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Behavioral swimming effects and acetylcholinesterase activity changes in *Jenynsia multidentata* exposed to chlorpyrifos and cypermethrin individually and in mixtures

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ABSTRACT

The pesticides cypermethrin (CYP) and chlorpyrifos (CPF) were found together in water bodies located in agricultural and urban areas. However, the impact to non-target biota from exposure to mixtures has received little attention. In the current study, we evaluated changes in swimming behavior and cholinesterase enzymes activity in *Jenynsia multidentata,* to investigate the possible effects of these insecticides individually and in mixtures. Moreover, differences between technical and commercial mixtures of the pesticides were evaluated. Females of *J. multidentata* were exposed over 96-h to CYP (0.04 and 0.4 $\mu g\,L^{-1}$), CPF (0.4 and 4 $\mu g\,L^{-1}$), individually and in a technical and commercial mixtures. Swimming behavior was recorded after 24 h and 96 h of exposure. Also, we measured cholinesterase enzymes activity in brain and muscle after 96 h of exposure. Exposure to CYP increased the exploratory activity of *J. multidentata* in the upper area of the aquarium. Fish exposed to CPF (4 $\mu g\,L^{-1}$) showed a decrease in swimming activity and an increase in the time spent at the bottom of the aquarium. Interestingly, fish exposed to the technical and commercial mixture of CYP and CPF displayed a different behavior based on the concentration of exposure. Low concentration of pesticides elicited an increase in *J. multidentata* swimming activity with preference for the upper area of the aquarium, and high concentrations caused decrease in swimming activity with preference for the bottom area of the aquarium. Based on the response of cholinesterase enzymes, acetylcholinesterase in muscle was more sensitive to exposure to CYP, CPF and their mixtures than in brain. A decrease in swimming behavior correlates significantly with the inhibition of acetylcholinesterase activity in muscle of *J. multidentata* exposed to high concentrations of pesticides.

These results draw attention to the need of more studies on the potential ecotoxicological impact of pesticides and its mixtures at environmental relevant concentrations.

1. Introduction

Cypermethrin (CYP) and chlorpyrifos (CPF) are two insecticides widely used both in households and in farming activities. These compounds can be applied individually or combined in order to maximize the performance of pest treatments in crops (CASAFE 2013/2015).

The presence of pesticide mixtures in natural environments is highly likely due to the current interest in the use of active ingredients combinations and the simultaneous implementation of different plant protection products in crops grown near a river (Brodeur et al., 2014). Studies conducted in bodies of water located in agricultural and urban areas found concentrations of CYP and CPF above the limits established by CCME (2012) and SRHN (2003) for the protection of aquatic biota (Bonansea et al., 2013; Jergentz et al., 2005; Marino and Ronco, 2005). However, more studies discussing the direct effect of mixtures of pesticides on non-target biota are needed, given that they are commonly applied

Abbreviations: 96-h LC_{50}, 96-h medium lethal concentrations; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChEs, cholinesterases; CPF, chlorpyrifos; CYP, cypermethrin; CYP+CPF, cypermethrin plus chlorpyrifos technical mixture; PRODUCT, cypermethrin plus chlorpyrifos mixture of a commercial products; SPE–SPME–GC–MS, solid phase extraction-solid phase microextraction-gas chromatography coupled to Mass Spectrometry

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simultaneously, and that this information is more environmentally relevant (Shinn et al., 2015).

Using fish as an experimental model helps to assess the toxicity of these compounds on aquatic organisms, allowing the early detection of the effect of pollutants. The native fish *Jenynsia multidentata* (Cyprinodontiformes, Anablepidae) is considered to be a good experimental model in biology and toxicology (Molero and Pisanó, 1987). It is a small (about 4 cm), freshwater, viviparous species with external sexual dimorphism. It is also easy to transport and keep in laboratory conditions. This fish species has been successfully used to evaluate the effects of lindane, glyphosate and endosulfan on different biological processes (Ballesteros et al., 2009b; Hued et al., 2012; Monserrat et al., 2014; Pesce et al. 2008).

Changes in behavior are among the most widely used biomarkers at individual level. This is mainly because this response integrates many cellular processes, which are essential to the viability of the organism, the population and the community. Therefore, observations of behavioral changes provide with a unique toxicological perspective, linking both biochemical and ecological consequences of environmental pollution (Little and Finger, 1990). Different investigations have demonstrated that pesticides exposure alters the swimming behavior on fishes (Ballesteros et al., 2009a; Khalil et al., 2013). However, little is known about the behavioral effects of its mixtures (Kumar et al., 2007).

On the other hand, measurement of the activity of cholinesterases (ChEs) is frequently studied as a sensitive biomarker of organophosphate and carbamate pesticides exposure. Cholinesterases can be initially distinguished by their specificity in relation to a substrate. Acetylcholinesterase (AChE) catalyses the hydrolyzes of the neurotransmitter acetylcholine, while butyrylcholinesterase (BChE) mainly hydrolyzes butyrylcholine (Rodriguez-Fuentes et al., 2013). AChE hydrolyzes acetylcholine in the cholinergic synapses, completing the communication process at this level. In consequence, its inhibition results in an accumulation of acetylcholine within the synaptic space, leading to overstimulation of cholinergic receptors followed by depression or paralysis and eventual death. Although its physiological function is unknown, BChE has a protective role participating in the elimination of anticholinesterasic compounds, such as organophosphate and carbamate pesticides, preventing these compounds from acting on AChE (Yang et al., 2013). Membrane AChE is the molecule with cholinergic activity in the nervous system, and the soluble or extracellular form of BChE has the main non-cholinergic function (Schegg et al., 1992). It has been shown that several pesticides can cause inhibition of cholinesterase activity in non-target organisms as fishes (Botte et al., 2012; Kumar et al., 2009; Sandrini et al., 2013). Some laboratory studies showed that the inhibition of AChE in fish can be associated with alterations in their swimming behavior, as is the case of *J. multidentata* exposed to sublethal concentrations of endosulfan, and *Gambussia affinis* exposed to chlorpyrifos (Ballesteros et al., 2009a; Rao et al. 2005).

