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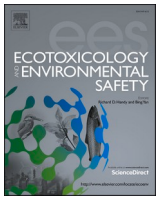

Reduced avoidance behaviour in *Daphnia magna* due to agrochemical-induced vulnerability

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ABSTRACT

The continuous discharge of agrochemicals used in intensive agriculture contaminates aquatic systems, harming aquatic biota and their processes. Although mobile organisms can avoid continuous exposure by moving to less-affected habitats, their capacity can be altered by pollutant exposure. Populations with a previous disturbance history, which show a lower ability to respond to subsequent stressors, are defined as vulnerable. Therefore, this study investigated the so far unknown escape capacity of a vulnerable zooplankton population previously exposed to a contaminated environment. To this end, agrochemically driven vulnerability was induced in populations of *Daphnia magna* by exposure to sublethal concentrations of glyphosate. Vulnerability was verified using a starvation test in which significant differences were observed between the control populations and populations with a disturbance history. Both the Control and Vulnerable populations were assessed for their avoidance capacity by exposing them to a glyphosate gradient using a Heterogeneous Multiple-Habitat Assay System (HeMHAS). The control populations showed a rapid reaction from the beginning of the assay, with avoidance rates increasing over 24 h, while vulnerable populations were unable to avoid contaminated habitats for up to 24 h. Therefore, we concluded that vulnerable populations have a lower capacity to avoid contaminated habitats. In heterogeneously contaminated habitats, a lower avoidance capacity is responsible for the differential spatial distribution of the affected species, which impacts the ecosystem structure. Additionally, agrochemically induced vulnerability and its effect on avoidance behaviour may affect ecosystem functioning through the altered spatial distribution of zooplankton populations.

1. Introduction

Human population growth has imposed a high demand for food that requires intensive agriculture, which is considered one of the main drivers of global change (Matson and Vitousek, 2006; Steffen et al., 2015). The natural processes of drift, runoff, and leaching imply that agrochemicals applied to intensive cultivation systems reach water bodies, damaging biota and their processes (Parra et al., 2005; del Arco et al., 2019; Kumar et al., 2021). Agrochemical contamination significantly affects the formation of chemical barriers that create discontinuities in aquatic systems (Araújo et al., 2019). This creates chemically fragmented habitats, where contamination levels in different areas limit the movement and displacement of organisms without a physical barrier (Fuller et al., 2015; McFadden et al., 2023). This habitat discontinuity can severely affect freshwater systems, as it can significantly alter species dispersal patterns, particularly affecting aquatic invertebrates (Brooks et al., 2013; Sondermann et al., 2015; McFadden et al., 2023).

Because the decrease in habitat connectivity poses a significant threat to biodiversity, it is essential to consider the impact of agrochemical pollution on the spatial distribution and habitat selection of aquatic species (Araújo et al., 2016; Alcívar et al., 2021). Efforts have been made to study the spatial avoidance behaviour of zooplankton populations in contaminated habitats by utilising a multicompartmental exposure system (HeMHAS) to simulate habitat connectivity (Araújo et al., 2018). This exposure system aids in obtaining more information on zooplankton trends, focusing on displacement in contaminated aquatic systems, thereby moving towards a more realistic approach regarding the chemical heterogeneity (as a gradient or patches of

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contamination) of habitats (Alcívar et al., 2021). Furthermore, through HeMHAS tests, it is possible to simulate scenarios of global change with individual or combined stressors, allowing the anticipation of the behaviour of zooplankton populations within contaminated aquatic systems (Mena et al., 2020; Moreira et al., 2021).

