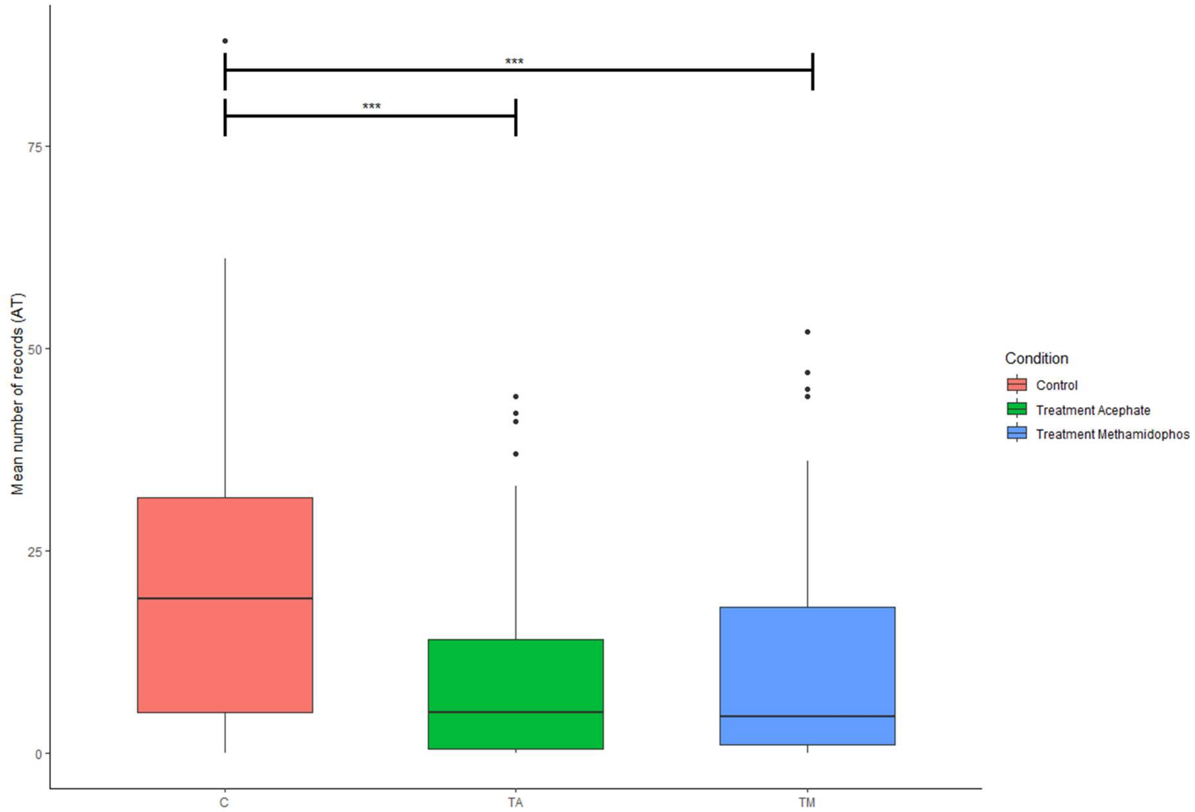

Figure 6: Mean record of “Active” behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (Acephate treatment - TA x Methamidophos treatment - TM x Control – C) during the exposure period of 28 days. Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

As for the "fleeing" behaviour, it is noticeable that a similar pattern to the "aggressive" behaviour was found. We recorded at least one fleeing individual for each fish that displayed aggressive behaviour. Thus, this behaviour was more often recorded in the TA treatment than in the C or TM conditions (Figure 7).

