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Abstract:	Brazil is the largest producer of sugarcane, a crop largely dependent on chemical control for its maintenance. The insecticide fipronil and herbicide 2,4-D stand out among the most commonly used pesticides and, therefore, environmental consequences are a matter of concern. The present study aimed to investigate the toxicity mechanisms of Regent® 800 WG (a.i. fipronil) and DMA® 806 BR (a.i. 2,4-D) pesticides using forced and non-forced exposures through an integrative approach: firstly, to assess whether contamination by fipronil and 2,4-D can trigger the avoidance behavior of the fish Danio rerio (zebrafish) and Hyphessobrycon eques (serpae tetra or mato-grosso). Additionally, the effects on fish were analyzed considering the swimming behavior together with a biomarker of neurotoxicity, the activity of acetylcholinesterase (AChE). In avoidance tests with pesticide gradients, D. rerio avoided the highest concentrations of the two compounds and H. eques avoided only the highest concentration of 2,4-D. The swimming behavior (distance moved) was reduced and AChE was inhibited when D. rerio was exposed to fipronil. The 2,4-D affected the swimming (maximum speed) of H. eques, but AChE was not altered. Avoidance response seemed not to have been affected by possible effects of contaminants on swimming behavior and Ache activity. This study showed the importance of knowing the avoidance capacity, swimming behavior and neurotoxic effects of pesticides on fish in an integrated and realistic context of exposure in environments contaminated with pesticides and can be useful as ecologically relevant tools for ecological risk assessment.
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Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- Toxicity and avoidance of fipronil and 2,4-D were studied in *D. rerio* and *H. eques.*
- Environmentally relevant concentrations of fipronil were repellent for *D. rerio.*
- *D. rerio* and *H. eques* avoided 2,4-D concentrations.
- Fipronil affected the swimming behavior of *D. rerio* and 2,4-D of *H. eques.*
- Fipronil inhibited the enzymatic activity of AChE in *D. rerio* but not in *H. eques*.

Fipronil and 2,4-D effects on tropical fish: Could avoidance response be explained by changes in swimming behavior and neurotransmission impairments?

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Abstract

Brazil is the largest producer of sugarcane, a crop largely dependent on chemical control for its maintenance. The insecticide fipronil and herbicide 2,4-D stand out among the most commonly used pesticides and, therefore, environmental consequences are a matter of concern. The present study aimed to investigate the toxicity mechanisms of Regent® 800 WG (a.i. fipronil) and DMA® 806 BR (a.i. 2,4-D) pesticides using forced and non-forced exposures through an integrative approach: firstly, to assess whether contamination by fipronil and 2,4-D can trigger the avoidance behavior of the fish *Danio rerio* (zebrafish) and *Hyphessobrycon eques* (serpae tetra or mato-grosso). Additionally, the effects on fish were analyzed considering the swimming behavior together with a biomarker of neurotoxicity, the activity of acetylcholinesterase (AChE). In avoidance tests with pesticide gradients, *D. rerio* avoided the highest concentrations of the two compounds and *H. eques* avoided only the highest concentration of 2,4-D. The swimming behavior (distance moved) was reduced and AChE was inhibited when *D. rerio* was exposed to fipronil. The 2,4-D affected the swimming (maximum speed) of *H. eques*, but AChE was not altered. Avoidance response seemed not to have been affected by possible effects of contaminants on swimming behavior and Ache activity. This study showed the importance of knowing the avoidance capacity, swimming behavior and neurotoxic effects of pesticides on fish in an integrated and realistic context of exposure in environments contaminated with pesticides and can be useful as ecologically relevant tools for ecological risk assessment.

Keywords: Pesticides; *Danio rerio*; *Hyphessobrycon eques*; Habitat disturbance; Acetylcholinesterase

1. Introduction

Brazil is one of the largest biofuel producers, mainly considering the sugarcane ethanol (Martinelli and Filoso, 2008). However, sugarcane crops are widely dependent on chemical control to maintain plantations. In this context, concerns about the risk faced by aquatic ecosystems around sugarcane areas have increased, for example by the input of chemicals, such as pesticides (Martinelli and Filoso, 2008). Fipronil and 2,4-D stand out for being prominent pesticides applied in sugarcane cultivation and have been constantly found in Brazilian water bodies (Albuquerque et al., 2016; CETESB, 2018). The 2,4-D was detected in aquatic environments located in areas with a predominance of sugarcane crops in the state of São Paulo, Brazil, at concentrations between 175.1 and 366.6 μg/L, and for fipronil, the concentrations observed ranged from 6 to 465 μg/L (CETESB, 2018). Both compounds are also used in several other agricultural crops, including rice, corn, soy and wheat, and their presence has also been reported in water bodies worldwide in concentrations ranging from 0.7 - 153 ng/L of fipronil (Fang et al., 2019) and from 0.062 - 12 μg/L of 2,4-D (Islama et al., 2018).

The insecticide fipronil acts directly on γ-aminobutyric acid chloride (GABA) channels in insects for disturbing neuronal signaling (Gunasekara and Troung, 2007). Studies have also indicated that GABA antagonists, such as fipronil, cause several effects, e.g., seizures, hyperactivity and death in fish (Beggel et al., 2012). Other studies suggest that fipronil induces the production of reactive oxygen species in fish cells, which can lead to increased oxidative stress and cell damage (Möhler et al., 2004; Ki et al., 2012; Margarido et al., 2013). Menezes et al. (2016) when exposing carp (*Cyprinus carpio*) and silver catfish (*Rhamdia quelen*) to 0.65 μg/L fipronil for 192 h observed inhibition of acetylcholinesterase (AChE). Gripp et al. (2017) showed that exposure to fipronil and its metabolites at environmentally relevant concentrations can damage the antioxidant

system and establish oxidative stress in tadpoles of the species *Eupemphix nattereri*, resulting in changes in their physiological conditions.

The herbicide 2,4-D interferes with the growth processes of broadleaf plants by an action similar to auxins (Walters, 2011). However, adverse effects of vertebrate exposure to the herbicide 2,4-D have also been reported (Zuanazzi et al., 2020). Pericardial edema was observed in zebrafish embryos when exposed to a concentration of 25 mg/L after 70 h (Li et al., 2017). Genotoxic effects occurred in the fish *Cnesterodon decemmaculatus*, where animals exposed to the range of 252-756 mg/L had an increased frequency of micronuclei after 48 and 96 h (Arcaute et al., 2016). The results obtained by da Fonseca et al. (2008) indicated that 2,4-D (1 or 10 mg/L) affects brain and muscle AChE activity and some blood and tissue metabolic parameters of *Leporinus obtusidens*. Gaaied et al. (2019) tested concentrations of 2,4-D from 0.02 to 0.8 mg/L on zebrafish (*Danio rerio*) embryos from 3 h post fertilization to 96 hpf and the effect of 2,4-D in ChE was translated by an inhibition of the enzyme activity in all treated groups. For amphibians, metamorphosis was delayed in tadpoles of *Physalaemus centralis* after 21 day-exposure to 2,4-D at concentrations of approximately 130 and 260 mg/L (Figueiredo and Rodrigues, 2014). *Lithobates catesbeianus* tadpoles distinguished gradients with increasing environmental concentrations of 2,4-D, so that the population preferentially moved towards the lowest concentrations (Freitas et al., 2019).

The evidence that fipronil and 2,4-D cause toxic effects on aquatic organisms has been shown by an exposure approach in which organisms are forcedly exposed to contaminants (forced exposure). In spite of the importance of this approach to predict the potential toxicity of chemicals, previous studies have indicated that different species of animals (fish, amphibians and some invertebrates) can detect and avoid contaminated environments, preventing them from suffering the toxic effects of the pollutant to which

they are exposed (reviews by Araújo et al., 2016 and Moreira-Santos et al., 2019 and references therein). These studies used unforced exposure systems, in which organisms move freely among many concentrations and select the most favorable one. This change in approach provides a new view of contamination risk focused on how the repellency of contaminants influences the spatial distribution of organisms (Araújo et al., 2016; Moreira-Santos et al., 2019).