Considered a fundamental model in the field of ecotoxicology, *Daphnia magna* is a non-target species used for the ecological risk assessment of agrochemicals in aquatic systems through standardised toxicity tests (Brock and VanWijngaarden, 2012). In spite of its broad use, the effects of toxic substances at sublethal concentrations on *D. magna* populations do not necessarily show a reduction in population's abundance (Bendis and Relyea, 2014). Although there are cases in which exposure to sublethal concentrations leads to decreased fitness (Dutilleul et al., 2017; Toyota et al., 2019; Hintz et al., 2019), the consequences of exposure to sublethal concentrations may depend, among other factors, on the toxic mode of action and the organism's life history. Thus, previous exposure could induce undetectable consequences that drive susceptibility to future stressful situations (Ribeiro and Lopes, 2013). In our previous studies, we showed that sublethal exposure of *D. magna* to agrochemicals over a specified period, regardless of their mode of action, induced populations that were less tolerant to subsequent stressors than populations without the mentioned disturbance history (López-Valcárcel et al., 2021, 2023, 2024); in these articles, we defined them as vulnerable population. In the context of global change scenarios, the subsequent stressors to which daphnids are exposed include factors such as low food availability, exposure to new toxins, high salinity, and changes in temperature. *D. magna* responses confirmed the vulnerability of these populations in all the mentioned cases through significant differences compared to daphnid populations without a previous disturbance history. A follow-up question is how agrochemically induced vulnerability might influence *D. magna* behaviour, affecting population dispersal in chemically fragmented habitats.

Glyphosate, one of the most widely used broad-spectrum herbicides, has been detected in both marine and freshwater environments (specifically in water) with concentrations ranging from 0.1 mg L^{-1} to 0.7 mg L^{-1} (Giesy et al., 2000; Kjaer et al., 2002; Peruzzo et al., 2018). In the present study, glyphosate was used to induce vulnerable *D. magna* populations. Subsequently, we characterised the differences in avoidance behaviour between populations with a disturbance history (vulnerable populations) and those without a disturbance history (control populations). These differences were tested in the Heterogeneous Multi-Habitat Assay System (HeMHAS) test (Araújo et al., 2018) by simulating a gradient of contamination with different glyphosate concentrations. This study hypothesised that *D. magna* populations with vulnerability induced by previous exposure to sublethal glyphosate concentrations would have a lower capacity to avoid habitats contaminated by glyphosate than *D. magna* control populations without a disturbance history. The objectives of this study were: a) to induce vulnerability through sublethal exposure to glyphosate in *D. magna*; b) to verify the existence of this vulnerability through a starvation test; and c) to evaluate and compare the capacity of vulnerable and control populations to avoid habitats contaminated by different glyphosate concentrations in a spatially connected landscape.

2. Materials and methods

2.1. Test organisms and culture conditions

The test organism utilised was *D. magna* originally sourced from the natural wetland "Laguna grande" (Baeza, Jaén, Spain). Specimens of the same clone were cultivated in the laboratory for several years. The microalga *Scenedesmus obliquus* (Turpin) Kützing 1833, obtained from the Chemical Engineering Laboratory of the University of Jaén, served as the primary nutritional resource. These algae were routinely maintained in the 3N-BBM*V (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified Bischoff and Bold, 1963) culture medium, with a pH range of 8.3–8.5 (adapted from CCAP, Scotland). Both *D. magna* and *S. obliquus* were cultivated in a chamber with controlled temperature conditions (ARALAB-600PHL-LED) and under identical settings (20 ºC and a light-dark cycle of 12/12 h). Commercial mineral water (< 4 g P/L) with a total hardness of 209 mg L^{-1}, enriched with thiamine (0.0075 g L^{-1}), vitamin B12 (0.010 g L^{-1}), biotin (0.0075 g L^{-1}), and sodium selenite (0.0010 g L^{-1}), was used following the cultivation protocol outlined by Díaz-Baez et al. (2004).