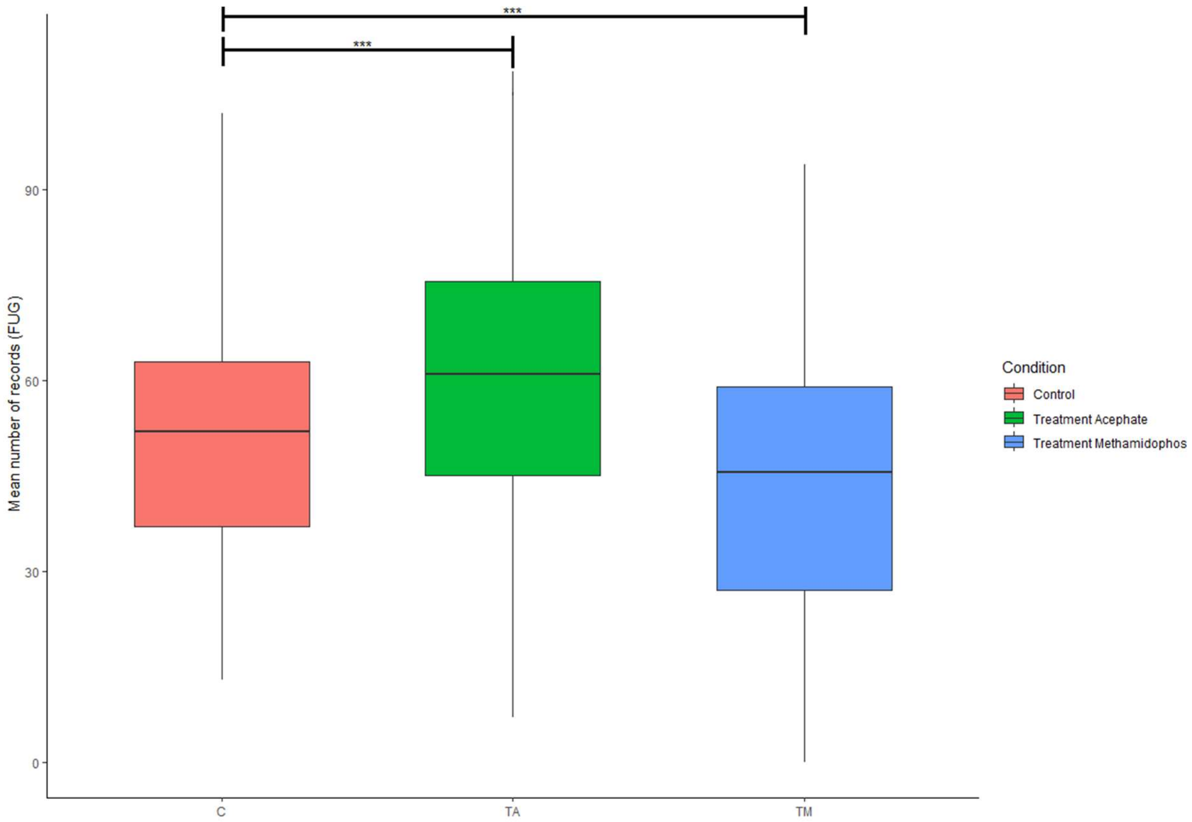

Figure 7: Mean record of "Fleeing" behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (Acephate treatment - TA x Methamidophos treatment - TM x Control – C) during the exposure period of 28 days. Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

The "abnormal body position" behaviour varied during the experiment. However, when comparing the groups, more records of this behaviour were seen in TA and TM than in the control.

During the study, the "piping" behaviour was also observed, and it was registered more frequently in the treatment groups, as evidenced by comparing TA and TM with C (Figure 8).