Considering these points, the present study aimed initially to assess whether the fish species *Danio rerio* (zebrafish) and *Hyphessobrycon eques* (serpae tetra or mato-grosso) detect and avoid the contamination gradient of Regent® 800 WG (a.i. fipronil) and DMA® 806 BR (a.i. 2,4-D) by moving to less contaminated habitats. Considering that both compounds might affect the cholinergic system in fish (da Fonseca et al., 2008; Menezes et al., 2016; Gaaied et al., 2019), we also evaluated if the pattern of avoidance response to avoid fipronil and 2,4-D could be closely related to the effects on swimming behavior (maximum speed and distance moved) and neurotoxicity (measured through enzyme activity of acetylcholinesterase - AChE). *H. eques* and *D. rerio* fish were used in the experiments because they are suitable for maintenance in laboratory conditions, they were sensitive in bioassays and have high ecological relevance, as they occur in freshwaters of the tropical region (Spence et al., 2008; Shukla and Bhat, 2017; Fujimoto et al., 2013). In addition, using the two species allows us to compare the responses of a biological model (*D. rerio*) and a native species still little used in ecotoxicological studies (Mansano et al., 2018). To the best of our knowledge, this is the first study that attempts to explain how impairments in swimming behavior and enzymatic changes on a neurotoxicity biomarker (AChE) might affect the ability of fish to avoid contamination. Thus, integrating two different exposure approaches: forced and un-forced exposure systems.

2. Material and methods

2.1 Test organisms and culture conditions

Adult individuals of the *H. eques* (4-6 months old, body weight 0.40 ± 0.07 g, body size 3.1 ± 0.16 cm) and *D. rerio* (4-6 months old, body weight 0.29 ± 0.06 g, body size 3.0 ± 0.23 cm) were acquired from a local commercial hatchery in São Carlos (SP, Brazil) and cultivated as described in Mansano et al. (2018) and ABNT 15088/2016 (ABNT, 2016) for *H. eques* and *D. rerio*, respectively. The use of the fish was approved by the Ethics Committee on the use of animals (CEUA/EESC-USP no.01/2020). The fish were acclimated for one week before beginning the tests and were kept in aquaria containing 50 L of natural well water. About 20% of the water in the aquaria was renewed every day. The animals were kept under a controlled temperature at 25 ± 2 ºC, pH (7.0 ± 1.0), dissolved oxygen (80% air saturation), a photoperiod of 12 h of light: 12 h of darkness and a density up to 1 animal per liter. The fish were fed daily, at least twice, with flaked fish food (Tetramin®) until one day before the beginning of the tests.

2.2 Chemicals and test concentrations

The commercial formulation DMA® 806 BR (purchased from Dow AgroSciences Industrial Ltda., Brazil) is 67% w/v active ingredient (a.i.) - 2,4-D acid equivalent, (80.6% w/v of - 2,4-D, dimethylamine salt) (41.9% w/v inert ingredients) and Regent® 800 WG (purchased from BASF, Brazil) is 80% w/v active ingredient (20% w/v of inert ingredients). In the sublethal toxicity and behavior tests with fish species, stock solutions of 1 g/L of 2,4-D (administered as DMA® 806 BR) and 3.2 mg/L of fipronil (administered as Regent® 800 WG) were used. The nominal test concentrations of each chemical were obtained by diluting their respective stock solutions in natural well water. The stock solutions and test concentrations were prepared immediately before testing.

151

152 *2.3 Chemical analysis of pesticides*

153 The stock solution and samples from the avoidance experiments for 2,4-D were diluted
154 in an H_2O/MeOH 70:30 v/v mixture and filtered through a syringe filter (PTFE 0.22 μm).
155 The stock solution of fipronil was diluted in a filtered H_2O/MeOH 70:30 v/v and filtered
156 (PTFE 0.22 μm), whereas samples from the avoidance experiments did not need dilution
157 were filtered through a syringe filter (PTFE 0, 22 μm) and then analyzed. The samples of
158 the behavior (swimming) experiments for 2,4-D and fipronil were prepared in the same
159 way as described previously for the escape experiments.
160 The quantification of the compounds was performed by liquid chromatography coupled
161 to tandem mass spectrometry (LC-MS/MS). An Agilent chromatograph model 1200 was
162 used, equipped with a binary pump, automatic injector and a thermostatic column
163 compartment. The chromatographic separation was performed with a Zorbax SB-C18
164 column (2.1x30 mm, the particle size of 3.5 μm) at 30 °C. The mobile phase consisted of
165 ultrapure water (A) and methanol (B), previously filtered through membranes with 0.2
166 μm porosity, containing 0.01% (v/v) NH4OH (an additive that favors the ionization of
167 the compounds). The instrumental quantification limits for 2,4-D and fipronil were 5 μg/L
168 and 0.5 μg/L, respectively. The linear working range was 5 to 300 μg/L for 2,4-D and 0.5
169 to 300 μg/L for fipronil.
170

171 *2.4 Unforced exposure system*

172 A multi-compartmentalized system was constructed by gluing 14 (2 bottles for each
173 compartment) 500 ml bottles (Indeplast®) in a row (Figure 1). The glue used was PU
174 Sealant General Purpose Geminni®. The compartments were opened at both ends so that
175 the fish could move freely between them. The connection between compartments was

performed using sections of transparent, non-toxic silicone hose. The system had seven compartments and a total volume of 2100 mL, with each compartment having a volume of 300 mL.

Figure 1

A spatial distribution control in the multi-compartmental experimental system was carried out in triplicate to validate the assumption that, in the absence of a chemical stressor, the fish had no spatial preference and would be randomly distributed among the seven compartments. Three fish of each species were transferred to each compartment containing only natural well water and their distribution was recorded after 10 hours (final experimental time) (n = 21). Avoidance control tests were performed by each species separately.

2.5 Avoidance tests

In the avoidance tests, only one species was introduced in the system, with a density of three individuals per compartment. Avoidance tests with a gradient of fipronil and 2,4-D were performed: 21 fish (3 fish per compartment). In each system, the same gradient of fipronil and 2,4-D were prepared from their respective stock solution with the following nominal concentrations: 6.25, 12.5, 25, 50, 75 and 100 µg for fipronil and 200, 400, 800, 1500, 2000 and 2500 µg a.i./L for 2,4-D. The tested concentrations were established using a literature review on the environmental concentrations of fipronil and 2,4-D in surface waters, as presented in the introduction. In addition, previous acute toxicity tests exposing *D. rerio* to Regent® (a.i. fipronil) and DMA® (a.i. 2,4-D) showed mean LC_{50}-96 h values of 172 ± 30 µg a.i./L for the insecticide and 483000 ± 18000 µg a.i./L for the herbicide (data not shown). Thus, experiments were carried out only with sublethal concentrations to fish.

To avoid mixing test solutions between compartments during the disposal of the concentrations, they were isolated from each other with non-toxic modeling clay. Afterwards, the fish were introduced and finally the plasticine plugs were removed. All tests (control without contaminants and avoidance with a gradient of pesticides) were performed in a dark room at 25 ± 2 °C. The distribution of individuals among the system compartments was initially recorded after 4 hours and every 2 h for 10 h of exposure (4, 6, 8 and 10 hours), in which the plasticine plugs were inserted in the connections between the adjacent compartments and allowed the quantification of fish in each compartment. The six clays were inserted simultaneously by three people. The exposure time (10 h) was established considering that it is enough time to detect avoidance response in fish and to prevent excessive mix among the concentrations (Araújo et al., 2014a). The organisms were not fed during the tests. All experiments were conducted in triplicate for each pesticide. At the end of the tests, the plasticine plugs were inserted between adjacent compartments to close the connections and the samples were then taken to determine the final concentrations of the pesticides.

2.6 Analysis of swimming behavior

Sublethal toxicity tests were performed for 10 h (total time of the avoidance experiment), exposing the fish *H. eques* and *D. rerio* to pesticides. The concentrations of the compounds were 50, 75 and 100 µg a.i./L for fipronil and 750, 1000 and 1500 µg a.i./L for 2,4-D. For both insecticide and herbicide, the tested concentrations are similar to the three highest concentrations tested in the avoidance experiment. The control consisted of natural well water, also used as dilution water.

