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Short and long-term exposure to the pesticides fipronil and 2,4-D: Effects on behavior and life history of *Daphnia magna*

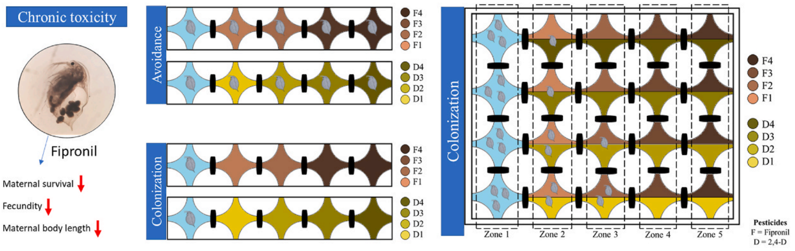
Raquel A. Moreira [a,*], Curro Polo-Castellano [b], Andrea Cordero-de-Castro [b], Mariana A. Dias [c], Thandy J.S. Pinto [a], Cassiana C. Montagner [c], Evaldo L.G. Espíndola [a], Julián Blasco [b], Cristiano V.M. Araújo [b]

[a] *NEEA/CRHEA/SHS and PPG-SEA, São Carlos Engineering School, University of São Paulo, Av. Trabalhador São Carlense, 400, 13.560-970, São Carlos, Brazil*
[b] *Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (ICMAN - CSIC). Campus Universitario Río San Pedro, 11519, Puerto Real, Spain*
[c] *Analytical Chemistry Department, Institute of Chemistry, University of Campinas, Campinas, São Paulo, Brazil*

HIGHLIGHTS

- Chronic toxicity and avoidance of fipronil and 2,4-D were studied in *D. magna*.
- The contamination by pesticides in the colonization of habitats by daphnids was studied.
- Environmentally relevant concentrations of pesticides were not repellent for *D. magna*.
- Daphnids did not move to contaminated zones in the colonization experiments.
- Negative consequences were observed, due to the chronic exposure to fipronil.

GRAPHICAL ABSTRACT



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ABSTRACT

The high levels of contamination in aquatic ecosystems caused by pesticides and the organisms' consequent continuous exposure to it has made them vulnerable to damage. However, mobile organisms can avoid this continued exposure to contaminants by moving to less disturbed habitats. Therefore, through the use of the Heterogenous Multi-Habitat Assay System (HeMHAS), our objective was to evaluate the ability of *Daphnia magna* to detect and avoid habitats contaminated by fipronil and 2,4-D, in a spatially connected landscape. Further, the role of contamination by these pesticides, isolated and in mixtures, concerning the colonization of habitats by daphnids was also evaluated. Given that not all organisms successfully escape contamination, the chronic toxicity of the same pesticides using different parameters for *D. magna* (maternal survival, fecundity and maternal body length) was also evaluated. When evaluating the avoidance response by *D. magna* exposed to pesticides, there was no preference for the less contaminated areas for both compounds. However, organisms did not move to contaminated zones in the colonization experiments, with no immigration of daphnids to the zones with intermediate and the highest levels of fipronil, nor to the highest concentration of 2,4-D. Finally, the colonization by daphnids was significantly prevented when exposed to a mixture of the pesticides, in which the areas with the highest combinations of pesticide concentrations were not colonized by *D. magna*. Regarding the long-term

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* Corresponding author. NEEA/CRHEA/SHS, São Carlos Engineering School, University of São Paulo, Av. Trabalhador São Carlense, 400, 13.560-970, São Carlos, Brazil.

E-mail address: raquel.moreira87@yahoo.com.br (R.A. Moreira).

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chronic effects, negative consequences were observed, particularly for maternal body length, fecundity and maternal survival, due to the exposure to fipronil. Considering that pesticides can limit the areas colonized by organisms by making them unattractive, the risk of local population extinction may be underestimated if only standard endpoints involving forced exposure are studied.

1. Introduction

Traditional intensive agricultural practices are known by multiple applications of pesticides, whose mixtures enter aquatic ecosystems potentially causing adverse effects on all biota (Albuquerque et al., 2016; Acayaba et al., 2020). The insecticide fipronil and the herbicide 2,4-D are used for many crops (sugarcane, rice, soybeans, wheat and corn), and their simultaneous occurrence has been reported in aquatic environments around the world in concentrations ranging from 0.5 to 10 μg/L of fipronil (Gan et al., 2012; Wu et al., 2014; Michel et al., 2016), reaching 465 μg/L in São Paulo, Brazil (CETESB, 2018) and 0.06–12 μg/L of 2,4-D (Islam et al., 2019), for which a high value of 366.6 μg/L has already been found in São Paulo, Brazil (CETESB, 2018).

Among aquatic biota, microcrustaceans are model organisms widely used in Ecotoxicology (Zhang et al., 2016), as disturbances at this trophic level can affect the food chain, posing a threat to aquatic life (Gebara et al., 2021). *Daphnia* sp. is a key freshwater crustacean with important ecological roles. They are filter-feeders that regulate the population density of different microorganisms, as algae, cyanobacteria, and protozoa (Stollewerk, 2010) and they are a food source for various species of predators. These ecological roles are crucial to ecosystem services, which are dependent on the preservation of habitats and maintenance of the natural ecosystem functions (Lomartire et al., 2021).

An important consequence of aquatic contamination by pesticides is the loss of habitat continuity (Mena et al., 2022), that in terms of environmental quality, results in an uneven distribution. If the levels of occurrence of contaminants in certain areas limit the displacement of animals, even if there is no physical barrier, then a habitat can be chemically fragmented (Maes et al., 2008; Fuller et al., 2015). The possibility of using the concept of a chemical barrier in ecotoxicological studies The use of the concept of chemical barrier in Ecotoxicology could help researchers to understand the role of contamination, which may disturb the functioning and balance within the ecosystem and interrupt spatial continuity, this can increase the vulnerability of populations and lead to local extinctions (Ribeiro and Lopes, 2013).