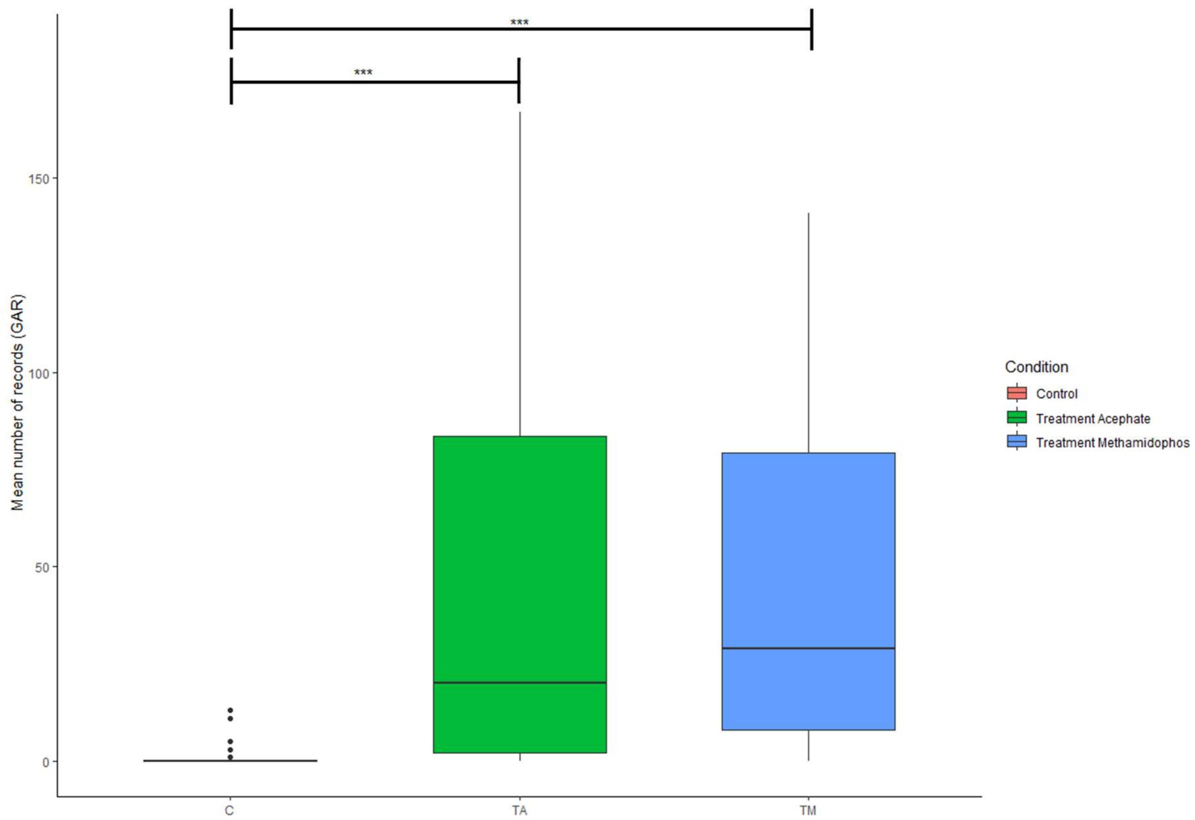

Figure 8: Mean record of “piping” behaviour displayed by individuals of the species *Danio rerio* in the different tested conditions (Acephate treatment - TA x Methamidophos treatment - TM x Control – C) during the exposure period of 28 days. Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

During the study, “freezing” behaviour was only observed a few times, only in TA and control groups. This behaviour was significantly more expressed by fish exposed to acephate. For all the remaining behaviours, no significant differences were found between the treatment and control groups.

The presence of pesticides in the water altered the use of the water column by the individuals. For the control group, during the 28 days, animals spent more time at the bottom of the aquarium than the surface. For the treatment groups, regardless of the type of the pesticide, all individuals started to use the surface of the aquarium more often as the time of exposure increased (Figures 9 to 11).