Each experimental unit consisted of a plastic container (Prafesta®) containing 1 L of the test solution and two test organisms, with three replicates per treatment (n = 6). The

sublethal tests were carried out under the same conditions as the organisms were exposed in the avoidance experiments (25 ± 2 °C and in a dark room). Basic physical-chemical parameters of water, including pH, electrical conductivity, temperature, and dissolved oxygen were measured at the beginning and at the end of the test.

At the end of 10 h of exposure, video analyses were performed to record the swimming behavior of three individuals from each concentration (1 fish out of the 2 from each replicate). The videos were shot in Full HD 1080p (30 fps) on a 16MP resolution camera (size 4:3). An animal was placed in a round transparent plastic container (15 cm) with the respective tested concentrations of insecticide and herbicide and filmed in high definition for 1 min, and observations were made under the light. The fish were acclimated for 10 minutes before the videos started. During the filming time, the organism was stimulated 3 times timed with a light touch with a Pasteur pipette. Videos were analyzed using the Kinovea software (2020) v.0.8.26 (https://www.kinovea.org/), adapting the methodology used by Khayrullin et al. (2016) with *Danio rerio*. This software was calibrated using the measures of the pot to measure the maximum speed (m/s) and distance moved (cm) as parameters of the behavior in swimming.

2.7 Acetylcholinesterase (AChE) activity

The fish used to assess the swimming behavior were later used to analyze AChE activity. After 10 h of exposure, groups of 6 fish per treatment were collected together in microtubes, quickly frozen in liquid nitrogen and stored at -80 °C until enzymatic analysis. To evaluate the activity of AChE, the fish were fully homogenized in a 1:10 (weight/volume) ratio in a cold solution (4 °C) of 0.1 M potassium phosphate pH 7.8 and for 10 min at 4 °C the samples were centrifuged at 10,000 g. The enzyme was analyzed in the supernatant fraction. The enzymatic activity of AChE was performed in a

251 microplate reader - Spectramax® Plus 384, following the methodology of Ellman et al.
252 (1961). The formation of a derivative thiol produced by the action of the enzyme is
253 measured on the substrate, which reacts with the 5,5'-Dithiobis-(2-Nitrobenzoic Acid)
254 (DTNB) that was monitored at 412 nm at 30 °C for 5 mi (11 cycles with a 30 s interval
255 between the readings). We used acetylthiocholine (CAS Number 1866-15-5, Sigma-
256 Aldrich) as a substrate to analyze AChE activity. Activities were expressed in nmol min^{-1}
257 mg protein. The protein concentration was determined by the Bradford method (Bradford,
258 1976) at 595 nm, and as a standard bovine serum albumin (BSA) was used.
259
260 *2.8 Statistical analysis*
261 In the multi-compartmented approach, a mixed-design ANOVA with repeated
262 measurements was used to assess the distributions of the fish along the compartments as
263 described by Silva et al. (2018) and the calculation of the avoidance response (in %) was
264 based on Moreira-Santos et al. (2008). A more detailed description has been provided in
265 Supplementary Material. Finally, the concentrations that trigger avoidance for 20, 50 and
266 80% of the populations (AC_{20}, AC_{50} and AC_{80}, respectively) were calculated by PriProbit
267 (Sakuma, 1998) considering the mean values of the four exposure times.
268 The analysis of the swimming behavior data (maximum speed and distance moved) and
269 the biochemical marker of neurotoxicity (AChE) were performed using the SigmaPlot
270 v11.0 software (Systat, 2008). Normality (Shapiro-Wilk) and homogeneity of data
271 (Levene) were verified and differences between treatments were assessed by analysis of
272 variance (ANOVA). In the case of data that met the criteria of normality and
273 homoscedasticity, Dunnett's post-hoc was performed. For data that did not meet these
274 requirements, the Kruskal-Wallis non-parametric test was used, followed by Dunn's post-

hoc test. All statistical tests were considered significantly different when $p < 0.05$ (Systat, 2008).

3. Results

3.1 Validation of tests, abiotic variables, and chemical analyses

The initial concentrations of the swimming behavior and AChE enzyme activity tests were quantified. They were: 47.6 ± 2.55, 62.2 ± 2.40 and 100.1 ± 0.74 μg a.i./L for the respective nominal concentrations of fipronil of 50, 75 and 100 μg a.i./L. and, for 2,4-D, the values of 1169.38 ± 147.62, 1423.1 ± 24.61 and 2083 ± 99.6 μg a.i./L were detected for the respective nominal concentrations of 1000, 1500 and 2000 μg a.i./L. In the controls, none of the compounds were quantified (< quantification limits). For the representation of results, nominal concentrations were chosen.

For avoidance tests, the final concentrations were quantified at the end of the experiment, after 10 h. The fipronil values detected in the system compartments were: 15.5 ± 0.19, 6.1 ± 0.27, 9.4 ± 0.75, 40.2 ± 2.3, 66 ± 4.69, 75 ± 2.22 and 97.5 ± 4.48 μg a.i./L for the control and the respective nominal concentrations of 0, 6.25, 12.5, 25, 50, 75 and 100 μg a.i./L. The final concentrations of 2,4-D detected in the control compartment and in the rest of the system were: 84.3 ± 1.16, 220 ± 3.2, 448 ± 1.9, 790 ± 25, 1345 ± 86, 1894 ± 10 and 2613 ± 47 μg a.i./L for the respective nominal concentrations of 2,4-D: 0, 200, 400, 800, 1500, 2000 and 2500 μg a.i./L. Considering that (i) the quantification of concentrations was performed at the end of the tests, which supposes a mix among the concentrations in relation to the initial concentration, (ii) those concentrations were very similar to the initial nominal concentrations (except in the control compartments), and (iii) the avoidance response after 4 h (minimal mix) already showed the trend of the

organisms to avoid the chemicals, and it was decided to use the nominal concentrations to represent the results.

The physical-chemical conditions of the water remained stable throughout the experiments for all treatments, with temperatures ranging from 23.6 to 24.7 °C; pH from 6.8 to 7.3; electrical conductivity from 25 to 44.4 µS/cm and dissolved oxygen from 6 to 7.0 mg/L.

The fish distributions in the avoidance control tests (using natural well water) were analyzed for the experiment (21 fish per system) to determine whether the distribution was random. No avoidance or preference for any side of the system was recorded in the organisms tested. The fish distribution was random, with no statistically significant differences in the percentage of organisms between the compartments ($F_{6, 14} = 0.09$, $p = 0.99$ for *D. rerio* and $F_{6, 14} = 0.04$, $p = 0.99$ for *H. eques*).

3.2 Avoidance responses to the pesticide gradients

The mean data of avoidance response to the gradient of fipronil and 2,4-D of the two species fish, *D. rerio* and for *H. eques*, and the four observation periods in the tests are shown in Table S1. For *D. rerio* (Tables S2 and S3), the avoidance percentage of the organisms was influenced by the different concentrations of fipronil ($F_{6, 14} = 16.693$, $p < 0.001$), but not by the different time intervals observed ($F_{2.137, 29.922} = 1.819$ after Greenhouse-Geisser correction, $p = 0.178$) (Tables S2). There was also no statistically significant effect for the interaction of the two fixed factors (time and compartment) ($F_{12.824, 29.922} = 0.636$ after the Greenhouse-Geisser correction, $p = 0.850$). Considering the mean organisms' distribution, the avoidance percentage represented in Figure 2A shows an avoidance higher than 40% from 50 µg/L, reaching almost 100% at 100 µg/L.

When *D. rerio* were exposed to the 2,4-D contamination gradient, only the concentrations of the herbicide influenced the organisms' distribution ($F_{6, 14} = 70.716$, $p < 0.001$); for the time and the interaction of the two factors (time and compartments), there were no statistically significant effects (time: $F_{2.425, 33.943} = 1.217$ after Greenhouse-Geisser correction, $p = 0.315$; time and compartment: $F_{14.547, 33.943} = 0.892$ after Greenhouse-Geisser correction, $p = 0.577$) (Tables S3). For both pesticides, the organisms tended to avoid the compartments contaminated with the highest concentrations (50, 75 and 100 µg a.i./L of fipronil) and (1000, 1500 and 2000 µg a.i./L of 2,4-D), moving towards lower concentrations and control. The avoidance percentage was more marked from 1000 µg/L, whose values varied between ca. 30 and 60%, and at 2000 µg/L, when a mean avoidance of 95% was recorded (Fig. 2B).