In this context, the general hypothesis of this study assumes that contamination by pesticides in aquatic environments can isolate daphnid populations, even if the mobility of the organisms is not spatially restricted. Thus, through the use of Heterogenous Multi-Habitat Assay System (HeMHAS), our primary objectives were: *i*) to evaluate the ability of *Daphnia magna* to detect and avoid habitats contaminated by gradients of fipronil and 2,4-D, singly, in a spatially connected landscape and *ii*) to evaluate the role of contamination by fipronil, 2,4-D, and mixtures of these pesticides (zones of contamination), in preventing the colonization of new habitats by *D. magna*, defining the threshold of contamination towards which daphnids will not migrate. Furthermore, considering that not all organisms escape from contamination and that the conditions do not always favor this flight, a forced exposure scenario was used to assess the toxicity of the pesticides fipronil and 2,4-D, on different life history traits in *D. magna* (maternal survival, fecundity and maternal body length) simulating situations, without the possibility to flee, when organisms are exposed to contamination.

2. Materials and methods

2.1. Test organisms and culture conditions

Daphnia magna was obtained from a culture at the University of Jaén, Spain. Cultures were kept according to US EPA (2022) in 1-L glass beakers (20–30 ind/L) containing commercial mineral water (<4 g P/L) with a total hardness of 209 mg/L, as described in Álvarez-Manzaneda et al. (2017), at 20 ± 2 °C and 16 h:8 h (light: dark). Food (*Scenedesmus* sp. 5×10^4 cells mL^{-1}) was provided *ad libidum* three times a week. Third to fifth brood neonates (≤24 h) were obtained from cultures as the test-organisms (OECD 202 2000; OECD 211 2012).

2.2. Chemicals and test concentrations

The following commercial formulations were used: Regent® 800 WG (a.i. fipronil), purchased from BASF, Brazil and DMA® 806 BR (a.i. 2,4-D), purchased from Dow AgroSciences Industrial Ltda., Brazil. Stock solutions of 1.6 mg/L of fipronil and 1000 mg/L of 2,4-D, administered as commercial formulations, were used for the behavioral tests and chronic toxicity with *D. magna*. The test concentrations were prepared by diluting the stock solutions with culturing water.

2.3. Chemical analysis of pesticides

For the chemical analysis, samples were initially diluted in methanol (1:1) and filtered in a PTFE syringe filter (pore size of 0.22 μm, diameter of 13 mm). The compounds were quantified by liquid chromatography coupled with mass spectrometry (LC-MS/MS) (Goulart et al., 2020). Fipronil and 2,4-D concentrations were determined by using external calibration curves. The instrumental quantification limits (LOQ) were determined by the signal-to-noise ratio (SNR) method using an SNR of 10:1. LOQs for fipronil and 2,4-D were, respectively, 0.1 μg/L and 0.5 μg/L. The linear working range was 0.1–100 μg/L for fipronil and 0.5–500 μg/L for 2,4-D.

2.4. Non-forced exposure system: HeMHAS - heterogeneous multi-habitat assay system

The HeMHAS consists of compartments of 320 mL each, with 24 compartments in total. It is a two-dimensional (unforced exposure) system that simulates various contamination scenarios (Araújo et al., 2018) (Fig. S1). The system is constructed of a thermoplastic material (polyoxymethylene) to reduce the adsorption of contaminants. In the present study, we used 20 compartments, due to the number of concentrations tested. HeMHAS has connectivity between all adjacent compartments, in which connections can be opened or closed, thus allowing the simulation of an environment composed by patches of contamination.

2.5. Habitat selection: avoidance tests

Gradients of 2,4-D and fipronil in the experiments with the single compounds were prepared with four nominal concentrations: 125, 250, 500 and 750 μg a.i./L for 2,4-D and 2.5, 5, 10 and 20 μg a.i./L for fipronil. The different concentrations were put into the compartments of HeMHAS, with the doors connecting the compartments closed to maintain the gradients. Then, *D. magna* (10 individuals) was inserted into each compartment (320 mL). As the pesticides gradient avoidance experiments were performed with 4 concentrations plus a control for each compound, 50 daphnids were used per replicate and 4 replicates were tested (Fig. 1). A fully non-contaminated experiment (control) was performed, with four replicates, to verify if daphnids in the absence of contamination present any trend in moving to certain compartments. For this, each compartiment of the HeMHAS was filled with 320 mL of

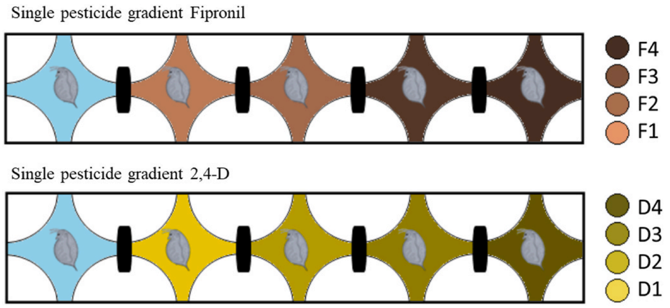

Fig. 1. Scheme of the experimental design for the avoidance experiments. The concentration of fipronil (F) and 2,4-D (D) increases horizontally. The concentrations of the F1–F5 and D1-D5 are shown in Table 1. Then, *D. magna* (10 individuals) was inserted into each compartment (320 mL).

culture water and ten daphnids were inserted into each compartment. In all the experiments (control and with pesticides), daphnids aged between 6 and 8 days were used and the number of organisms in each HeMHAS compartment was registered at a time interval of 30 min during the first 4 h and then after 24 h.