2.2. Selected glyphosate concentrations

Throughout the experiment, sublethal concentrations of the commercial liquid herbicide Roundup® Bayer glyphosate were employed. Based on previous studies, sublethal concentrations of Roundup have been shown to cause no significant effects on reproduction or survival of *Daphnia magna* in chronic exposure scenarios up to 0.1 mg L^{-1} (Cuhra et al., 2013; Álcivar et al., 2021). Additionally, environmental surveys have documented glyphosate concentrations ranging from 0.1 to 0.7 mg L^{-1} in surface waters affected by agricultural runoff (Giesy et al., 2000; Peruzzo et al., 2018), supporting the ecological relevance of the selected concentration. To utilise existing tools for Ecological Risk Assessment (ERA), we chose an herbicide concentration of 0.1 mg L^{-1} , which aligns with environmentally relevant concentrations and is consistent with sublethal thresholds reported in chronic toxicity studies.

The concentrations reported in mg L^{-1} refer to the full formulation of Roundup, which includes both glyphosate as the active ingredient (AI) and co-formulants. The herbicide stock solution (100 mg L^{-1}) was prepared using Milli-Q water. Aliquots of the stock solution were added to the aquariums and gently stirred to ensure homogeneous chemical distribution in the water column. Although we used the full formulation of Roundup in all the tests, we used the term "glyphosate" in the text for simplicity, given that it is the main active ingredient of the formulation.

2.3. Induction and verification of vulnerability

2.3.1. Microcosm setup

Six glass aquaria (2 L) were prepared at 20 ºC. The aquariums underwent a 2-week stabilization period to ensure successful and balanced population growth under these conditions. Weekly samples of daphnids and microalgae were collected to measure their abundance and assess population growth. Daphnids were counted using a stereoscopic microscope (Leica M712), and microalgal cell density was estimated using a Sedgewick Rafter chamber with a microscope (Leitz DMIL). Additionally, to assess the status of *S. obliquus*, the colony formation rate was measured (Galotti et al., 2018). Colonies were defined regardless of whether the colony (Cenobia) was composed of two, four, or eight cells. Physicochemical parameters, dissolved oxygen (DO), and pH were recorded weekly using a multiparametric probe (YSI) (Table 1).

2.3.2. Vulnerability induction: Glyphosate exposure period

After the stabilization period, 2 treatments, with 3 replicates each, were established: (C), Control population, without glyphosate, and (V), Vulnerable population, with glyphosate at 0.1 mg L^{-1}. The population was identified as vulnerable based on the protocol used in previous studies (López-Valcárcel et al., 2021, 2023, 2024). The experiment lasted for 21 d, and the medium was renewed weekly to maintain the initial concentration. The abundance of *D. magna*, algal abundance, and colony formation were quantified weekly by extracting 200 mL from each microcosm. Physicochemical parameters, DO, and pH were measured weekly (Table 1). Nominal concentrations of the Roundup formulation were used in this study. These values represent the full formulation, which includes both glyphosate as the active ingredient (AI) and co-formulants.

2.3.3. Vulnerability verification: Starvation test

Adults from the Control and Vulnerable populations (C and V) were

Table 1
D. magna abundance (individuals L^{-1}), phytoplankton parameters: cell count (cells mL^{-1}), and colony formation (single cell/colony percentage), and physico-chemical properties parameters: pH and DO (ppm) means and standard deviation during the microcosm experiment.

Population Treatments	Days			
	0	7	14	21
***D. magna* abundance**				
C	18 ± 3.60	19 ± 2.64	20.33 ± 6.50	19.33 ± 2.51
V	15 ± 4.35	19 ± 7.93	20.66 ± 6.11	21 ± 7
Algae abundance				
C	52.66 ± 8.50	57.33 ± 7.50	46 ± 12.52	49 ± 13.07
V	52.00 ± 7.00	44.33 ± 6.11	58.66 ± 14.01	55.66 ± 8.50
Colony formation				
C	6.60 ± 11.50	12.20 ± 5.70	20 ± 17.30	13.30 ± 15.20
V	16.66 ± 20.80	16.60 ± 15.20	13.30 ± 20.00	20 ± 10
pH				
C	8.55 ± 0.10	8.55 ± 0.23	8.67 ± 0.10	8.56 ± 0.07
V	8.57 ± 0.12	8.58 ± 0.18	8.63 ± 1.17	8.61 ± 0.10
DO				
C	6.37 ± 0.23	6.43 ± 0.32	6.40 ± 0.14	6.40 ± 1.52
V	6.48 ± 0.27	6.74 ± 0.44	6.46 ± 0.40	6.48 ± 1