Figure 2

For tests with *H. eques*, when exposed to the fipronil contamination gradient, the avoidance percentage was not determined by the concentration gradient ($F_{6, 14} = 2.192$, $p = 0.106$). Moreover, no statistically significant effects were observed for the different observation times and the interaction of the two factors (time and compartments) (time: $F_{1.806, 25.282} = 2.285$ after Greenhouse-Geisser correction, $p = 0.127$; time and compartment: $F_{10.835, 25.282} = 0.551$ after Greenhouse-Geisser correction, $p = 0.848$) (Table S4). Regarding the percentage of avoidance response, it was <12% in all the concentrations, except at 100 µg/L when it reached a maximum of 45% (mean avoidance of 33%) (Fig. 3A).

However, *H. eques* showed avoidance patterns dependent on the concentration gradient of 2,4-D ($F_{6, 14} = 8.176$, $p < 0.001$), but the different time intervals observed ($F_{1.367, 19.138} = 2.030$ after correction Greenhouse-Geisser, $p = 0.168$) and the interaction of the two fixed factors (time and compartment) ($F_{8.202, 19.138} = 1.428$ after the Greenhouse-Geisser

348 correction, $p = 0.247$) did not show any significant statistical differences (Table S5). Fish
349 (40% of the population) tended to avoid only the compartments with the highest
350 concentration (2000 µg a.i./L of 2,4-D) and the population was distributed along the
351 remaining concentrations (Fig. 3B).

352 **Figure 3**

353 *3.3 ACs in avoidance tests*

354 The values of AC_{20}, AC_{50} and AC_{80} of both pesticides for both species are shown in Table
355 1. These values were normally calculated from the mean avoidance percentage from the
356 four observation periods; however, ACs values were calculated considering the mean
357 avoidance percentage from the exposure times of 4 and 10 h (initial and final distribution)
358 because, in addition to showing a real decision about avoiding, the responses observed in
359 the intermediate times (6 and 8 h) were statistically lower and different, which show an
360 inconsistence and highly variable response. In general, *D. rerio* presented the lowest
361 values of ACs, indicating a higher capacity to detect and avoid both pesticides than *H.*
362 *eques*.

363 **Table 1**

364 *3.4 Swimming behavior of D. rerio and H. eques*

365 The average distance moved by *D. rerio*, compared to the control, was significantly
366 altered (decreased) when exposed to the three tested concentrations of fipronil (One Way
367 ANOVA; $F_3 = 6.833$; $p < 0.05$; Dunnet test, $p < 0.05$; Fig. 4A). However, the average
368 maximum speed was not affected in any of the treatments with the insecticide to which
369 the *D. rerio* fish were exposed (Kruskal-Wallis; $H_3 = 5.442$, $p = 0.142$, Fig. 4A).
370 Regarding the exposure of *D. rerio* to 2,4-D, the average distance moved (One Way
371 ANOVA; $F_3 = 1.398$ $p = 0.312$; Fig. 4B) and maximum speed (One Way ANOVA; $F_3 =$
372 2.584, $p = 0.126$; Fig. 4B) were not changed in relation to the control.

Figure 4

For the species *H. eques*, the average distance moved (One Way ANOVA; $F_3 = 2.482$, $p = 0.135$; Fig. 5A) and the average maximum speed (One Way ANOVA; $F_3 = 0.842$, $p = 0.508$; Fig 5A) were not affected when the fish were exposed to fipronil in relation to the control group.

When *H. eques* fish were exposed to 2,4-D, a frenetic movement due to an over-excitement was observed at the highest concentration, manifested by a significant increase in their maximum speed (0.40 ± 0.06 m/s) when compared to the control (0.18 ± 0.04 m/s) (One Way ANOVA; $F_3 = 4.583$; $p < 0.05$; Dunnet test, $p < 0.05$; Fig. 5B). However, no statistical differences were observed in relation to the control for the average distance moved (One Way ANOVA; $F_3 = 2.277$, $p = 0.157$; Fig. 5B), probably due to the high variability among replicates, as suggested by high standard error values.

Figure 5

3.5 Acetylcholinesterase (AChE) activity

The effect of 2,4-D and fipronil on AChE activity, after 10 h of exposure, is shown in Figure 6. Considering the comparison with the control group, the two highest concentrations of fipronil caused statistically significant inhibition of the enzyme activity in *D. rerio* (One Way ANOVA; $F_3 = 19.1$; $p < 0.05$; Dunnet test, $p < 0.05$; Fig. 6A). However, exposure of *D. rerio* to 2,4-D did not cause changes in the enzymatic activity of AChE (One Way ANOVA; $F_3 = 1.508$; $p = 0.243$; Fig. 6B). For *H. eques* exposed to both fipronil (One Way ANOVA; $F_3 = 3.058$; $p = 0.053$) and 2,4-D (One Way ANOVA; $F_3 = 0.131$; $p = 0.941$), no statistically significant differences were observed in relation to the control (see Figure S1).

Figure 6

4. Discussion

4.1 Avoidance response

In the present study, it was observed that the fish *D. rerio* and *H. eques* are able to detect and avoid compartments contaminated with high concentrations of the herbicide 2,4-D and that *D. rerio* is also capable of detecting and avoiding environmentally relevant concentrations of fipronil. Therefore, comparing the avoidance responses of the two species, *D. rerio* was more sensitive than *H. eques*.

Insecticide and herbicide perception can be beneficial for animals if there is a chance to escape to non-contaminated environments. However, the lack or reduced ability to perceive increasing contamination gradients, such as that observed for *H. eques* (which avoided only the highest concentration of the herbicide), might lead the organisms to occupy equal places with concentrations that could cause deleterious effects. This scenario was observed in tadpoles of *Lithobates catesbeianus* that did not avoid the lowest concentration (around 200 μg/L) of 2,4-D, and signals of stress due to toxicity were observed (Freitas et al., 2019). The difference regarding the magnitude of the avoidance observed by Freitas et al. (2019) and the current study could be attributed to the differences in the sensitivity of the organisms. Sometimes, compounds impair the ability of organisms to perceive increasing contamination gradients due to lethargic effects caused by toxicity (Gutierrez et al., 2012; Araújo et al., 2014b; Díaz-Gil et al., 2017).

Effects of 2,4-D on fish are found in the literature, e.g., *D. rerio* larvae showed a 61% reduction in survival and 24% in prey capture capacity when exposed to concentrations of 16 μg/L of the commercial formulation of herbicide (DMA4®IVM) (Dehnert et al., 2019). Neskovid et al. (1994) observed that *Cyprinus carpio* exposed to 2,4-D (150000 μg/L) for 14 days showed enzymatic dysfunctions such as the increased activity of glutamate oxaloacetate transaminase and decreased alkaline phosphatase, in addition to

causing morphological changes in the gills, liver, and kidneys. Likewise, previous studies reported the high toxicity of fipronil to fish, for example, DNA damage in erythrocytes of *Rhamdia quelen* (0.10 - 0.23 μg/L) (Ghisi et al., 2011), in the malformation of the zebrafish embryo (96 h after fertilization - hpf), mRNA transcription (≥ 31 μg/L) (Beggel et al., 2012) and miRNA transcription (520 μg/L) (Wang et al., 2010). Yan et al. (2016) also suggested the hepatoxicity of fipronil (400 μg/L) in *Danio rerio* due to variations in the saturated fatty acid content and in the primary bile acid synthesis process. Larvae of the Japanese medaka (*Oryzias latipes*) were exposed to fipronil (3, 10 and 30 μg/L), until 28 days post-hatching and the GABA blocker resulted in the down-regulation of follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone receptor (*lhr*) (Sun et al., 2014). Beggel et al. (2010) compares the sublethal toxicity of the fipronil, to their commercial formulation Termidor® to larval fathead minnow (*Pimephales promelas*), to determine effects on growth and swimming performance after short-term (24 h) exposure. Fipronil and Termidor led to a significant impairment of swimming performance at 142 μg/L and 148 μg/L respectively, with more pronounced effects for the formulation.