The aim of this study was to assess changes in swimming behavior and cholinesterase enzymes activity in *Jenynsia multidentata* exposed to cypermethrin and chlorpyrifos, to discern the possible effects of these insecticides individually and in mixtures. Moreover, differences between technical and commercial mixtures of the pesticides were evaluated.

2. Materials and methods

2.1. Chemicals

Cypermethrin (99%) and CPF (98%) reference standards were purchased from Sigma-Aldrich (USA). Also a commercial formulation of CYP 25% (Glacoxan, Punch Química S.A., Argentina) and CPF 40% (Clorfox, GLEBA S.A., Argentina) were used.

2.2. Fish

Female adults of *J. multidentata* were captured by a backpack electrofisher equipment from an unpolluted area of Yuspe river, Córdoba (Bistoni and Hued, 2002). Fish were transported to the laboratory and acclimated to laboratory conditions for 2 weeks previous to the experiments. They were maintained in a temperature controlled room at 21 ± 2 °C and 12 h:12 h light/dark photoperiod.

2.3. Experiments

All experiments were conducted in 3 L glass aquarium (1 fish per liter) containing aquarium prepared water (distilled water containing 100 mg L^{-1} sea salt, 200 mg L^{-1} $CaCl_2$, and 103 mg L^{-1} $NaHCO_3$; Best et al., 2002). Aquarium water was maintained at temperature 23.5 ± 0.1 °C, pH 7.92 ± 0.06, dissolved oxygen 6.73 ± 0.09 mg L^{-1} and conductivity 948 ± 33 µS cm^{-1}. Fish were fed *ad libitum* once a day with commercial fish pellets (TetraMin, USA) and the remainder food was removed after feeding. Pesticides were added, dissolved in acetone. Acetone was also added to control groups.The final concentration of dissolvent was the same in all the treatments and was always lower than 0.05%. Exposure medium was renewed daily during the assay. Each exposure was carried out three times, meaning 9 specimens by condition.

2.4. Experimental design

After acclimatization period, fish (mean standard length: 4.2 ± 0.5 cm and mean body weight: 0.8 ± 0.3 g) were exposed over 96 h to low and high concentrations of pesticides as follow: 0.04 and 0.4 µg L^{-1} of CYP; 0.4 and 4 µg L^{-1} of CPF; 0.04 µg L^{-1} CYP + 0.4 µg L^{-1} CPF and 0.4 µg L^{-1} CYP+4 µg L^{-1} CPF in a technical mixture (CYP+CPF); as well as 0.04 µg L^{-1} CYP+0.4 µg L^{-1} CPF and 0.4 µg L^{-1} CYP+4 µg L^{-1} CPF in a mixture of a commercial products (PRODUCT). After 24 h and 96 h of exposure, the animals were carefully removed from the exposure tanks and placed individually in the filming tank, where their behavioral activity was recorded for 10 min. A digital camera was connected to a laptop for recording the videos and the behavioral parameters were afterward analyzed using appropriate video-tracking software (ANY-maze, Stoelting CO, USA). After 96 h exposure and behavior record the animals were sacrificed and, brain and muscle were taken and stored at −80 °C to measure AChE and BChE activity.

The measurement of CYP and CPF in the exposition medium was performed by solid phase extraction- solid phase microextraction-gas chromatography coupled to Mass Spectrometry (SPE–SPME–GC–MS) according to Bonansea et al. (2013). The CYP and CPF concentrations were determined in water samples collected in the aquariums 30 min after the addition of the pesticides and previous to the daily medium renewal. Initial concentrations were: CYP = 0.04 ± 0.01 µg L^{-1} and 0.38 ± 0.03 µg L^{-1}; CPF = 0.31 ± 0.03 and 3.4 ± 0.3 µg L^{-1}. The decay in pesticides concentrations after 24 h was 49% and 81% for CYP and CPF, respectively. Insecticides concentrations in control aquariums were below the detection limits of the method (CYP = 0.2 ng L^{-1}; CPF = 0.2 ng L^{-1}). The exposure concentrations were selected taking into account three main criteria: 1. Concentrations of CYP and CPF in natural freshwaters (CYP = 0.05–3.5 µg L^{-1}; CPF = 0.21–2 µg L^{-1}; Bonansea et al., 2013; Jergentz et al., 2005; Marino and Ronco, 2005); 2.96 h medium lethal concentrations (96-h LC_{50}) available for *Poecilia reticulata*, which belongs to the same order than *J. multidentata* (Cyprinodontiformes; CYP 96-h LC_{50} = 21.4 µ

g L^{-1}; Polat et al. 2002; and CPF 96-h LC_{50}=176 μg L^{-1}; Sharbidre et al., 2011); the sublethal concentrations 0.2 and 2 % of the 96-h LC_{50} were chosen; 3. Exposure to equitoxic mixture as well as in a proportion usually used for agricultural purposes (0.002 and 0.02 Toxic Units according to 96-h LC_{50} for *Poecilia reticulata* and a proportion of 5 CYP: 50 CPF; SENASA, 2015).