2.6. *Habitat selection: colonization tests*

For the colonization tests with the compounds acting alone, the fipronil and 2,4-D gradients were prepared with the same concentrations adopted for the avoidance test. After placing the concentrations of the pesticides into the system, 50 daphnids were put in the control compartment (the first compartment without contamination) to assess what was the highest concentration organisms would still colonize. Each experiment was performed in triplicate, totaling 150 daphnids per experiment. In these experiments, the daphnids could only colonize the compartments linearly, extending in one dimension.

A fully non-contaminated control system was also analyzed, with three replicates, to verify the colonization pattern in the pesticide absence (linear one-dimension displacement). For this, each compartment was filled with 320 mL of culture water. Then, 50 daphnids were inserted into the first clean area, totaling 150 daphnids into the system.

For the colonization tests using a mixture of pesticides, a full factorial experimental design, which included a set of 16 combinations. In this second scenario, all the connections between the compartments were opened, from which the organisms could transit throughout system (2D: longitudinal and transverse displacement). The proportions of fipronil and 2,4-D in the mixtures are shown in Table 1. A total of 180 daphnids were exposed to system (45 daphnids in each of the four clean areas; 2 replicates). The organisms were always introduced into the first compartment without contamination. In order to establish contamination zones with mixtures of both pesticides, five contamination zones were established (Fig. 2), as follows: Zone 1: control (dechlorinated water without contamination at the beginning of the experiments); Zone 2: Combination of mixtures (M1-M4); Zone 3: Combination of mixtures (M5-M8); Zone 4: Combination of mixtures (M9-M12) and Zone 5: Combination of mixtures (M13-M16).

A second control test (2 replicates) was performed for the mixtures of pesticides (contamination zones). The system compartiments were filled with 320 mL of clean culture water and 45 daphnids were inserted into each one of the four clean areas (Zone 1), totaling 180 daphnids within the system. While the first control test was used to refute the presence of any other factor influencing the organisms' behavior, the daphnia distribution obtained for each zone (Z1-Z5) during the control test simulating the experiment with the mixtures was used to compare with the results obtained in the respective zones during the experiments with mixtures of both pesticides.

In all the experiments, the number of daphnids colonizing each compartment was registered in a 30 min interval during the first 4 h period and after 24 and 48 h. Daphnids aged between 6 and 8 days were used. All tests were maintened under constant lighting. The longer exposure time for the colonization test was used considering that this period is sufficient for the daphnids to reach all the compartments available to them in the system, in a contamination-free scenario. Food was not provided during the experiment. Water samples were taken to determine the concentrations of pesticides at the beginning and at the experiment ends.

Table 1
Labels adopted for the avoidance test treatments. Treatments with F refer to fipronil (Regent), and D to 2,4-D (DMA) and M to mixtures.

Treatments	Concentrations	
	Regent (μg a.i./L)	DMA (μg a.i./L)
C	0	0
F1	2.5	0
F2	5	0
F3	10	0
F4	20	0
D1	0	125
D2	0	250
D3	0	500
D4	0	750
M1	2.5	125
M2	2.5	250
M3	2.5	500
M4	2.5	750
M5	5	125
M6	5	250
M7	5	500
M8	5	750
M9	10	125
M10	10	250
M11	10	500
M12	10	750
M13	20	125
M14	20	250
M15	20	500
M16	20	750

2.7. *Chronic toxicity tests*

The chronic tests were carried out according to OECD 211 (2012) protocol, using the following nominal concentrations: 0, 1.25, 2.5, 5, 10, and 20 μg a.i./L for fipronil, administered as Regent® 800 WG, and 0, 62.5, 125, 250, 500, and 750 μg a.i./L for 2,4-D, administered as DMA® 806 BR. One neonate (<24 h) was inserted in each of ten replicates (100 mL) per test concentration and control (culturing water). The light and temperature conditions and feeding regime were the same as the culture. The test solutions were fully renewed every other day when the mother's survival and the number of neonates produced were recorded. Maternal body length (mm), maternal survival (days), and fecundity (neonates per female) were the parameters evaluated. *D. magna* body length was measured only in the experiment ending by using a stereomicroscope and a micrometer ruler. The length was determined as a line from the top of the head to the tip of the carapace - the rear end of the organism.

2.8. *Statistical analysis*

Avoidance responses for the experiments with the isolated design over time were analyzed by ANOVA with repeated measures. Time was treated as a within-effect factor and each compartment as a between-effect factor. Mauchly's test was used to assess the repeated measures' sphericity. In the case of non-compliance, the degrees of freedom were adjusted by the Greenhouse-Geisser correction. For the colonization assays, the organisms' distribution was compared with the responses recorded in the compartments without contamination during the control test by the Student's T-Test. The data normality and homocedasticity were assessed by the Shapiro-Wilk and Levene tests, respectively.