The comparison of the concentrations used in other studies in which toxicity was observed lead us to think that if *D. rerio* does not avoid contamination by 2,4-D, some toxic effects could be expected to occur. However, this comparison might be made with caution: for instance, avoidance by *D. rerio* to 2,4-D was significant from 750 μg/L, a concentration that for the same species seems to affect the capacity of larvae to capture prey (Dehnert et al., 2019). Therefore, it is plausible to hypothesize that, if the exposure to the gradient of contamination was extended for a long time, without causing physiological impairments, fish would be able to detect it and show a time-delayed avoidance: a time-correlated avoidance (Araújo et al., 2018).

447 The capacity of *D. rerio* to escape from contamination to prevent toxicity has been
448 reported in other studies: for instance, zebrafish avoided effluents of acid mine drainage
449 (Moreira-Santos et al., 2008), the fungicide pyrimethanil (Araújo et al., 2014a) and a
450 copper gradient (Silva et al., 2018; Islam et al., 2019). This type of response is directly
451 dependent on the capacity of organism to detect and correctly interpret the risk that the
452 exposure might suppose, which is dependent on bioavailability and toxicity of the
453 compounds (Solomon et al., 2009). The detection and escape of the highest
454 concentrations of fipronil (environmentally relevant concentrations) by the fish might be
455 related to the molecular structure of fipronil, which consists of a carbon atom linked to
456 three fluorine atoms (CF3) in positioning 4- (tri-fluor-methyl-sulfinyl-pyrazole). This
457 favors the dissolution of fipronil in organic matrices (lipids, oils and organic solvents)
458 and bioconcentration in the adipose tissue (Sun et al., 2016), causing quick adverse effects
459 at low concentrations. In fact, this rapid bioaccumulative potential of fipronil and its
460 metabolites in several aquatic animals, including fish of the species *Pampus*
461 *argenteus*, *Pseudosciaena crocea*, and *Larimichthys polyactis*, have already been attested
462 (Zhang et al., 2018).
463
464 *4.2 Swimming behavior and neurotoxicity*
465 The analysis of swimming has been considered a sensitive endpoint to detect the sublethal
466 effects of pollutants in fish (Kane et al., 2004; Eissa et al., 2010). Thus, it is known that
467 pesticides can cause abnormal swimming behavior or impaired swimming ability in fish
468 and other aquatic organisms (Altenhofen et al., 2017; Freitas et al., 2019). In the present
469 study, *D. rerio* exposed to fipronil (50, 75 and 100 µg a.i./L) did not show changes in
470 swimming activity concerning the average maximum speed; however, the distance moved
471 was lower in all exposure concentrations. This response might be possibly associated with

472 the neurotoxic effect of fipronil, in which changes (inhibition) in AChE activity were
473 observed for the two highest concentrations (75 and 100 µg a.i./L).
474 Changes in swimming behavior in adults of *D. rerio* exposed to fipronil single and in a
475 mixture with glyphosate in concentrations of 9 and 18 µg/L were observed by Chaulet et
476 al. (2019). Behavioral changes in *D. rerio* embryos were also observed when exposed to
477 insecticide fipronil (Stehr et al., 2006 and Yan et al., 2016). In accordance with Stehr et
478 al. (2006), 333 µg/L fipronil causes abnormalities in the morphology of the muscle fibers
479 of the larvae (2-48 hpf), resulting in bilateral contractions of the axial muscles and leading
480 to irreversible degeneration of the notochords. Moreover, according to Yan et al. (2016),
481 the spinal deformities observed in *D. rerio* exposed to 400 µg/L of fipronil may be related
482 to the interruption of the aminoacyl-RNA transporter biosynthesis in the embryos, which
483 may be related to malformations. Reactions of behavioral changes in swimming have also
484 been reported in other teleosts such as carp (Qureshi et al., 2016) and Nile tilapia (El-
485 Muhr et al., 2015) exposed to fipronil. According to Huang et al. (2019), these changes
486 occur when fipronil binds to the GABA receptor chlorine channels, preventing its closure,
487 leading to neuronal over-stimulation and triggering hyperactivity, spasms, convulsions
488 and, in more severe cases, death. Besides, Wang et al. (2016) pointed out that fipronil can
489 act through disturbances in several metabolic pathways. Fipronil can also be a potential
490 neurotransmission disruptor, such as glycine (Grillner, 2003), which is an inhibitory
491 neurotransmitter in the central region of the nervous system, mainly in the spinal cord
492 (López-Corcuera et al., 2009).
493 Only fish of the species *H. eques* submitted to a higher concentration (2000 µg/L) of the
494 herbicide 2,4-D showed changes in swimming activity, in which the average maximum
495 speed was altered (increased), but with no change in the average distance moved. This
496 response indicates that fish had an erratic and frenetic movement due to an over-

497 excitement of the higher concentration (2000 µg/L). Unlike what was observed in the
498 present study, Gaaied et al. (2019) reported (i) inhibition (decrease) of the swimming
499 distance in *D. rerio* embryos (at 120 hpf) in low doses (20 µg/L) of that same herbicide
500 and (ii) effects on the cholinesterase activity (at 96 hpf), demonstrating that 2,4-D can
501 alter the cholinergic system by affecting ChE activity that may be involved in the
502 locomotion decrease of exposed larvae. In the present study, no changes were observed
503 in the enzymatic activity of AChE in any 2,4-D concentration, probably due to the short
504 exposure period (10 h) and the experiments being carried out with adult fish, that are less
505 sensitive than embryos for this parameter (Sanches et al, 2018 and references therein).
506 In summary, avoidance can be recorded even if no evident toxicity signal is detected,
507 therefore it might be used as a warning response able to detect the minimal evidence of
508 risk (toxicity). Thus, avoidance has been considered an environmentally realistic endpoint
509 as it prevents animals from being continuously exposed and suffering the toxic
510 consequences (Oliveira et al., 2013). The present study makes the importance clear of
511 integrating both exposure approaches (forced and non-forced) to obtain a more complete
512 view on the possible risks that contaminants can cause to aquatic ecosystems. In addition,
513 our results are a matter of concern for biota conservation, as low concentrations of
514 pesticides may lead to the displacement of fish to more favorable areas when available,
515 but in a scenario where agricultural practices and production will increase in the coming
516 years, the existence of available decontaminated areas, suitable to accommodate aquatic
517 life, is uncertain.
518
519 **5. Conclusions**
520 Both species, *H. eques* and *D. rerio*, were able to detect 2,4-D and move to less
521 contaminated compartments. However, only *D. rerio* avoided environmentally relevant

concentrations of fipronil. Fipronil, in environmentally relevant concentrations, affected swimming (distance moved) only in *D. rerio*, and 2,4-D affected only swimming (maximum speed) by *H. eques*. The enzymatic activity of AChE was altered (inhibited) only when *D. rerio* was exposed to fipronil, with no changes observed when fish are exposed to 2,4-D. From the avoidance responses to pesticides observed and the sublethal effects described, it can be concluded that avoidance tends to occur in a short time, showing that this endpoint is a sensitive tool to detect sublethal effects of pollutants in fish. Avoidance response seemed not to have been affected by possible effects of contaminants on swimming behavior and Ache activity.

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Conflict of Interest The authors declare that they have no conflict of interest.

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749 **Figure Captions**

750

751 **Figure 1**: Schematic diagram of the unconfined multi-compartment experimental system.
752 The second panel is an overhead view.

753

754 **Figure 2**: Avoidance (%) of *Danio rerio* with 21 fish (with mean values + SE of three
755 repetitions for the four observation times) at a concentration gradient of Regent® (a.i.
756 fipronil) (A) and DMA® 806 BR (a.i. 2,4-D) (B). Different letters above the lines indicate
757 significant statistical differences, considering the means of the four observation times (p
758 < 0.05).