2.5. Swimming behavioral

The swimming behavioral test was performed during the same time frame each day (between 09:00 am and 2:00 pm). The apparatus consisted of a rectangular glass tank (30 × 15 × 10 cm), which was virtually divided into three horizontal areas: bottom, middle, and top. The locomotor of *J. multidentata* was measured by the total tank behavior, which included the total distance traveled, mean speed, time the individual is moving and immobile episodes. Additionally, in order to assess the fish preference for the different areas of the aquarium, the number of entries in the top (up) or bottom (down) areas were considered, as well as the time the fish spent in each area. The number of entries and the time spent in the middle area of the aquarium are not considered because this is a transition area hardly ever explored by the fish (Rosemberg et al., 2011). These measures were calculated based on segmentation, in periods of 2 min during the total recording time (10 min). The last 6 min of recorded swimming activity were considered for the analysis.

2.6. Enzyme extraction and measurement

Enzyme extracts were prepared according to Wiegand et al. (2000) with few modifications. Thus, brain and muscle tissues (n=6) were homogenized using a glass homogenizer in 0.1 M potassium phosphate buffer, pH 6.5, at 4 °C. Then samples were centrifuged at 4 °C for 10 min at 13,000*g*. Supernatants were removed and centrifuged at 4 °C for 1 h at 105,000*g*. Supernatants were conserved to soluble enzyme activity determination. Obtained pellet was resuspended in 20 mM potassium phosphate buffer, pH=7, and was used to membrane fraction enzyme activity determination. Enzyme extracts were frozen and maintained at −80 °C until analysis.

The soluble and membrane fraction were used to assess BChE and AChE activity, respectively. Activity of AChE was measured according to Ellman et al. (1961), with acetylthiocholine as a substrate while BChE activity was measured with butyrylcholine as a substrate. The enzymatic activity was calculated in terms of the protein content of the sample (Bradford, 1976), and is reported in nanokatals per milligram of protein (nkat mg $prot^{-1}$). Each enzymatic assay was carried out by triplicate.

2.7. Statistics

All values are expressed as mean ± standard deviation. Normal distribution of data was analyzed by Shapiro Willks test, while Levene test was used to test the homogeneity of variance. To evaluate the differences of enzymes activity measured, one-way analysis of variance (ANOVA) followed by Tukey test was performed to compare among treatments. When the data showed abnormal distribution, they were subjected to a non parametric statistical analysis (Kruskal-Wallis) followed by Dunn test. Spearman correlation test was used to establish the association between different variables. The InfoStat/P software (Di Rienzo et al., 2011) was employed in all cases. Significance was accepted for $p < 0.05$. Mixed Linear Model was performed with transformed variables to compare the results of the behavioral test (SPSS™ software SPSS, USA, $p < 0.05$). Joint action analysis for each treatment was calculated using Relative Interaction Index (RII) according to Mansour and Refaie (2000). For this analysis biochemical and behavoiral variables were included. Relative interaction index was calculated according to the following equation: (Mixture value+Control value)/(CYP value+CPF value). In cases of positive effect on the baseline values (increase or more release of an enzyme activity): RII=1 for additive effects, RII ≤ 1 for antagonism, or ≥1 for potentiation. In cases of negative effects on the baseline values (decrease or inhibition of an enzyme activity): RII=1 for additive effects, RII ≤ 1 for potentiation, or ≥1 for antagonism. For accuracy, a "safety factor" of ± 0.05 is added to the indices values when ranking the joint action (Mansour et al., 2008).

3. Results and discussion

3.1. Swimming behavior

Locomotor activity was evaluated 24 and 96 h after exposure. The results obtained for total distance traveled, mean speed, time the individual is moving and immobile episodes are shown in Fig. 1.

After 24 and 96 h of exposure of *J. multidentata* to low and high concentrations of CYP, no significant differences in the swimming behavior of exposed fish were observed when compared to control fish. Fish exposed to low concentrations of CPF only showed a significant decrease in the number of episodes when the organisms remained immobile after 96 h of exposure (Fig. 1H), with no significant differences in the other parameters of swimming activity evaluated. No differences were observed after 24 h of exposure. On the other hand, fish exposed to high concentrations of CPF showed significant changes in the decrease of the distance traveled, the speed and the time the fish remains mobile after 24 and 96 h of exposure. There was also a significant increase in immobile episodes after 24 h of exposure. These results reveal that exposure to high concentrations of CPF causes a significant decrease in the swimming activity of *J. multidentata*.

Fish exposed to low concentrations of the technical mixture of CYP+CPF showed a significant increase in the distance traveled and mean speed after 96 h, with no differences in mobile and immobile episodes compared to control organisms. Furthermore, high concentrations of exposure showed a significant decrease in the distance traveled, speed and mobile episodes after 24 and 96 h of exposure, and a significant increase in the number of immobile episodes after 24 h. This denotes a differential effect in the swimming activity of the fish based on the concentration of exposure to the technical mixture, with lower concentrations resulting in an activation effect on swimming behavior and higher concentrations causing an inhibition effect.

Fish exposed to a low concentration of the PRODUCT showed a significant increase in the distance traveled and speed after 96 h of exposure. After 24 h, however, there were no significant differences for these parameters, or for mobile and immobile episodes after 24 and 96 h of exposure. High concentrations resulted in significantly decreased distance traveled, swimming speed and time when the individual remains mobile after 24 and 96 h of exposure. The number of immobile episodes decreased significantly after 96 h, but no differences were observed after 24 h. The behavior of the fish exposed to the PRODUCT was similar to the one displayed after exposure to the technical mixture, where low concentrations resulted in activation and high concentrations in inhibition, but in this last treatment, this effect is observed after 24 h of exposure, and on a scale similar to the one observed with the technical mixture after 96 h of exposure. It was also noted that a longer exposure time caused a significantly greater number of immobile episodes.