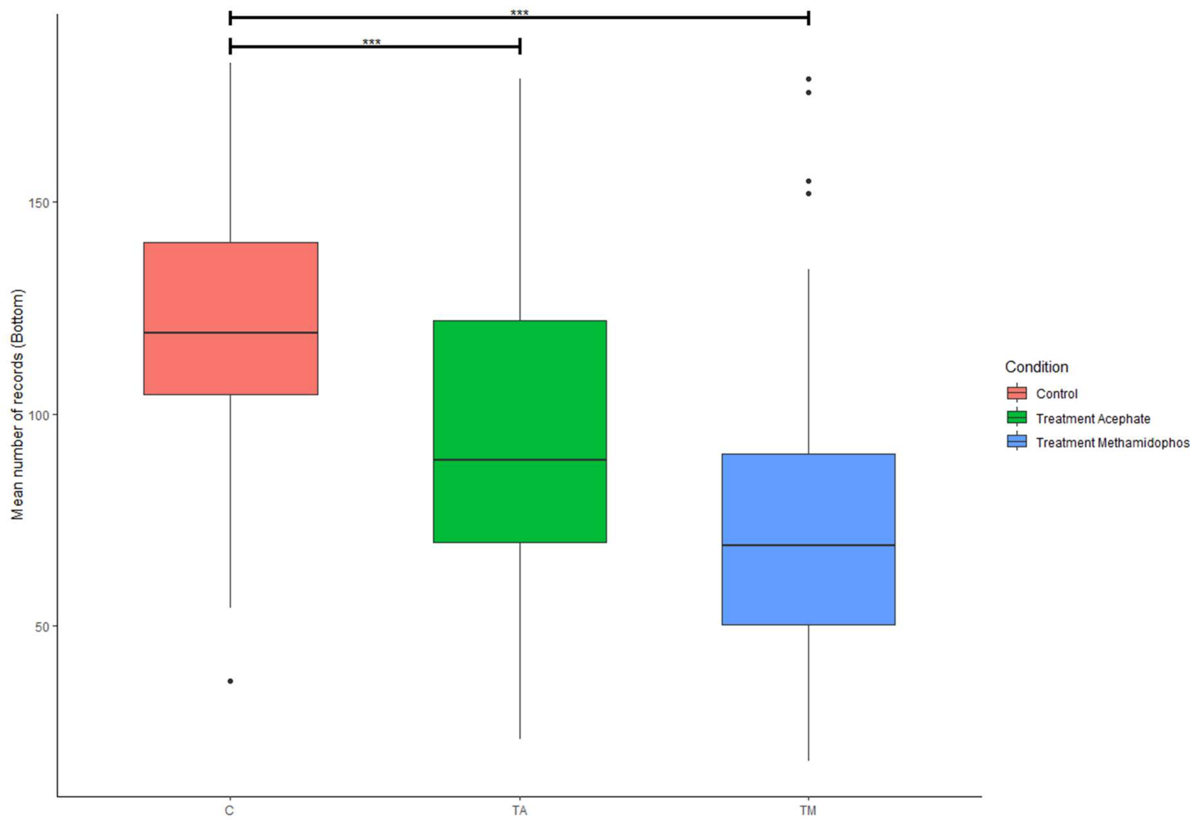

Figure 9: Mean number of records for individuals of the species *Danio rerio* observed using the bottom of the water column in each tested condition for a period of 28 days (Acephate treatment - TA x Methamidophos treatment - TM x Control – C). Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