759

760 **Figure 3**: Avoidance (%) of *Hyphessobrycon eques* with 21 fish (with mean values + SE
761 of three repetitions for the four observation times) at a concentration gradient of Regent®
762 (a.i. fipronil) (A) and DMA® 806 BR (a.i. 2,4-D) (B). Different letters above the lines
763 indicate significant statistical differences, considering the means of the four observation
764 times ($p < 0.05$).

765

766 **Figure 4**: Swimming behavior of *Danio rerio* exposed to Regent® (a.i. fipronil) (A) and
767 DMA® 806 BR (a.i. 2,4-D) (B) indicating maximum speed (m/s, light gray) and distance
768 moved (cm, dark gray). Bars represent the mean + SE of three repetitions for each
769 treatment. The asterisk indicates a significant difference between the tested concentration
770 and control ($p < 0.05$).

771

772 **Figure 5**: Swimming behavior of *Hyphessobrycon eques* exposed to Regent® (a.i.
773 fipronil) (A) and DMA® 806 BR (a.i. 2,4-D) (B) indicating maximum speed (m/s, light

774 gray) and distance moved (cm, dark gray). Bars represent the mean + SE of three
775 repetitions for each treatment. The asterisk indicates a significant difference between the
776 tested concentration and control ($p < 0.05$).
777
778 **Figure 6**: AChE activity (nmol/min/mg protein) of *Danio rerio* after 10 h of exposure to
779 Regent® (a.i. fipronil). AChE activity is expressed as mean ± SE (n = 6). The asterisk
780 indicates a significant difference between the control and the tested concentrations ($p <$
781 0.05).
782

Graphical Abstract

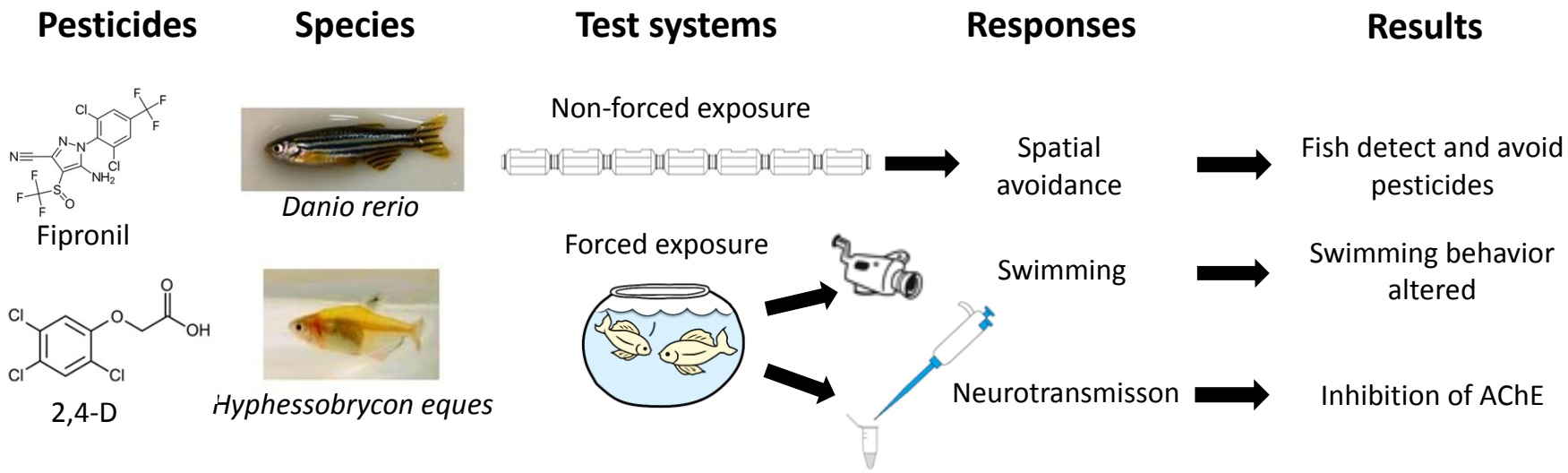

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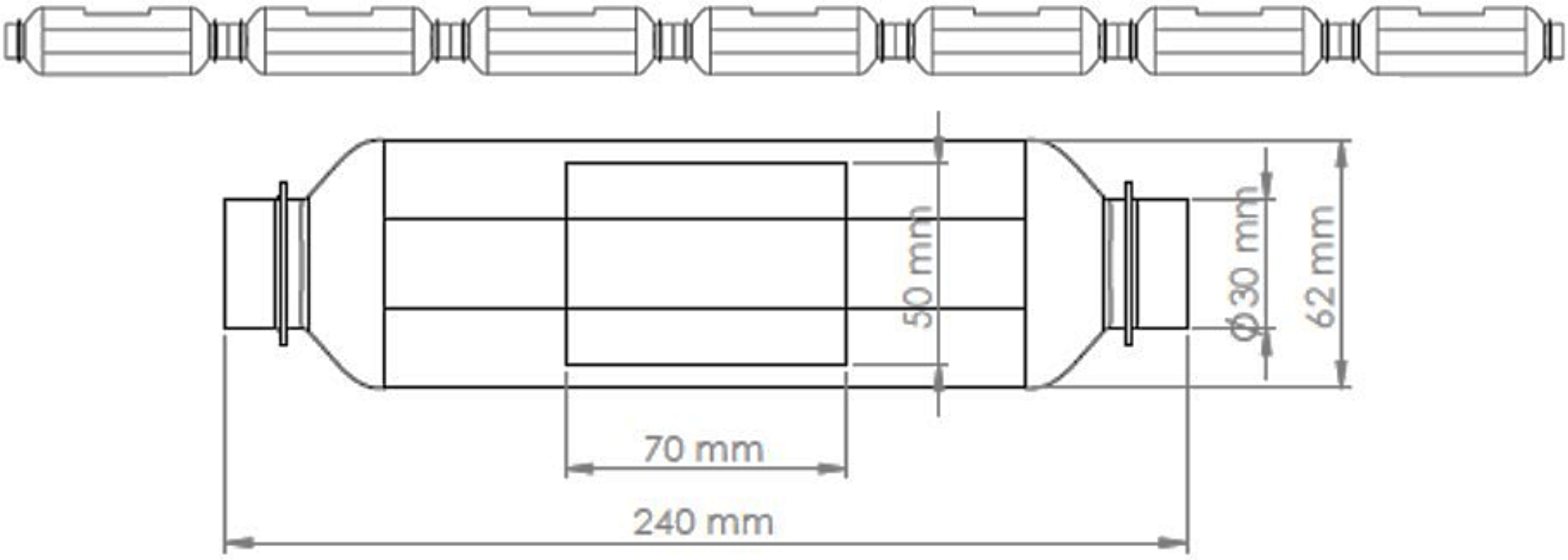
Fig. 1