In addition to the previous analysis, the spatio-temporal

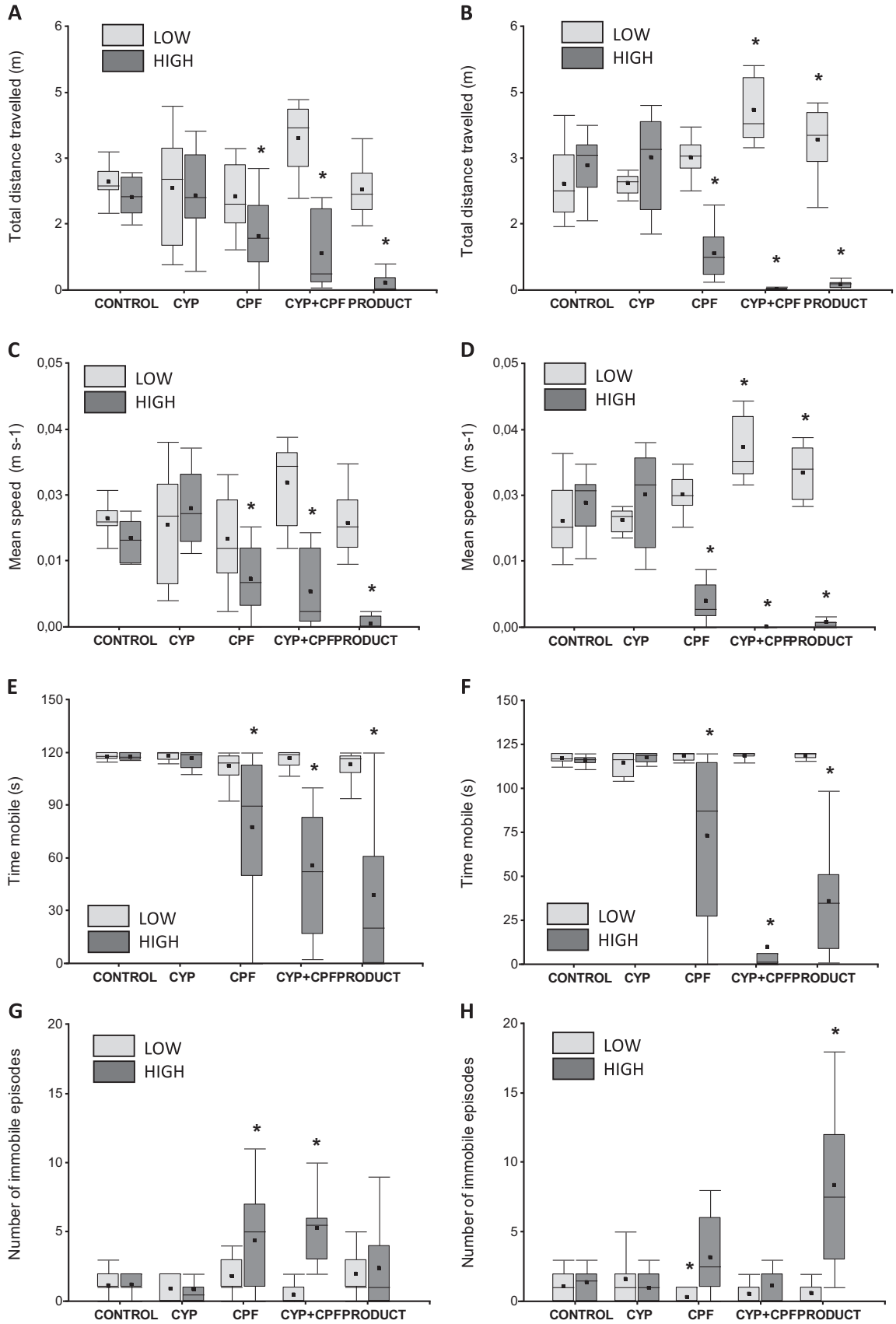

Fig. 1. Total distance traveled, mean speed, time of moving and immobile episodes of *Jenynsia multidentata* after exposure to low (light gray) and high (dark gray) concentrations of cypermethrin and chlorpyrifos, single and in mixture, for 24 h (A, C, E and G) and 96 h (B, D, F and H). Data are expressed as mean ± 1 SD ($n=9$). Significant differences between exposed and control groups are indicated with asterisks ($p < 0.05$).