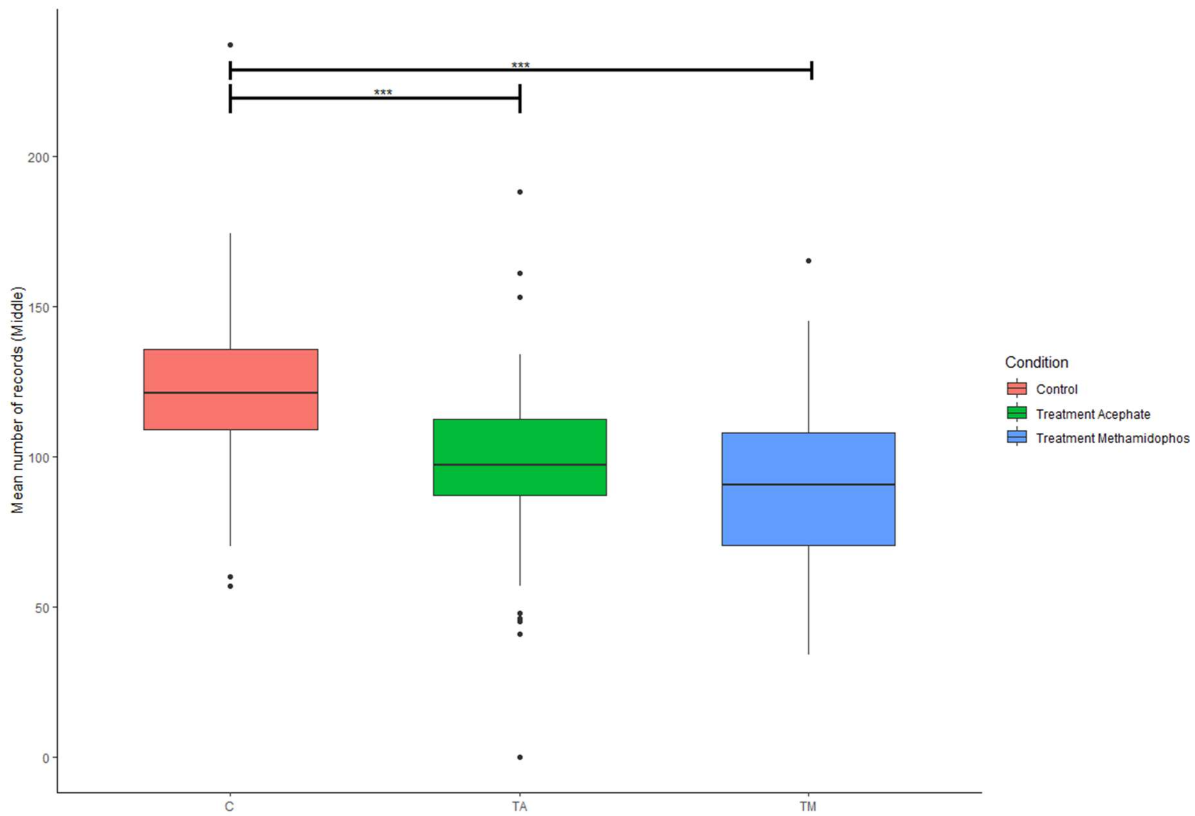

Figure 10: Mean number of records for individuals of the species *Danio rerio* observed using the middle of the water column in each tested condition for a period of 28 days (Acephate treatment - TA x Methamidophos treatment - TM x Control – C). Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

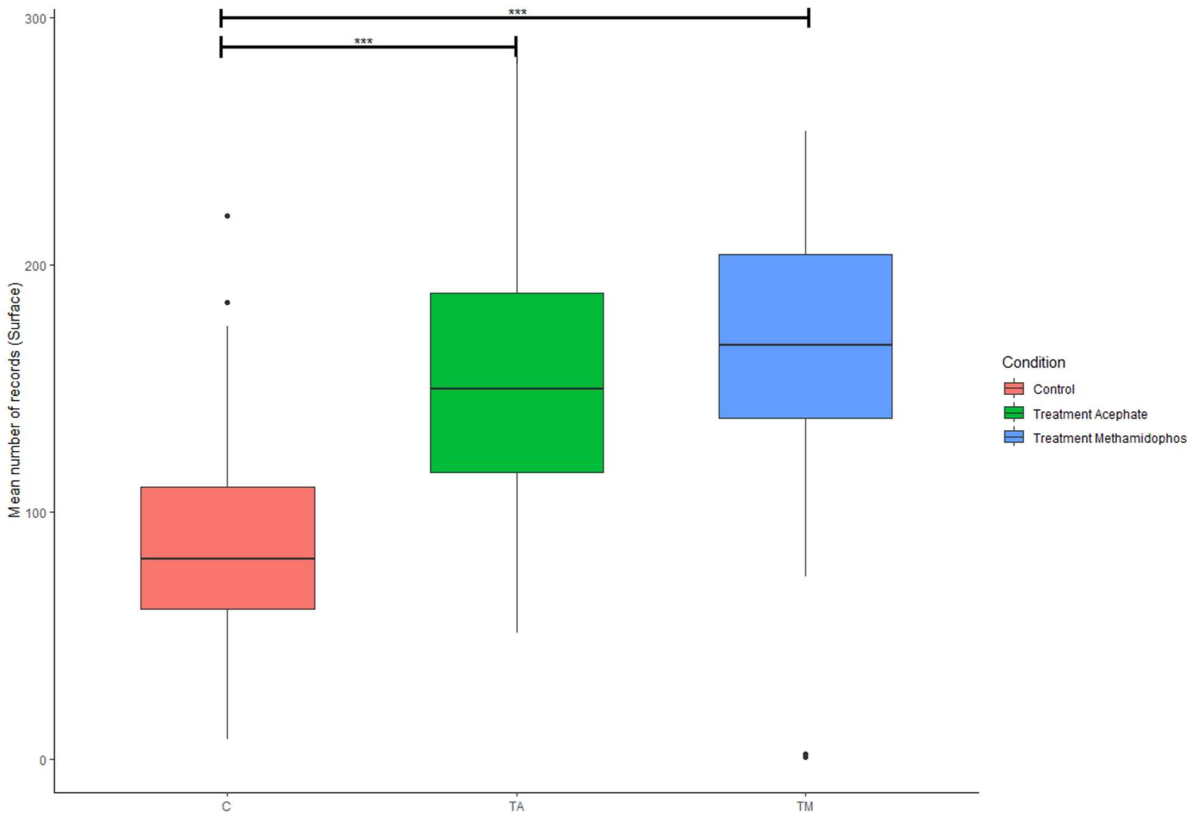

Figure 11: Mean number of records for individuals of the species *Danio rerio* observed using the surface of the water column in each tested condition for a period of 28 days (Acephate treatment - TA x Methamidophos treatment - TM x Control – C). Tick line represents median, whiskers show maximum and minimum values, box represents interquartile ranges. ** indicates significance value where p = 0.01; *** indicates significance value where p = 0.001.