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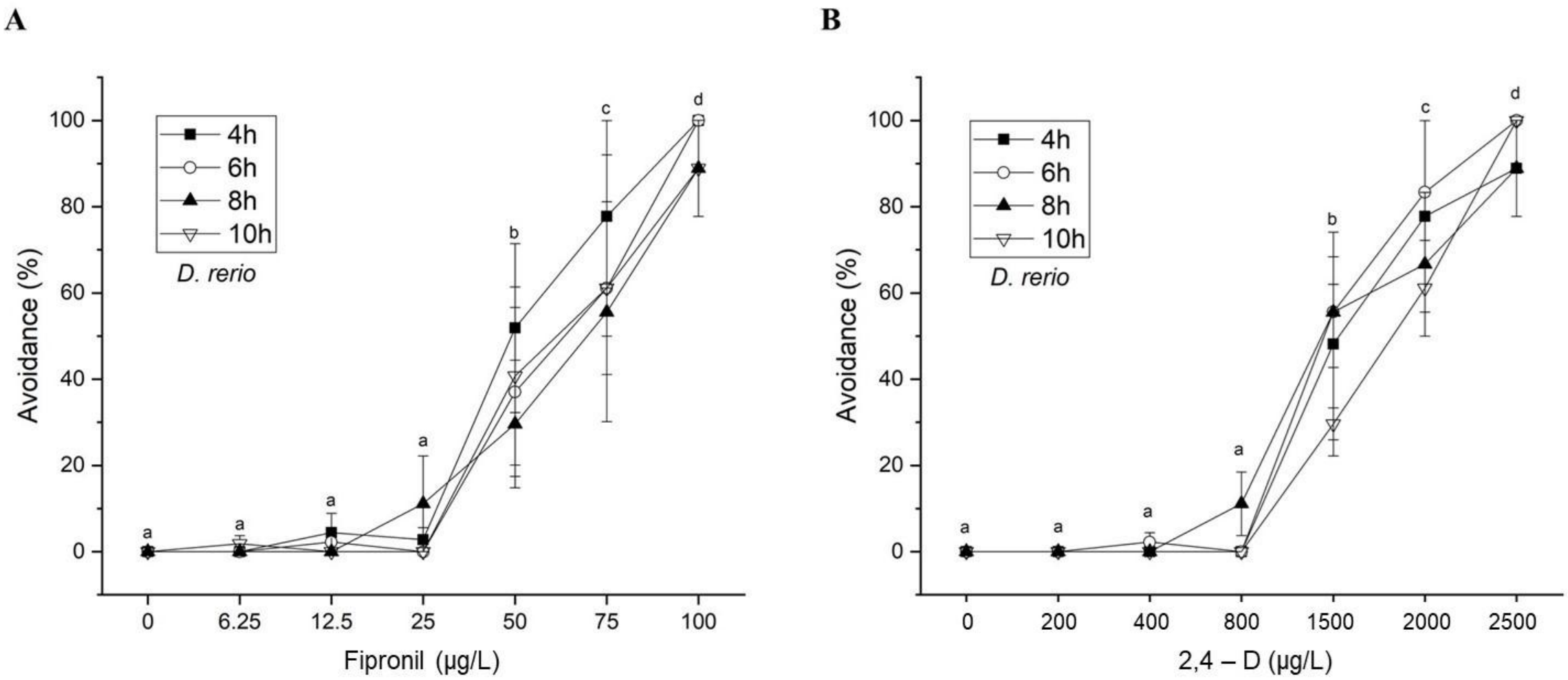