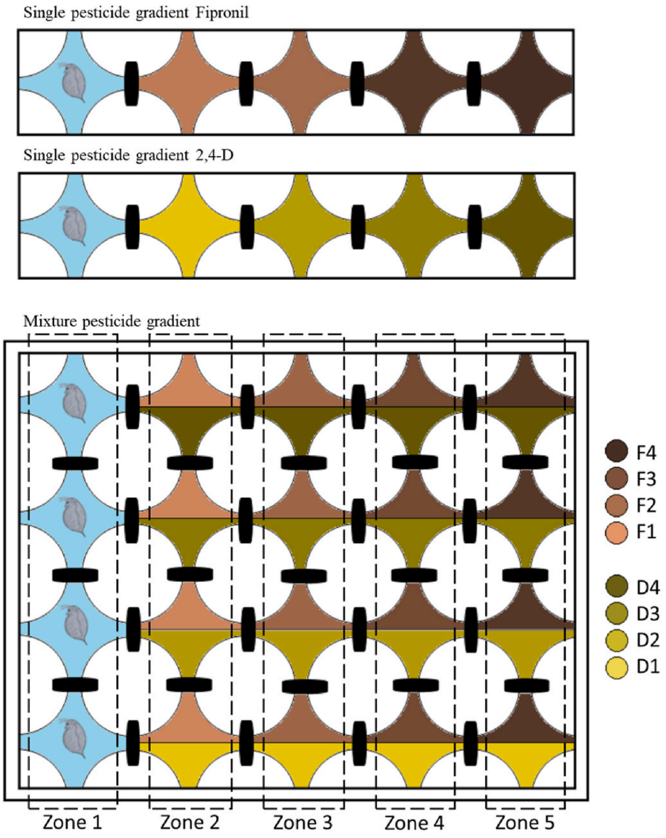

Fig. 2. Experimental design for the mixtures of pesticides (contamination zones). While the concentration of fipronil (F) increases vertically, the concentration of 2,4-D (D) increases horizontally. Zone 1: uncontaminated zone (cultivation water); Zone 2: Combination of mixtures (M1-M4); Zone 3: Combination of mixtures (M5-M8); Zone 4: Combination of mixtures (M9-M12) and Zone 5: Combination of mixtures (M13-M16). The proportions of the M1-M16 mixtures are shown in Table 1. Fifty and forty-five daphnids were introduced into the control compartment (the first compartment without contamination) to assess the maximum concentration the organisms would colonize, when the compounds act singly and in mixtures, respectively.

The maternal survival in the chronic tests was verified by using the Fisher's exact test. For the other chronic parameters, the data normality and homocedasticity were first cheched, as previously described. One-way ANOVA followed by the Fisher's post-hoc test was used for normally distributed data. In the contrary (non-normal data), the Kruskal-Wallis test with the Dunn method (multiple comparison) was used (Zar, 1996). All analyses were carried out with a confidence interval of 95% ($p < 0.05$) in the software Statistica 7.0 (Statsoft, 2004).

3. Results

3.1. Chemical analyses and validation of tests

In the supplementary material (Tables S1, S2 and S3) the results obtained related to quantified concentrations of fipronil and 2,4-D of the samples of the different experiments with the compounds acting alone are presented, being: chronic tests, avoidance and colonization, respectively. Table S4 shows the quantified concentrations of mixtures of both pesticides. Nominal concentrations were used to represent the results, due to the concentrations being similar to nominal concentrations, except in the control compartment.

The distributions of daphnids in the control tests for avoidance (using only cultivation water) were analyzed for the experiments with single compounds. No preference for any area of the experimental system or avoidance was recorded in the test organisms. There was no statistically significant difference in the percentage of organisms in the compartments, therefore, the distribution of daphnids was random. ($F_{4,15} = 2.145$, $p = 0.125$).

Control tests for colonization were also carried out (using only cultivation water) for the single and mixed contaminant experiments. We used the results obtained for each zone (Z1-Z5) from the control test to compare with the results obtained in the zones characterized by the different mixture combinations (Z1-Z5), as described previously in Section 2.8.

3.2. Individual pesticide gradients: avoidance responses

The average percentage of organisms obtained from the fipronil and 2,4-D gradients for *D. magna* are shown in Fig. 3. It is observed that the different fipronil concentrations did not influence the percentage of organisms in each compartment, nor by the different observation times ($F_{4,15} = 2.639$, $p = 0.07$) (Fig. 3A). The same occurred for the 2,4-D gradient. No statistically significant differences were observed regarding the mean percentage of daphnids either for the 2,4-D gradient or for the different observation times ($F_{4,15} = 1.03$, $p = 0.42$) (Fig. 3B). For the observation time variable, no statistically significant differences were presented. Thus, we chose to present the mean of all the times in an integrated way.

3.3. Colonization by *D. magna* of the pesticide-contaminated areas

The percentage of organisms in the colonization tests with *D. magna*, in the contamination gradients of fipronil, 2,4-D and areas of a mixture of these pesticides are presented in Fig. 4. The percentage of organisms in each zone was determined by the concentration gradient and observation time. Thus, for the gradient of fipronil at 24 h, the F2 and F4 zones showed significant differences compared with the control zones ($p < 0.05$) and, at 48 h, the CT, F2 and F4 zones showed significant differences with the control zones ($p < 0.05$) (Fig. 4A).

As for the 2,4-D gradient, in relation to the average percentage of daphnids, the D4 zone presented statistical difference in relation to the control zone ($p < 0.05$) at 24 h; however, at 48 h, no zone showed a statistically significant difference in relation to the control zone ($p > 0.05$) (Fig. 4B).

Finally, for the gradients of mixtures (contamination zones), at 24 h, Z5 was significantly different from the control zone and at 48 h, Z3, Z4 and Z5 were different from the control zones, when analyzing the average percentage of daphnids per zone ($p < 0.05$) (Fig. 4C).

3.4. Chronic toxicity

After 21 days of exposure to the 2,4-D, the body length (maternal) of *D. magna*, was not significantly affected ($p > 0.05$; Fig. 5A). However, exposure to fipronil affected (decreased) the maternal body length of the daphnids (F3 - 5 μg/L, F4 - 10 and F5 - 20 μg/L) ($p < 0.05$; Fig. 5B).