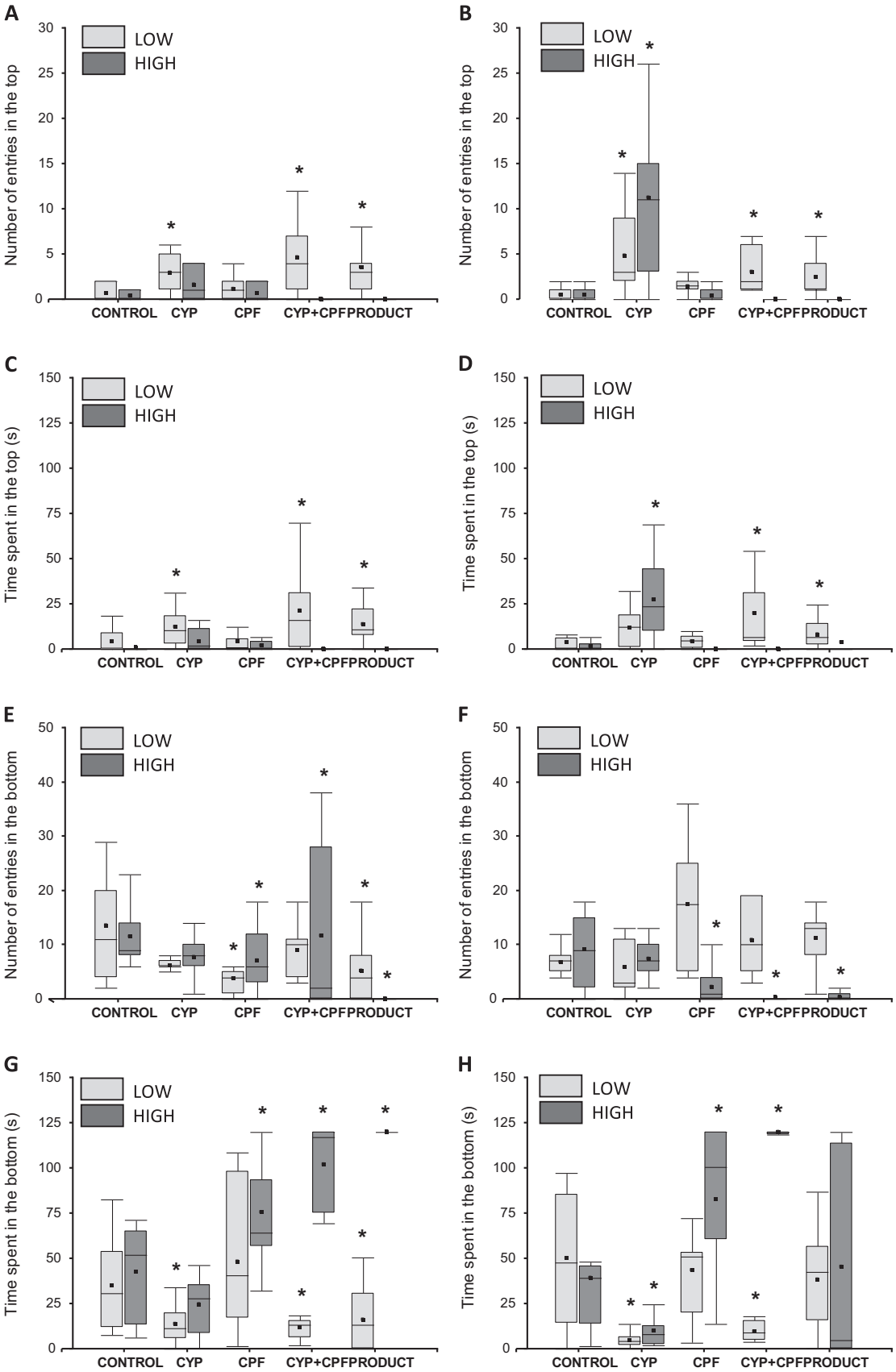

Fig. 2. Number of entries in the top area, time spent in the top area, Number of entries in the bottom area and time spent in the bottom area of *Jenynsia multidentata* after exposure to low (light gray) and high (dark gray) concentrations of cypermethrin and chlorpyrifos, single and in mixture, for 24 h (A, C, E and G) and 96-h (B, D, F and H). Data are expressed as mean ± 1 SD (n=9). Significant differences between exposed and control groups are indicated with asterisks ($p < 0.05$).

behavior of *J. multidentata* was evaluated in control fish and fish exposed to the different treatments during 24 and 96 h. In order to assess the fish preference for the different areas of the aquarium, the number of entries in the top (up) or bottom (down) areas were considered, as well as the time the fish spent in each area. The number of entries and the time spent in the middle area of the aquarium are not considered because this is a transition area hardly ever explored by the fish (Rosemberg et al., 2011). Fig. 2 shows the number of entries and the time spent in the top area as well as the number of entries and the time spent in the bottom area.

Control fish showed preference for the bottom area, spending most of the time in this area (Fig. 2), with an average stay of 37.8 and 46.0 s in the bottom area, and 2.9 and 3.3 s in the top area after 24 and 96 h of exposure, respectively. Moreover, they have a greater number of entries to the bottom area, with an average of 14.8 and 10.4 events, in relation to the number of entries to the upper areas, with an average of 1.3 and 3.0 events, after 24 and 96 h of exposure, respectively.

When compared to control, fish exposed to low concentrations of CYP showed a significant increase in the number of entries to the top area after 24 and 96 h, and the time spent in this area after 24 h. There were no differences in the time spent in the top area after 96 h. Furthermore, there was a significant decrease in the time spent in the bottom area after 24 and 96 h of exposure. When exposed to high concentrations of CYP, fish showed a significant increase in the time spent in the top area and the number of entries to this area after 96 h of exposure. These differences were not observed after 24 h, but there was a significant decrease in the time spent in the bottom area when compared to the control after 96 h of exposure. These results reveal that both concentrations of this insecticide increase the exploratory activity of *J. multidentata* in the upper area of the aquarium.

Fish exposed to low concentrations of CPF did not show significant differences in the time spent in the top area and the number of entries to this area after 24 and 96 h of exposure. There were also no significant differences in the time spent in the bottom area after 24 and 96 h of exposure, nor in the number of entries to this area after 96-h of exposure. However, there was a significant decrease in entries to the bottom area after 24 h of exposure. Fish exposed to high concentrations of CPF did not show significant differences in the top area after 24 and 96 h of exposure. In the bottom area, however, there was an increase in the time spent and a decrease in the number of entries to this area after 24 and 96 h of exposure. These results suggest that fish exposed to high concentrations of CPF leave the bottom area less frequently.