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Fig. 3

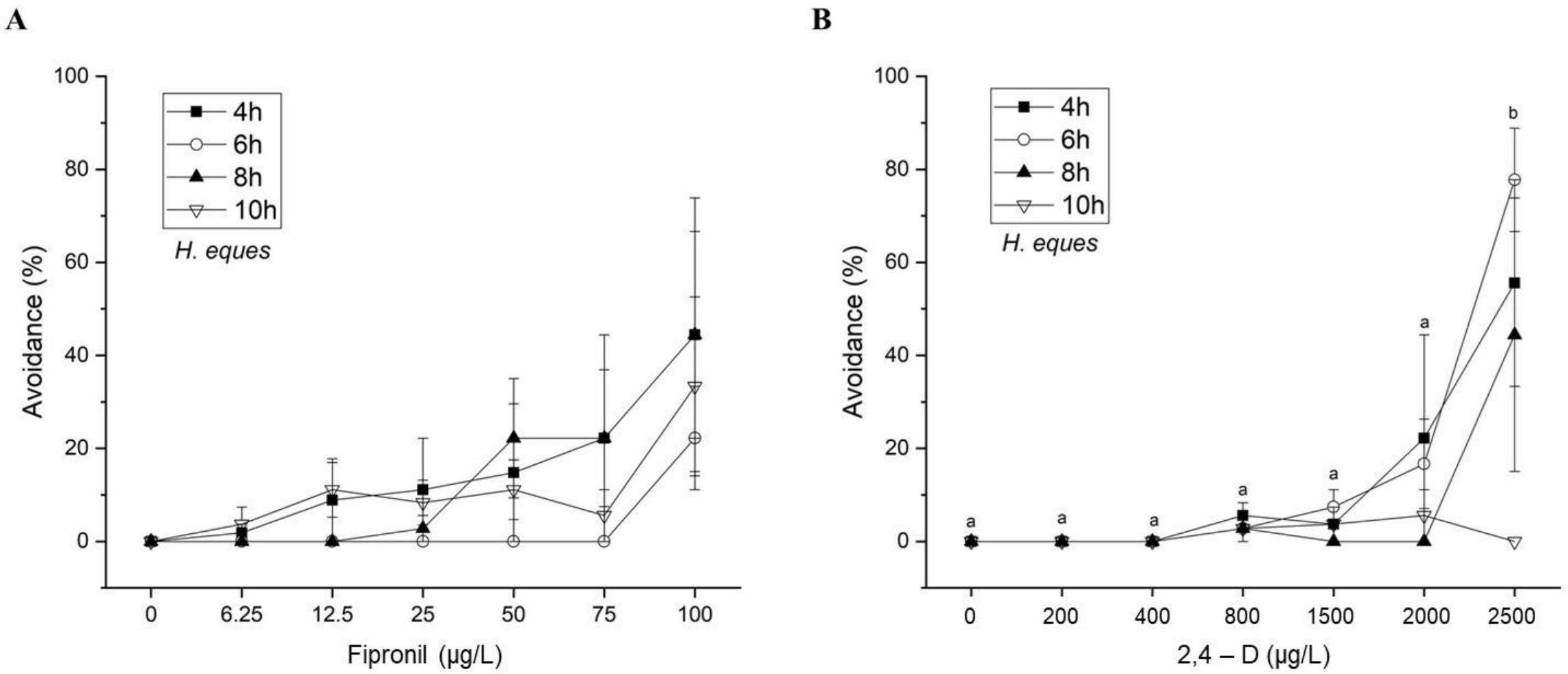

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Fig. 4

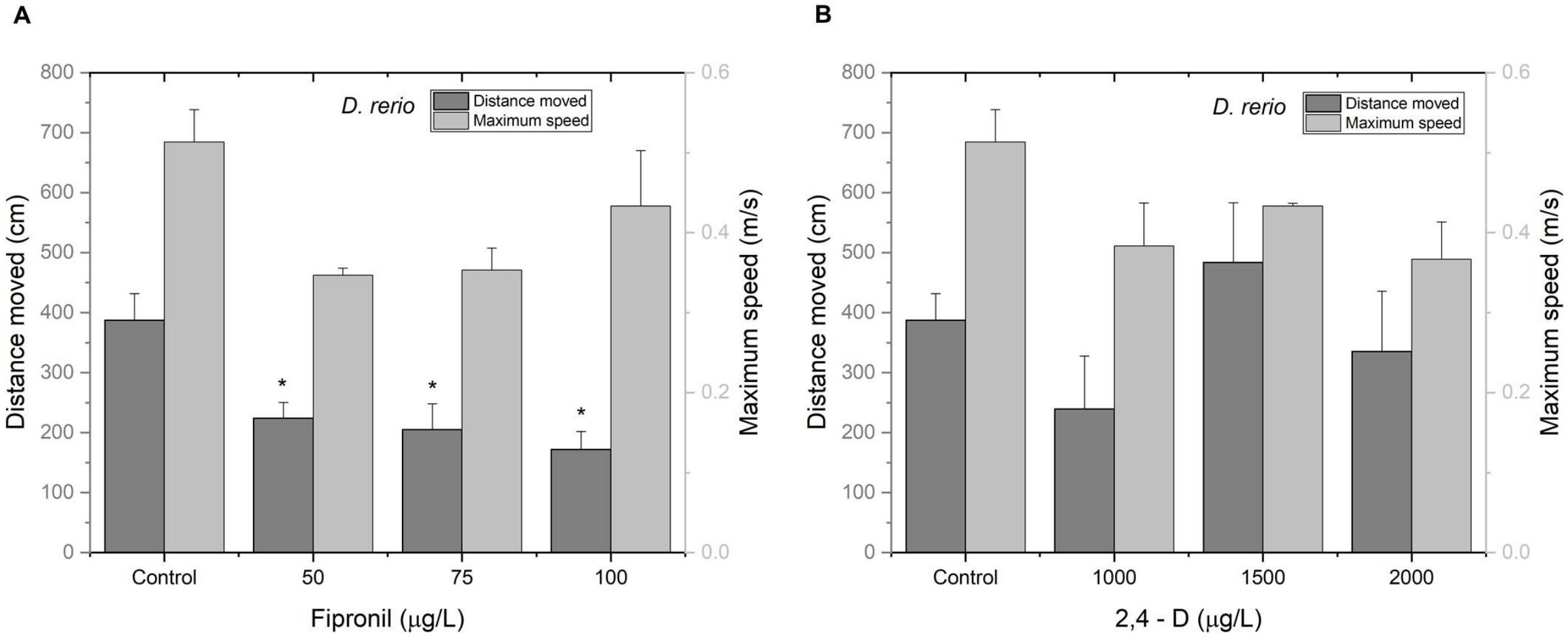

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Fig. 5

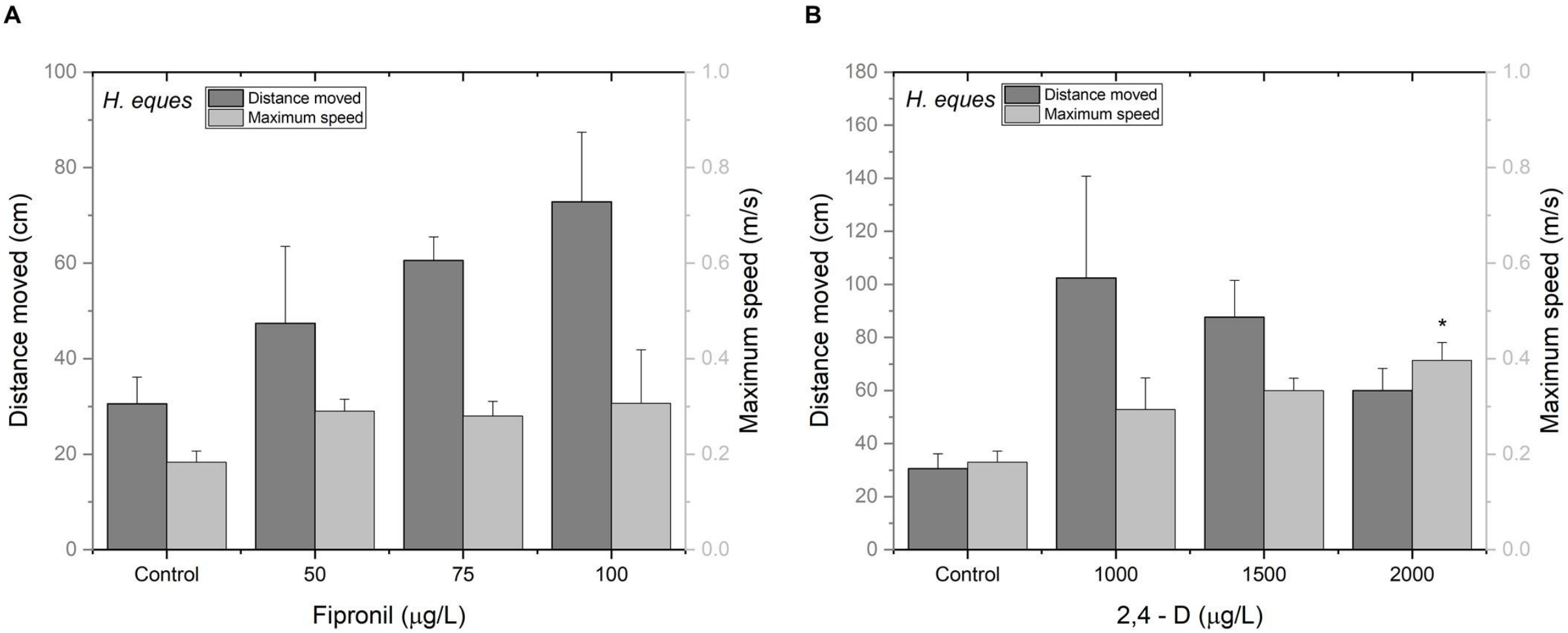

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Fig. 6

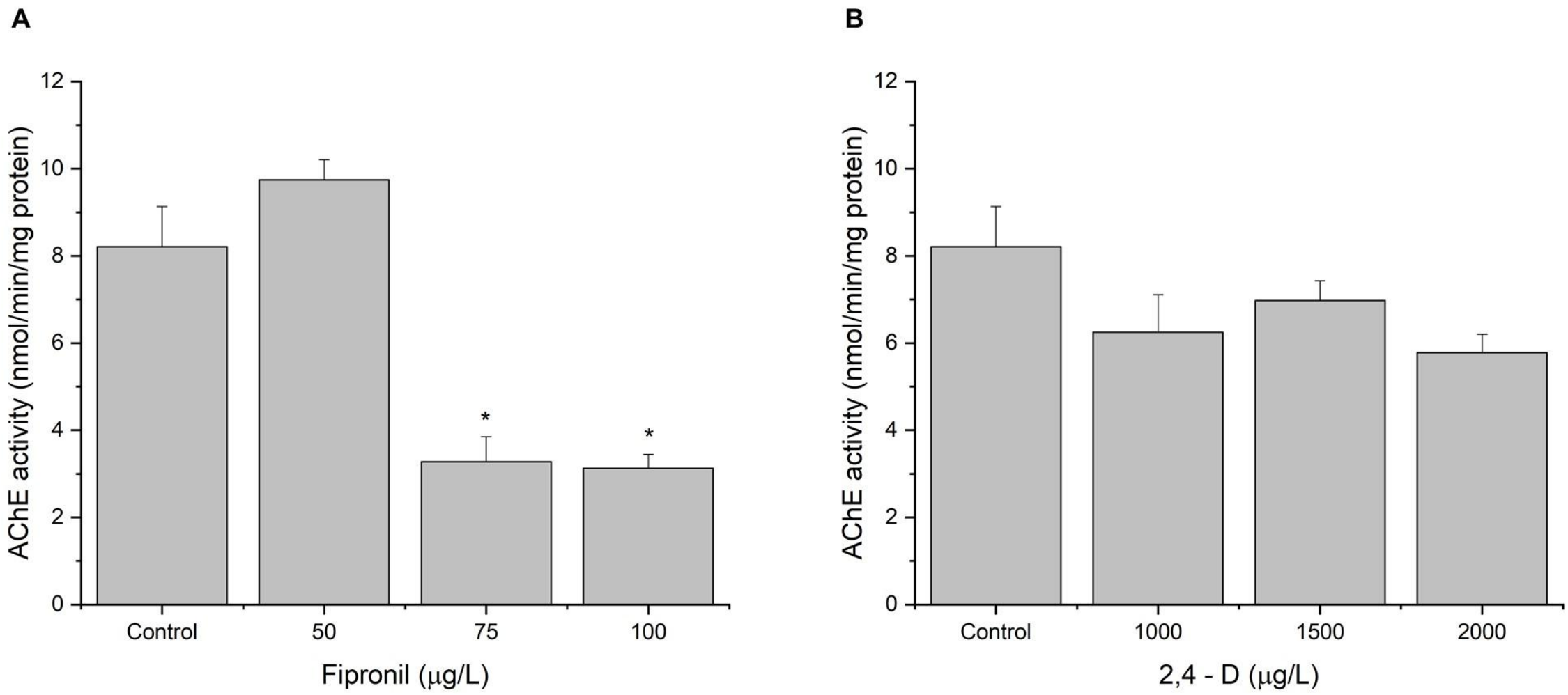

Table1
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Table 1. Values of the concentrations (in µg/L) and respective confidence intervals triggering avoidance response to 20, 50 and 80 (AC_{20}, AC_{50} and AC_{80}, respectively) of the fish populations (*Danio rerio* and *Hyphessobrycon eques*) exposed to gradients of fipronil and 2,4-D.

Pesticide	Species	AC_{20}	AC_{50}	AC_{80}
Fipronil	*D. rerio*	36 (nc)	56 (nc)	88 (nc)
	H. eques	71 (44-178)	>100 (nc)	>100 (nc)
2,4-D	*D. rerio*	1128 (657-1390)	1537 (1181-1857)	2093 (1744-3016)
	H. eques	1991 (1004-4497)	>1500 (nc)	>1500 (nc)

nc: not calculated. Values >100 or >1500 indicate that the AC values were higher than the highest tested concentration.

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