4. Discussion

The exposure of adult zebrafish to acephate and its by-product, methamidophos caused behavioural alterations in the studied population. Our results corroborate the initial hypotheses that pesticide exposure would affect zebrafish behaviour, reducing activity levels, increasing lethargy, causing an increase in abnormal behaviours and piping, and changing the water column use.

Although acephate and methamidophos possess different toxicity levels, our study revealed that both have similar impacts on the behaviour of adult zebrafish, not corroborating our initial hypothesis. In the present study, acephate and methamidophos caused alterations in behaviours related to swimming activity. When in the presence of pesticides, although swimming alternate between fast and slow swimming, zebrafish decreased their overall swimming activity, becoming more inactive and lethargic; a similar response was also observed in the swimming capacity of zebrafish and serape tetra *Hyphessobrycon eques* (Steindachner, 1882) in contact with acephate (MOREIRA et al., 2021). Similarly, when exposed to acephate (<10mgL), zebrafish larvae decreased locomotion activity, as acephate inhibited acetylcholinesterase action on the synaptic clefts (LIU et al., 2018).

The behaviour “inactive” was exhibited more frequently by groups exposed to acephate; this behaviour can be related to stress markers and acetylcholinesterase inhibition. The acetylcholinesterase inhibition can directly affect the synaptic clefts on muscle tissue, causing acetylcholine to accumulate on those areas and a inhibition on the muscle activity and swimming activity paralysis, causing an increase in inactivity (COSTA-SILVA et al., 2015). The increased records of “active” on the control groups further corroborate that acephate and methamidophos impair overall activity on adult zebrafish.

Abnormal behaviours were more frequently detected in the fish exposed to acephate and methamidophos. Among those, swimming in circles at the bottom of the aquarium and weavers were more frequent displayed by the individuals. This behavioural modification can also be related to acetylcholinesterase inhibition on the synaptic clefts in the muscle tissue, as the accumulated acetylcholine in these regions can cause hyperactivity, coordination lost, seizures e paralysis (ZHANG et al., 2017). In addition, the “abnormal body position” was also observed more often on these groups. This behaviour can be a result of alterations on neurological phenotype of the zebrafish caused by pesticide exposure resulting in morphological alterations in the brain and subsequent cognitive problems (KALUEFF et al., 2013).

The “abnormal swimming” behaviour was only observed in the treatment groups. When exposed to pesticides, adult zebrafish suffer with an increase in oxidative stress, and with neurological alterations as result of acetylcholinesterase inhibition (CHEN et al., 2021; WU et al., 2021b). Additionally, “abnormal swimming”, includes alterations on the swimming behaviour through the loss of motor skills, which can be explained by the inhibition of neurotransmitter acetylcholine.

The "freezing" behaviour, a complete cessation of the fish activity, except for eyes and breathing, could be considered the last stage of contamination caused by acephate and methamidophos. However, only a physiologic and behavioural-associated study can reach this conclusion. In adult zebrafish, when exposed to fipronil (WU et al., 2021a, 2021b), an increase in oxidative stress, inflammation and apoptosis of cerebral tissue cells was observed, which could lead to an increase in "freezing" behaviour. Evaluating the impacts caused by both pesticides in cerebral tissue could be a practical approach to understanding the increase in fish's "freezing" behaviour.