Fish exposed to the technical mixture of CYP+CPF displayed a different behavior based on the concentration of exposure. Low concentrations of the mixture resulted in a significant increase in the time spent and the number of entries to the top area after 24 and 96 h of exposure. There was also a significant decrease in the time spent in the bottom area after 24 and 96 h. Conversely, there were no significant differences in the number of entries to this area in neither of the exposure periods. These results denote greater exploratory activity in the upper area of the aquarium. With high concentrations of the technical mixture, there were no significant differences in the time spent and the number of entries to the top area after 24 and 96 h. However, there was a significant increase in the time spent in the bottom area after 24 and 96 h of exposure, as well as a significant increase in the number of entries to the bottom area after 24 h, and a decrease in the number of entries to this area after 96 h. These results indicate that fish exposed to high concentrations of the technical mixture of CYP and CPF prefer the bottom area of the aquarium, which was more clearly observed after 96 h, when exposed fish spent most of the time in the bottom of the aquarium and the number of entries to the bottom and top areas is zero, indicating there were no transitions during the 6 min recorded.

Fish exposed to low concentrations of the PRODUCT showed a significant increase in the time and the number of entries to the top area after 24 and 96 h of exposure. After 24 h of exposure, there was also a significant decrease in the time spent and the number of entries to the bottom area, whereas after 96 h, there were no differences in this area. For the fish exposed to high concentrations of the commercial product mixture, the time spent and the number of entries to the top area were approximately zero after 24 and 96 h of exposure. In contrast, in the bottom area, after 24 h of exposure there was an increase in the time spent and a decrease in the number of entries, which were approximately zero, while after 96 h of exposure, there was only a significant decrease in the number of entries to this area, but the time spent did not differ from the control. Data belong to two different groups of individuals comprised of fish which remained in the upper area throughout the recording time and fish which stayed at the bottom of the aquarium, almost immobile in both cases. Consequently, fish exposed to the PRODUCT during 24 h show a behavior similar to that observed for the technical mixture of these compounds, with an increase in exploratory activity of the top area after exposure to the low concentrations studied. Fish exposed to high concentrations spent all the time in the bottom area without transitioning to other areas. Fish exposed to the commercial product mixture over longer periods of time (96 h) showed very little mobility and, therefore, the area they occupied when entering the recording aquarium – at the top or the bottom – was the place where they remained throughout the recording.

After exposure to low and high concentrations of CYP, *J. multidentata* only showed preference for the top area of the aquarium. These results could be considered as an escape conduct of the fish, similarly reported for *Labeo rohita* and *Poecilia reticulata* species after the first hours of exposure to concentrations of CYP near LC50 for each species (Marigoudar et al., 2009; Yilmaz et al., 2004). Fish exposed to high concentrations of CPF showed a decrease in swimming activity and an increase in the time spent at the bottom of the aquarium. These results of hypoactivity are consistent with those reported for medaka species (*Oryzias latipes*) exposed to 18 μg L^{-1}, 55 μg $L^{-1}$and 166 μg L^{-1} of chlorpyrifos over 96 h (Khalil et al., 2013). There were also similar changes in the behavior of zebrafish larvae exposed to 35 μg L^{-1} and 3.5 μg L^{-1} of CPF during development (Richendrfer et al., 2012). After exposure to technical mixtures and low concentrations of PRODUCT, there was an increase in swimming activity with preference for the upper area of the aquarium, and after exposure to high concentrations, there was a decrease in swimming activity with preference for the bottom area of the aquarium. As long as we know, there were no previous studies reporting results in mixtures of these insecticides.

Alterations in behavior observed after the different treatments show a potential risk for species exposed to polluted water, since both hyperactivity and hypoactivity can lead to a reduction in survival of individuals. Increased swimming activity enhances the vulnerability of an organism to becoming prey, while decreased swimming activity is associated with decreased ability to get food (Christensen et al., 2005). Furthermore, occupying the top area of the aquarium, a behavior observed in exposed fish, could indicate that the individuals travel to the surface for aerial breathing, in order to avoid contact of the pesticide with gills (Santhakumar et al., 2000).

3.2. Activity of AChE and BChE

Table 1 show the results obtained for the activity of microsomal AChE and cytosolic BChE enzymes. These activities were evaluated

Table 1
Activity of acetylcholinesterase and butyrylcholinesterase (nkat mg protein^{-1}) in brain and muscle of *Jenynsia multidentata* after exposure to sublethal concentrations of cypermethrin and chlorpyrifos single and in mixtures for 96 h. Data are expressed as mean ± 1 SD. Different letters, when indicated, mean significant differences among treatments ($p < 0.05$).

	Brain				Muscle			
	AChE		BChE		AChE		BChE	
	Low	High	Low	High	Low	High	Low	High
CONTROL	16.5 ± 5.5^{ab}	18.4 ± 2.0	16.9 ± 2.6^{c}	19.7 ± 5.1	26.5 ± 2.0^{b}	26.5 ± 2.2^{c}	2.4 ± 0.2^{a}	2.4 ± 1.3^{b}
CYP	17.4 ± 7.1^{abc}	17.6 ± 2.6	6.1 ± 2.9^{a}	15.4 ± 8.0	25.1 ± 8.1^{b}	6.4 ± 1.4^{a}	2.5 ± 0.5^{a}	2.1 ± 0.6^{b}
CPF	13.6 ± 2.5^{a}	16.1 ± 3.0	7.1 ± 3.5^{a}	16.9 ± 3.7	27.2 ± 2.1^{b}	8.8 ± 2.2^{b}	4.3 ± 1.4^{b}	2.2 ± 0.5^{b}
CYP+CPF	24.7 ± 9.3^{c}	15.1 ± 3.5	10.5 ± 2.9^{b}	22.8 ± 3.4	25.3 ± 0.8^{a}	6.3 ± 1.2^{a}	2.3 ± 0.4^{a}	0.9 ± 0.2^{a}
PRODUCT	19.3 ± 4.1^{bc}	23.7 ± 10.5	14.2 ± 4.0^{bc}	22.3 ± 12.9	27.0 ± 2.0^{b}	12.3 ± 2.2^{b}	2.7 ± 0.6^{a}	2.3 ± 0.6^{b}