Regarding the fecundity of *D. magna*, 2,4-D also did not cause any significant differences after 21 days of exposure ($p > 0.05$; Fig. 5C); however, the total number of neonates per female decreased significantly after an exposure period to fipronil (21 days) for all treatments when compared with the control ($p < 0.05$; Fig. 5D). For maternal survival of *D. magna*, 2,4-D did not cause any significant differences in the organisms' survival after 21 days of exposure ($p > 0.05$; Fig. 5E), whereas a significant effect ($p < 0.05$; Fig. 5F) caused by fipronil (F4 - 10 μg/L and F5 - 20 μg/L) was observed, for the F4 and F5 treatments, whose survival was 40% and 50%, respectively.

4. Discussion

The effects of contaminats on the distribution of organisms in the landscape is a subject to which little focus has been given in ecotoxicological studies so far. This approach provides an idea concerning the

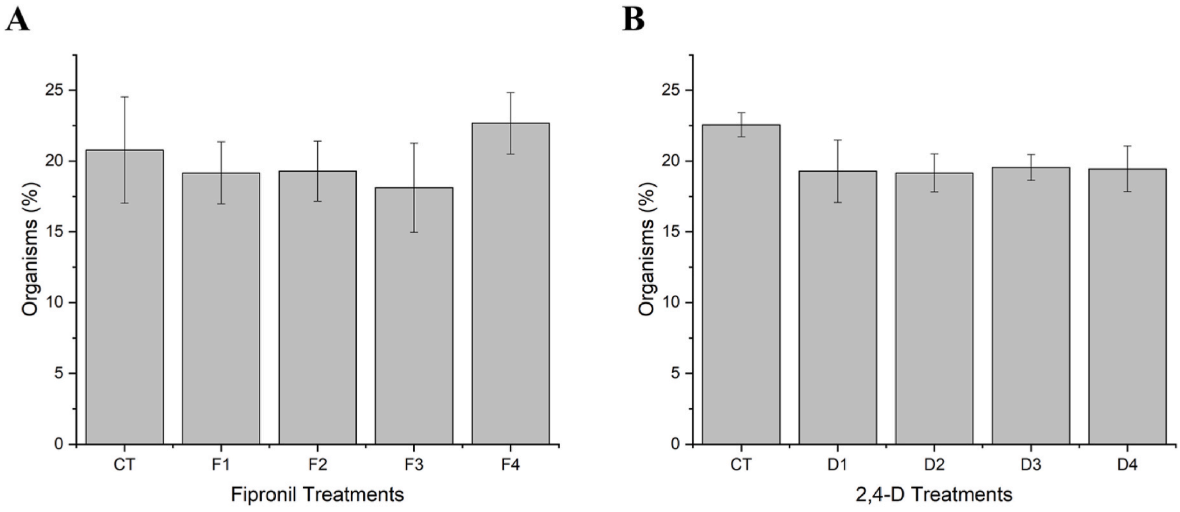

Fig. 3. 6Distribution (in %; with mean values ± SE of the three replicates) of daphnids along a concentration gradient of Regent® 800 WG (a.i. fipronil) and DMA® 806 BR (a.i. 2,4-D), considering the means of the six observation times. Fipronil treatments were: 0 (CT), 2.5 (F1), 5 (F2), 10 (F3) and 20 μg ai/L (F5). 2,4-D treatments were: 0 (CT), 125 (D1), 250 (D2), 500 (D3) and 750 μg a.i./L (D4).