isolated for the starvation test to obtain the neonates (< 24 h). The experiment took place in 50 mL containers, and populations were named according to their maternal population: (C) neonates from a population without glyphosate exposure and (V) neonates from a population under glyphosate exposure (replicates per treatment, n = 5; 5 neonates/container). The culture conditions were the same as those in the previous stages; however, no food was added throughout the experimental period until 100 % mortality was reached. Individual survival was assessed daily until death of the last individual.

2.4. Avoidance test

HeMHAS was used (Araújo et al., 2018). This system consists of 15 compartments in an arrangement of 3 × 5 compartments, each with a capacity of 320 mL. Such systems were employed to simulate different pollution scenarios, with the possibility of escaping through doors connecting the experimental compartments. These doors can be opened or closed, allowing the creation of different connectivity scenarios.

This assay comprised two independent tests with adult daphnids: in the first, only daphnids from the control population (C) and in the second, only organisms from the population with induced vulnerability (V) were used. (Fig. 1). The treatments used in the tests were 0, 0.1, and 1.0 mg L^{-1}. To simulate the glyphosate gradient, the doors connecting the compartments of the HeMHAS were closed until the beginning of the exposure period, and different concentrations were placed in the compartments. Then, 10 adult *D. magna* were introduced into each compartment (Fig. 1). Fifty daphnids were used per replicate, and there were 3 replicates per test. Afterwards, the doors were opened until the end of the experimental exposure period, and data (number of organisms per compartment) were collected every half hour during the first 4 h and