For the present study, the "aggressive" behaviour was registered more often in the treatment groups when compared to the control, which does not corroborate the results found by other authors that evaluated aggressiveness associated with organophosphate pesticides in aquatic environments and our initial hypothesis. Most studies observed a decrease in the aggressiveness of fish, not only zebrafish but also other species, such as *Astyanax altiparanae* (Garutti & Britski, 2000), popularly known as tetra (CRUZ, 2002). The “fleeing” behaviour was also seen more often in treatment groups, as for every individual that displays aggressive behaviour, we could also register a fleeing one (DAHLBOM et al., 2011), observed that zebrafish are indeed a very territorial fish and boldness is a dominance related behaviour commonly seen in zebrafish. This decrease in aggression is often associated with the necessity to alter the use of energy in order to return to a homeostatic state, and the need to perform more essential behaviours, such as inactive, swimming fast and swimming slow (BURTON; BURTON, 2018; JACQUIN et al., 2020; KRISTIANSEN et al., 2020). In the present study, aggressiveness increased in the treatments, being more exhibited by fish exposed to acephate. NUNES et al. (2020) observed in his study that when exposed to permethrin in the larval stage, adult zebrafish presented an increase in aggressiveness parameters caused by the pesticide's neurotoxic effects, which can also be observed in acephate and methamidophos, as all of them cause motor dysfunction. Additionally, as a response to the stress caused by the presence of the pesticides, and as the fish were captive without a fleeing chance, this could have explained the increase in the aggressiveness observed in this study.

The "piping" behaviour is usually related to a stress response from the fish when exposed to pesticides that inhibit acetylcholinesterase (ULLAH, 2015), such as acephate and methamidophos. Additionally, due to acetylcholinesterase inhibition, we can observe decreased operculum movement and oxygen absorption by the gills (ZHANG et al., 2017). Therefore, the fish reach the water's surface to perform the piping behaviour to supply the oxygen necessities. The observations of pipping behaviour in this study corroborates the predictions on hypothesis one, where the presence of both pesticides increases the display of abnormal behaviours and gasping for air and the previous results found in the literature.

The water column usage by the zebrafish individuals was also affected by pesticide exposure. During the exposure period, the treatment groups (TM and TA) showed a higher frequency of recordings of the usage of the surface area in their tanks; as for the control group, a higher use of the middle and the bottom of the tanks were observed. Therefore, we can conclude that when under the presence of acephate and methamidophos, adult zebrafish increase the expression of the “piping” behaviour and shift the usage of the water column to allow more use of the surface. Similar results have been found with other fish species such as carp (*C. carpio*) when exposed to chlorpyrifos (HALAPPA; DAVID, 2009), and rohu carp *Labeo rohita* (Hamilton, 1822) and barbel fish *Tor putitora* (Hamilton, 1822) when exposed to cypermethrin (MARIGOUDAR; AHMED; DAVID, 2009; ULLAH, 2015). Despite these pesticides belonging to different chemical classifications, the displayed behaviour, and the process behind it are very similar.

5. Conclusion

The results showed that acephate and its by-product methamidophos altered the behaviour of adult zebrafish individuals, increasing the recording of abnormal and stress related behaviours. Also, the presence of the pesticides altered the use of the water column by the zebrafish, with the tested population showing a tendency to stay next to the surface of the water. The increase on the fleeing behaviour could be related to an avoidance response, a typical behaviour observed in fish exposed to contaminated environment. As most of the behavioural changes observed could be related to changes in physiological parameters, it is possible that prolonged exposure to the pesticides would result in permanent damage to the biological functioning of the species. In a natural environment, the observed changes in behavioural patterns would severely affect the species and fish populations; the loss of swimming capacity and the need for more surface use would increase the chance of predation and neurological alterations could impair cognitive process and motility, further compromising the fish ability to survive, breed and migrate. These associated events, with time, could lead to a local extinction (i.e., extirpation) event related to the exposure to pesticides such as acephate and methamidophos. Studies investigating the effects of pesticides on fish behaviour highlight the usefulness of this method as an important bioindicator to the presence of chemicals in the aquatic environment and the potential effects of these substances on wild populations.

Due to acephate frequent used in Brazilian agriculture, it is important to conduct new studies concerning the effects of those pesticides in the Brazilian ichthyofauna and aquatic environment. This is especially significant as Brazil is home to one of the most diverse ichthyofauna's in the world. Finally, we highlighted the novelty of this study by evaluating the effects of these pesticides on the behaviour of adult zebrafish. This type of study helps in the fulfilment of gaps in ecotoxicological studies.

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