CYP: cypermethrin, CPF: chlorpyrifos, CYP+CPF: cypermethrin plus chlorpyrifos technical mixture, PRODUCT: cypermethrin plus chlorpyrifos mixture of a commercial products, Low: cypermethrin: 0.04 μg L^{-1} and chlorpyrifos: 0.4 μg L^{-1}, High: cypermethrin: 0.4 μg L^{-1} and chlorpyrifos: 4 μg L^{-1}, AChE: acetylcholinesterase, BChE: butyrylcholinesterase.

in muscle and brain of exposed fish to low and high concentrations of pesticides.

Exposure of *J. multidentata* to low concentrations of CYP only induced a significant 64% inhibition of BChE in brain compared to control, which shows an interaction of the insecticide with the protection enzyme, avoiding inhibition of AChE. In contrast, exposure to high concentrations of CYP did not show a response from the protection enzyme, and the insecticide caused a significant 67% inhibition of AChE in muscle compared to control. Pyrethroids are known to interact with aromatic aminoacids of ChEs, resulting in the inhibition of these enzymes (Rao et al., 2005). Similarly, studies on exposure to cypermethrin of fish *Channa punctatus* and *Cyprinus carpio* showed inhibition of AChE activity in muscle of exposed fish compared to control fish (Kumar et al., 2009, Reddy and Philip, 1994).

Fish exposed to low concentrations of CPF showed a significant 58% inhibition of BChE in brain, and a significant increase in BChE activity in muscle when compared to control. This increase can be due to the de novo synthesis of this enzyme by the compensation metabolism, which results from the presence of anticholinesterasic compounds to compensate for functional defects in the cholinergic system (Yang et al., 2013). The protection enzyme response in both of the organs evaluated could be associated with the lack of effects observed on AChE. Furthermore, high concentrations of CPF showed a 12% inhibition of AChE in brain, although this difference was not significant compared to control. There was also a significant 76% inhibition of AChE in muscle compared to control, showing that at high concentrations of the insecticide, the protection activity of BChE was insufficient to avoid inhibition of AChE in both of the evaluated organs.

Results obtained in *J. multidentata* are similar to those reported in other studies on the effect of chlorpyrifos, where there was inhibition of AChE in brain for *G. affinis*, and in muscle for *Acanthochromis polyacanthus*, when compared to control fish (Botte et al., 2012, Kavitha and Rao, 2008).

Fish exposed to low concentrations of CYP+CPF showed a significant increase of AChE in brain, and 38% inhibition of BChE in the same organ when compared to control. Also, the mixture caused a significant 5% inhibition of AChE in muscle compared to control. High concentrations of the technical mixture, on the other hand, induced a significant 76% inhibition of AChE in muscle, and 61% inhibition of BChE in the same organ, when compared to control. Additionally, the effects on the fish exposed to the technical mixture were observed at lower concentrations than on the fish exposed to each insecticide individually. Although the insecticides reacted to the protection enzyme with both concentrations of the technical mixture, this was insufficient since there was inhibition of AChE. There was also an increased response of AChE in brain, which can derive from an acceleration in the synthesis of the enzyme caused by a previously undetected inhibition (Yang et al., 2013).

Fish exposed to low concentrations of the PRODUCT only showed a non-significant 15% inhibition of BChE in brain compared to control. On the other hand, fish exposed to high concentrations only showed a significant 54% inhibition of AChE in muscle compared to control. In comparison with the responses found for the technical mixture, the commercial mixture had fewer effects on the ChEs enzymes. This may be because the effects caused by complex mixtures, which include not only active ingredients (CYP and CPF) but also adjuvant compounds and detergents, are different from the effects studied with pure compounds.

Moreover, according to the response observed in both organs with all the treatments, it can be concluded that in *J. multidentata*, AChE activity in muscle is more sensitive to exposure to CYP, CPF and their mixtures than in brain. This is consistent with the results observed in other species, where AChE in muscle was a better indicator of exposure to organophosphate pesticides than AChE activity in brain (Fulton and Key, 2001).

It can be interesting to consider the results from swimming behavior and AChE activity, since both hypoactivity and hyperactivity can be associated with inhibition of AChE function (Marigoudar et al., 2009). *J. multidentata* showed that changes in the behavior of exposed fish were consistent with inhibition of AChE in muscle, and no differences of AchE in brain. The activity of AChE showed a significant positive correlation with total distance traveled ($r=0.43$, $p < 0.001$), mean speed ($r=0.47$, $p < 0.001$) and time of moving ($r=0.42$, $p < 0.001$), while a significant negative correlation was observed for immobile episodes ($r=-0.22$, $p=0.020$). This same species showed a similar result when exposed to sublethal concentrations of endosulfan, where inhibition of AChE in muscle was consistent with a decrease in swimming activity (Ballesteros et al., 2009a). In contrast, studies on *G. affinis* exposed to CPF showed a decrease in the swimming behavior associated with inhibition of AChE in brain (Rao et al., 2005).