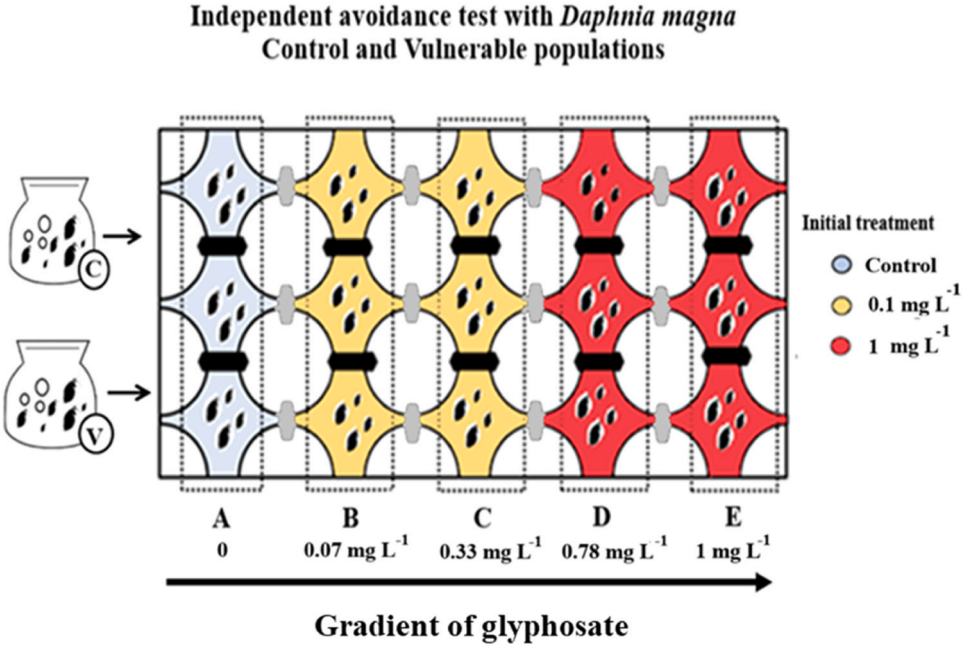

Fig. 1. Scheme of the avoidance tests conducted in a non-forced exposure system (HeMHAS). Two assays were performed: Avoidance test using *D. magna* Control (C) and Vulnerable populations (V), where daphnids from populations were introduced into the system. Each row in this system represents a replicate of the exposure circuit. The grey doors are open doors allowing connection between compartments, while black doors are closed doors to separate the experimental replicates. The letters below refer to the compartments, and each compartment has an initial treatment. Each color appearing in the compartments has been assigned to an initial treatment: Blue: Control treatment; Yellow: Glyphosate: 0.1 mg L^{-1}; Red: Glyphosate: 1 mg L^{-1}. The values below the compartments represent the assumed final concentrations/compartment.

at 24 h to check the trend of avoidance behaviour over time. The daphnids used for these tests were 6–8 days old, and the tests were conducted under constant illumination. No food was provided during the experimental period.

2.4.1. Glyphosate concentrations in HeMHAS

The glyphosate concentrations that formed the contamination gradient at the end of the tests were defined by assuming a homogeneous mix of concentrations in adjacent compartments. Therefore, the intermediate concentrations (compartments B–D) were defined as the mean value between the initial concentration of the respective compartment and the mean value of the adjacent concentrations. Thus, for the gradient that was initially formed by 0, 0.1, 0.1, 1.0 and 1.0 mg L^{-1}, assuming a mix of concentrations, the final defined concentrations were 0, 0.07, 0.33, 0.78 and 1.0 mg L^{-1}. The concentrations in the two extreme compartments were maintained as the initial reference, representing the lowest and highest concentrations in the system (see details in the Supplementary Material) (Fig. 1).

2.5. Data analysis

Data analysis was conducted using the R-Studio (RStudio Team, 2022) and R (R Core Team, 2022) software. R packages used for data management, graphics, and statistical analysis included "Metrics" (Hamner and Frasco, 2018), "DescTools" (Signorell, 2022), "dplyr" (Wickham et al., 2022), "ggplot2" (Wickham, 2016), "cowplot" (Wilke, 2020), and "forcats" (Wickham, 2021). For the microcosm experiments, a generalised linear model (GLM) was used to compare *D. magna* abundance, algal cell abundance, colony cell count, dissolved oxygen (DO), and pH between the microcosms in different populations (C and V) during the experimental period. Two-level factors (C and V) were considered. A GLM was chosen instead of a linear regression because GLMs provide a framework for handling a broader range of outcome distributions, variable relationships, and variance structures, whereas linear regression is more suited to continuous outcomes with normally distributed errors and constant variance. This flexibility made the GLM a more appropriate choice for our data analysis (Zuur et al., 2009).

For the starvation test, a GLM with two factor levels (C and V) was used. Additionally, the median lethal time (LT_{50}) was calculated for the two populations.

For avoidance tests, two different statistical analyses were conducted. First, a GLM was performed using the combined data from experiments conducted with control populations (C, no disturbance history) and populations with induced vulnerability (V). The purpose of this study was to assess whether there were differences in avoidance capacity between Control and Vulnerable populations. This GLM included a five-level factor (A, B, C, D, and E), analysing abundance as the dependent variable, and compartment (A, B, C, D, and E), time (0.5, 4, and 24 h), and vulnerability (Control and Vulnerable) as the independent variables. Each compartment is associated with the following nominal glyphosate concentrations: A: 0 mg L^{-1}, B: 0.07 mg L^{-1}, C: 0.33 mg L^{-1}, D: 0.78 mg L^{-1}, and E: 1.0 mg L^{-1}(Fig. 1). Separate GLMs were conducted for the control populations (C, no disturbance history) and populations with induced vulnerability (V). An analysis was performed for each of the time points at which data were collected (0.5, 4, and 24 h) to evaluate variations in abundance across different compartments (A, B, C, D, and E). In the case of significant differences, Tukey's post-hoc test was used to identify specific treatment pairs with mean differences. In addition, the percentage of avoidance per treatment in both the Control and Vulnerable populations was calculated. The percentage of avoidance (% avoidance) was determined by dividing the number of avoiders in each compartment by the expected number of organisms in each compartment and multiplying the result by 100 using the following formula:

This calculation was performed for each replicate of each treatment group to obtain the mean avoidance percentage.