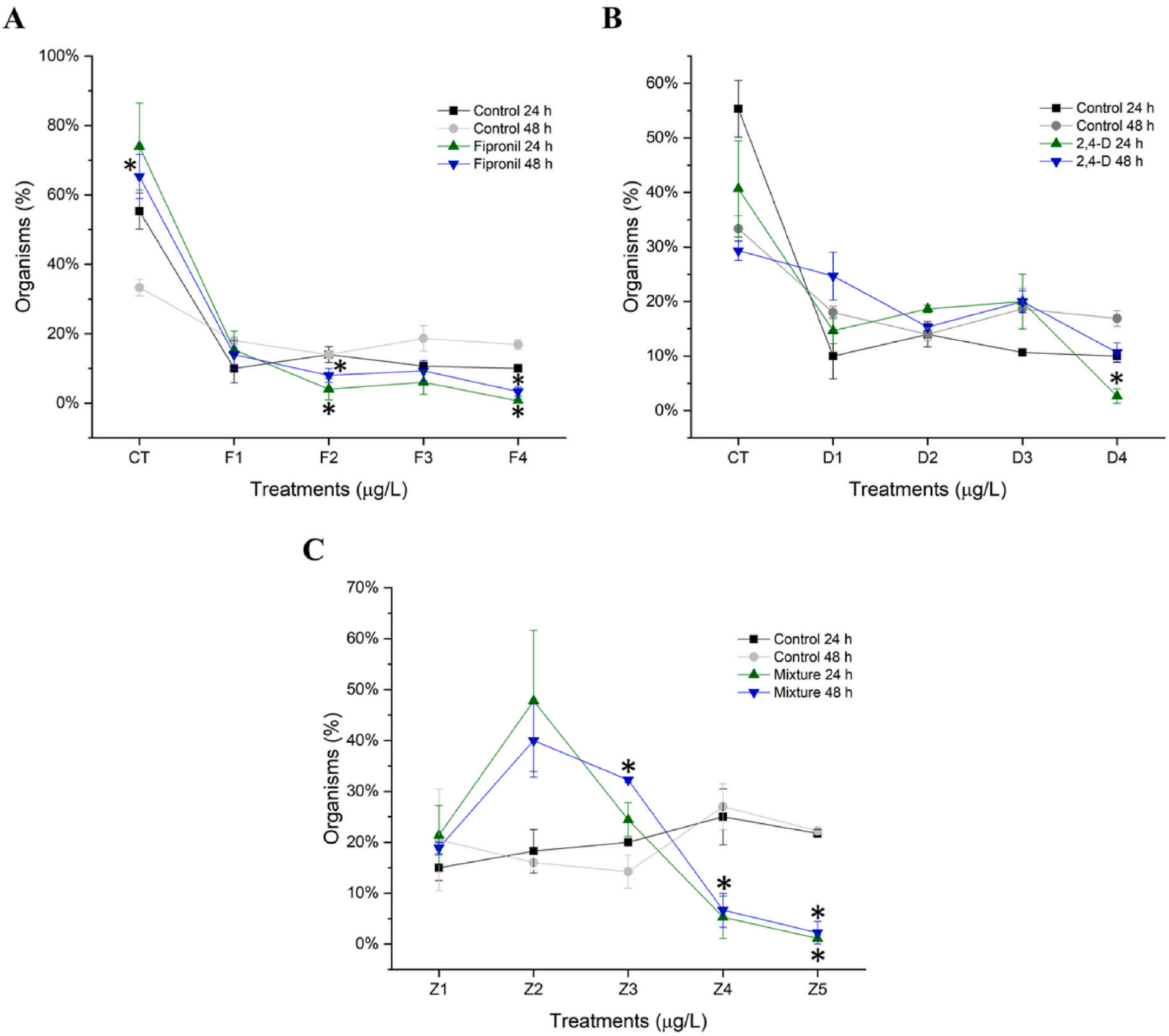

Fig. 4. 7Distribution (in %; with mean values ± SE) of daphnids in a gradient of different concentrations of Regent® (a.i. fipronil) (A), DMA® 806 BR (a.i. 2,4-D) (B) and pesticide mixtures (C). Asterisks indicate statistically significant differences regarding the preference (percentage of organisms) in the contaminated zone(s) when compared to the control zone(s), considering the observation times at 24 h and 48, respectively ($p < 0.05$). The legend for each zone is given in Fig. 1.

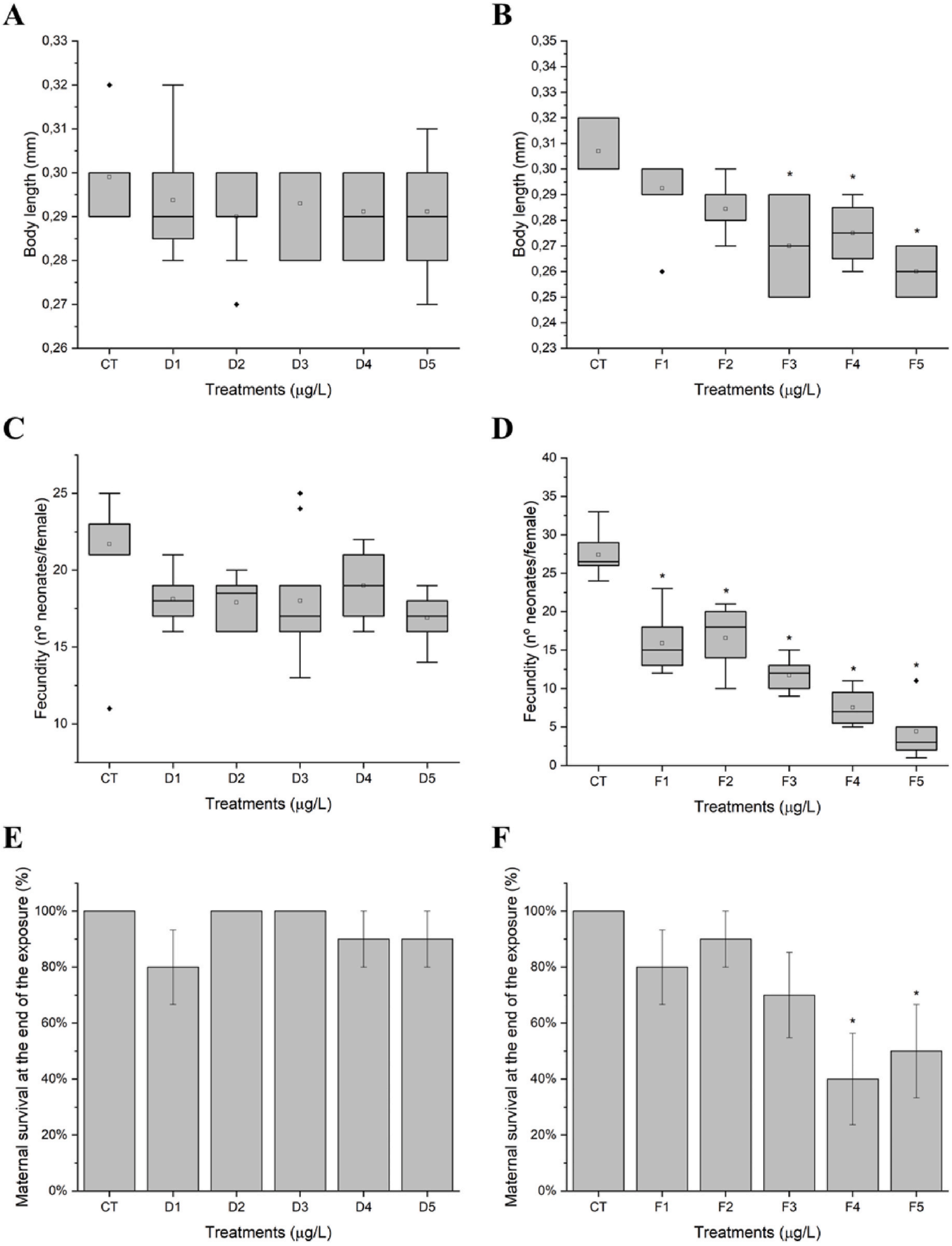

Fig. 5. Maternal body length (A–B), fecundity (C–D) and maternal survival (E–F) (n = 10) of *Daphnia magna* after 21 days of exposure to sublethal concentrations of Regent® 800 WG (a.i. fipronil) and DMA® 806 BR (a.i. 2,4-D), in chronic toxicity tests. Asterisks indicate significant difference from the control ($p < 0.05$).