3.3. Joint action analysis

Table 2 presents the joint action analysis and RII of the mixture of CYP and CPF on the studied variables. In general the tested mixtures, both technical and commercial, showed different types of interaction. Potentiating and antagonistic interactions were found more frequently than additive interactions. Moreover, potentiation prevailed over antagonism at higher concentrations of both technical and commercial mixtures of CYP and CPF.

According to Elhalwagy and Zaki (2009, and authors therein

Table 2
Joint intoxication on different variables measured in *Jenynsia multidentata* after exposure to sublethal concentrations of cypermethrin and chlorpyrifos single and in mixtures for 96 h.

Parameters	CYP+CPF						PRODUCT					
	Low			High			Low			High		
	Effect	**RII**	**Joint action**	**Effect**	**RII**	**Joint action**	**Effect**	**RII**	**Joint action**	**Effect**	**RII**	**Joint action**
AChE (Brain)	Increase	1.33	Potentiation	Decrease	0.99	Additive	Increase	1.15	Potentiation	Increase	1.25	Potentiation
BChE (Brain)	Decrease	2.08	Antagonism	Increase	1.32	Potentiation	Decrease	2.36	Antagonism	Increase	1.30	Potentiation
AChE (Muscle)	Decrease	0.99	Additive	Decrease	2.16	Antagonism	Increase	1.02	Additive	Decrease	2.55	Antagonism
BChE (Muscle)	Decrease	0.69	Potentiation	Decrease	0.77	Potentiation	Increase	0.75	Antagonism	Decrease	1.09	Antagonism
Total Distance	Increase	1.19	Potentiation	Decrease	0.75	Potentiation	Increase	1.06	Potentiation	Decrease	0.77	Potentiation
Mean speed	Decrease	1.00	Additive	Decrease	0.51	Potentiation	Decrease	1.00	Additive	Decrease	0.52	Potentiation
Time mobile	Increase	1.01	Additive	Decrease	0.66	Potentiation	Increase	1.01	Additive	Decrease	0.80	Potentiation
Immobile episodes	Decrease	0.87	Potentiation	Decrease	0.61	Potentiation	Decrease	0.89	Potentiation	Increase	2.38	Potentiation
Entries in the top area	Increase	0.57	Antagonism	Decrease	0.04	Potentiation	Increase	0.47	Antagonism	Decrease	0.04	Potentiation
Time spent in the top area	Increase	1.48	Potentiation	Decrease	0.06	Potentiation	Increase	0.75	Antagonism	Increase	0.20	Antagonism
Entries in the bottom area	Increase	0.76	Antagonism	Decrease	0.98	Additive	Increase	0.77	Antagonism	Decrease	0.99	Additive
Time spent in the bottom area	Decrease	1.23	Antagonism	Increase	1.72	Potentiation	Decrease	1.82	Antagonism	Increase	0.91	Antagonism

CYP+CPF: cypermethrin plus chlorpyrifos technical mixture, PRODUCT: cypermethrin plus chlorpyrifos mixture of a commercial products, Low: cypermethrin: 0.04 $\mu g\,L^{-1}$ and chlorpyrifos: 0.4 $\mu g\,L^{-1}$, High: cypermethrin: 0.4 $\mu g\,L^{-1}$ and chlorpyrifos: 4 $\mu g\,L^{-1}$, AChE: acetylcholinesterase, BChE: butyrylcholinesterase, RII: relative interaction index.

referenced) the toxic potency of organophosphate and pyrethroid insecticides is closely related to their rate of metabolic elimination. Pyrethroids are biotransformed by two pathways: cytochrome P450 dependent on oxidation and esterase-mediated hydrolysis. B-esterases can be inhibited by organophosphate pesticides due to irreversible covalent formation, thus decreasing the rate of pyrethroids metabolism in the organism. On the other hand, organophosphate pesticides require activation to its respective oxon for its cholinergic toxicity. This oxidation could be mediated by cytochrome P-450 or be enhanced by an oxidative environment. Thus, this oxidation could be attributed to the presence of CYP, which is known capable of generating an increase in reactive oxygen species (Marigoudar et al., 2012).

4. Conclusions

The results of this study demonstrated that:

- The swimming behavior of *J. multidentata* showed similar alterations after 24 and 96 h of exposure to the insecticides, with greater differences after 96 h.
- CYP exposure only increases the exploratory activity of *J. multidentata* in the upper area of the aquarium and inhibits AChE activity in muscle.
- Fish exposed to CPF reveals a decrease in the swimming activity of *J. multidentata* after 24 and 96 h, particularly those exposed to high concentrations. At high levels of the insecticide, the protection activity of BChE was insufficient to avoid inhibition of AChE in both of the evaluated organs.
- Fish exposed to low concentrations of CYP and CPF in the form of technical mixture and commercial product showed hyperactivity, while high concentrations of exposure resulted in hypoactivity.
- Although there was a protective response of BChE when fish were exposed to the technical mixture, this was insufficient since there was also inhibition of AChE. Even when a similar response was observed, the commercial mixture shows fewer effects on the ChEs enzymes than the technical mixture.
- Based on the response of cholinesterase enzymes in *J. multidentata*, AChE in muscle is more sensitive to exposure to CYP, CPF and their mixtures than in brain.
- A decrease in swimming behavior could be associated with the inhibition of AChE activity in muscle of *J. multidentata* exposed to high concentrations of pesticides.
- The combined effect of the two insecticides had varied effects on most investigated variables; nevertheless, potentiation prevailed over antagonism at higher concentrations of both technical and commercial mixtures.

Overall, these results draw attention to the need of more studies on the potential ecotoxicological impact of pesticides and its mixtures at environmental relevant concentrations.

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