potential of contaminants to condition the migratory movements and colonization of ecosystems. The major contribution of the current research consisted in including a non-forced approach, which is focused on the contamination-driven organisms' spatial distribution, with forced exposure, representing the traditional approach that is focused on unavoidable exposure to a contaminant. We have sought to link the effects of both pesticides fipronil and 2,4-D on daphnids in a heterogeneous, spatially connected landscape, in the context of emigration/avoidance and immigration/colonization, with any potential effects on organisms that are unable to migrate from habitats and suffer long-term exposure due to chronic toxicity.

It was seen that *D. magna* did not avoid the concentrations of both the insecticide and the herbicide, after 24 h of exposure. Some studies have shown that these organisms, particularly daphnids, can potentially avoid contamination, as has been observed for *D. longispina* in a copper gradient (Lopes et al., 2004), *D. magna* exposed to industrial effluents and atrazine (Rosa et al., 2008, 2012) and *D. magna* exposed to saline intrusion into freshwater environments (Venâncio et al., 2020). Therefore, the absence of avoidance could be related to: *i*) the concentrations were too low to trigger avoidance, *ii*) the mechanisms by which the organisms should sense both chemicals were not activated or *iii*) neurotoxic effects prevented "normal" avoidance behavior.

The neurotoxicity of fipronil is widely known, therefore very low concentrations of the insecticide can affect the homeostasis of animals (Chevalier et al., 2015). For instance, the results of Bownik and Szabelak (2021) indicated that fipronil reduced the swimming speed and distance covered, as well as in physiological activities such as heart rate, claw activity, and forelimb movements in *D. magna* in a concentration- and time-dependent manner at all concentrations used (0.1–100 μg/L). Fipronil is a phenyl pyrazole with effects on the central nervous system, leading to the death of target organisms by hyperexcitation and paralysis (Tingle et al., 2003). The herbicide 2,4-D belongs to the chemical group of phenoxy acetic acids and acts on dicotyledonous plants, deregulating their cell division, causing subsequent death (Jervais et al., 2008). Although the targets of 2,4-D are plants, other species suffer effects at low concentrations of the compound. The swimming activity of the epibenthic macroinvertebrate *Hyalella meinerti* was diminished due to exposure to 2,4-D (1385 μg/L) throughout all experimental periods, up to 89 days after contamination of mesocosms by the compound (Pinto et al., 2021b). The same study discusses the deficiencies in swimming ability and its implications regarding a decrease in foraging which, consequently, disrupts the growth and reproduction due to the lack of energy. Unlike our results, other species detected and avoided areas contaminated with the compound in non-forced exposure experiments. Tadpoles of *Lithobates catesbeianus* and the fish *Hyphessobrycon eques* detect gradients of 2,4-D, 200–700 μg/L, and 200–2500 μg/L respectively, so that organisms preferred to move to the lowest concentrations (Freitas et al., 2019; Moreira et al., 2021).

Regarding colonization behavior, our results show that colonization by *D. magna* is partially prevented when the concentration of 2,4-D increases and the zone with the highest concentration of the herbicide (D4 - 750 μg/L) was not colonized in 24 h of exposure; although, at 48 h, the zone was colonized even if only by a reduced number of organisms. For fipronil, daphnids did not colonize the intermediate (F2 - 5 μg/L) and highest (F4 - 20 μg/L) zone of the contamination gradient at 24 and 48 h of exposure. Furthermore, when evaluating the potential for colonization of contamination zones (mixtures of pesticides) the results showed that colonization by daphnids was significantly prevented. At 24 h, zone 5 (high fipronil concentration combined with different 2,4-D concentrations) was not colonized and, at 48 h, zones Z3, Z4, and Z5 were also not colonized by *D. magna*. Therefore, the hypothesis posited that contamination of aquatic environments by the pesticides fipronil and 2,4-D can isolate populations of daphnids, even if the mobility of organisms is not spatially restricted, was partially supported.

This difference between the ability to avoid and the colonization response, as observed in our results, does not support the avoidance-recolonisation theorem (stressor-driven emigration could foresee the potential of a population to establish in recovering habitats) (Araújo et al., 2018). This difference could be related to the stimulus received by daphnids when moving from a clean area to a contaminated. Possibly, with no attractive stimulus in increasing pesticides concentrations, organisms do not have any incentive to explore and colonize new environments, such as discussed by Araújo et al. (2018) and Islam et al. (2019). Additionally, the way both assays began could explain some differences between the avoidance and colonization behaviors. In the avoidance experiments, the organisms are initially in direct contact with the contaminant (see Fig. 1); therefore, they can sense the chemicals and feel stimulated to move to a most favorable area. However, if this initial contact impairs their ability to flee by causing some lethargy, the avoidance will be less intense. Considering that the avoidance-recolonisation theorem postulates that both responses are inversely correlated (Araújo et al., 2018), the colonization expected would be more intense as there was no avoidance. Here, the absence of any stimulus to colonize areas that provide aversive chemical signs might have conditioned the behavior of daphnids making them reluctant to colonize the highest concentrations. As this approach is very novel, more researches are needed to study the link that could exist between avoidance and colonization resposnes.

The daphnids' response to the exposure scenario for the mixture demonstrates potentiated effects of the compounds on the parameter evaluated: the immigration/colonization ability. Commonly, in natural environments, antagonisms and synergisms interactions are frequent, as stimuli and stressors occur normally together (Moe et al., 2013; Alkimin et al., 2020). When evaluating studies with zooplanktonic species, especially cladocerans, there are important responses that also indicate synergetic effects on behavioral (Andrade et al., 2018), morphological (Moreira et al., 2020), physiological (Barata et al., 2012; Silva et al., 2021) parameters and other life-history characteristics (Mansano et al., 2020), which are related to the presence of pesticide mixtures. Therefore, the synergism observed in the current study was expected, although there was no evidence of this interaction for the non-forced exposure approach.

For the chronic endpoints evaluated in the present study, no statistically significant effects (survival, maternal growth, and fecundity after 21 days of exposure) in relation to the control was recorded in *D. magna* exposed to 2,4-D. Although 2,4-D is an "old" molecule (introduced on the market in 1940), few studies have evaluated its chronic toxicity to cladocerans. For *D. magna*, the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) have been established as 79 and 151 mg/L, respectively (US EPA, 1992). Matsumoto et al. (2009) obtained a LOEC value of 10 mg/L, and Oris et al. (1991) obtained a median effect concentration (EC_{50}) of 86.8 mg/L after 4 days of exposure, both for *Ceriodaphnia dubia*. Silva et al. (2020) obtained an EC_{50} value of 69.8 mg/L for *C. silvestrii*, after 7 days of exposure to DMA® 806 BR (a.i. 2,4-D). As the data of chronic effects have been observed at higher concentrations than those used in the current study, the absence of any chronic effect here observed is in accordance with previous studies.

The concentrations of fipronil tested caused high toxicity to *D. magna*, in which survival was largely affected when exposed to F4 (10 μg/L) and F5 (20 μg/L) concentrations. For the daphnids that survived 21 days of exposure to fipronil, maternal body growth was affected at the highest concentrations (F3 - 5 μg/L, F4, and F5) and fecundity was decreased at all concentrations tested. The negative effect of low fipronil concentrations may also be found in the literature for *D. magna*, whose lethal tests recorded LOEC and NOEC values of 19 and 27 μg/L, respectively (US EPA, 1992) and for *D. pullex*, with NOEC and LOEC values of 30 and 50 μg/L, respectively, for 10 days exposure (Stark and Vargas, 2005). For *C. silvestrii*, the value of 1.6 μg/L was recorded when exposed to Regent® 800 WG (a.i. fipronil) (Silva et al., 2020).

The approach based on the organisms' attributes has a key role in predicting the effects on the structure of communities (McGill et al., 2006) and on the functioning of environments, since the loss or alteration of functional attributes in zooplanktonic species, such as reproduction and size, can result in changes in ecosystem processes (Violle et al., 2007, Sodré and Bozelli, 2019) and then in the generation of ecosystem services (Hooper et al., 2005). Body size is an integrative characteristic that defines many ecosystem processes carried out by zooplankton, such as growth and reproduction (Litchman et al., 2013). Respiration and excretion use to increase when the body size increases, thus improving the role of zooplankton in carbon and nutrient cycles (Hébert et al., 2016); this contribution is therefore diminished, with effects occurring on their functional attributes, as observed when *D. magna* were chronically exposed to fipronil.

Finally, habitat disturbance due to contamination is a serious problem of concern due to the structural and functional effects produced on communities and ecosystems (Molles, 2015). It is a widely known problem, but non-forced exposures are not yet used in environmental risk assessments. The current study has showed the importance of considering pesticides not only as potentially toxic compounds, but also as habitat disturbers by affecting the spatial distribution of species. We have shown that the integration between non-forced and forced exposure approaches might improve our understanding of: *i*) how contaminants condition the distribution of organisms in the ecosystems by triggering the avoidance behavior or preventing the colonization of new habitats (is the environment suitable to accommodate life?) and *ii*) the potential consequences caused by continuous exposure in cases in which organisms cannot flee (immobilization of organisms or the absence of chemical heterogeneity with areas to flee to).

5. Conclusions

Negative effects on survival parameters, maternal body length, and fecundity of *D. magna* occurred when the daphnids were chronically exposed to fipronil, although no such effects were observed when they were exposed to 2,4-D. When evaluating the detection and avoidance capacity of *D. magna* exposed to pesticides in a non-forced way, there was no preference for the less contaminated areas for both pesticides. However, when colonization was evaluated, the daphnids initially placed in clean/contamination-free zones did not migrate to the zones with intermediate and the highest concentrations of fipronil. Finally, colonization by daphnids was significantly prevented when exposed to a mixture of pesticides (contamination zones), of which the zones with the highest combinations of pesticide concentrations (Z3, Z4, and Z5) were not colonized by *D. magna*. Therefore, the current study shows the importance of including unforced exposure approach in ecological risk assessment, given that the presence of pesticides might make habitats unfavorable to inhabit, thereby disrupting their normal functioning, even before chronic effects occur.

Credit author statement

Raquel A. Moreira: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft., **Curro Polo-Castellano**: Investigation., **Andrea Cordero-de-Castro**: Investigation., **Mariana A. Dias**: Investigation., **Thandy J. S. Pinto**: Formal analysis., **Cassiana C. Montagner**: Resources; Methodology., **Evaldo L.G. Espindola**: Conceptualization; Writing – review & editing; Project administration; Funding acquisition, **Julián Blasco**: Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition., **Cristiano V.M. Araújo**: Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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