3. Results

3.1. Vulnerability induction: microcosm setup

During the glyphosate exposure period, no differences were detected among the treatments (C and V) for any of the measured parameters. No significant differences were observed in *D. magna* abundance ($\chi2 = 26.054$, df = 22, $p = 0.9006$). Similarly, there were no differences in phytoplankton abundance between populations ($\chi2 = 42.701$, df = 22, $p = 0.7335$), colony formation (percentage of individual cells/colonies) ($\chi2 = 28.841$, df = 22, $p = 0.5310$), pH ($\chi2 = 0.044697$, df = 22, $p = 0.8087$), nor dissolved oxygen (DO) ($\chi2 = 0.34109$, df = 22, $p = 0.2872$). The sublethal glyphosate concentrations used in this study had no significant effects on the selected biological or physicochemical parameters (Table 1).

3.2. Vulnerability verification: starvation test

The starvation test revealed that the population exposed to sublethal glyphosate concentration (V) was more susceptible to starvation than the Control population (C) ($\chi2 = 55.97$, df = 155, $p < 0.001$). Individuals from the Vulnerable population (V) died approximately 5 days earlier than individuals from the control population (C). The LT_{50} in (V) was 10.7 ± 2.16 days, while the LT_{50} in (C) was 16.3 ± 0.83 days (Fig. 2).

3.3. Avoidance test

3.3.1. Comparison of the avoidance test in Control and Vulnerable populations

Significant differences were found in the abundance of daphnids concerning the independent variable "Compartment" ($\chi2 = 88.129$, df = 86, $p < 0.001$), as well as, significant differences related to the independent variable "Vulnerability" ($\chi2 = 37.739$, df = 80, $p = 0.002$). No significant differences were observed concerning the independent variable "Time" ($\chi2 = 25.200$, df = 80, $p = 0.168$). These results indicate that there are differences in the avoidance capacity of contaminated habitats, depending on the level of toxicity (glyphosate concentration) and whether the population has had a previous disturbance history (Fig. 3).

3.3.1.1. Avoidance test in Control populations. At 0.5 h from the start of the test, the number of organisms in each compartment with different glyphosate concentrations was different ($\chi2 = 44.5$, df = 12, $p < 0.001$). The significance results of Tukey's post hoc test were as follows: A-D ($p = 0.008$), A-E ($p = 0.008$), B-D ($p = 0.03$), and B-E ($p = 0.003$) (Fig. 3). After 4 h of exposure, the significant differences remained ($\chi2 =$

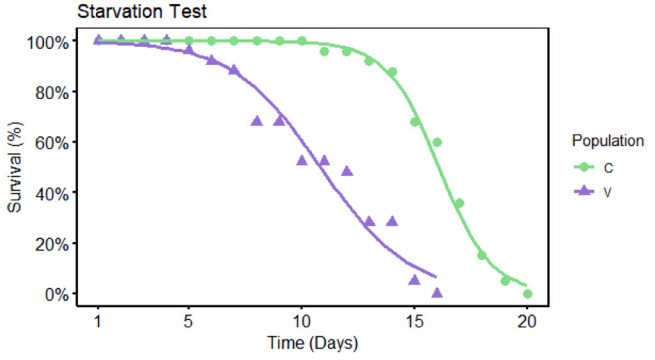

Fig. 2. Starvation test graph comparing the *D. magna* survival percentage belonging to the Control (C) and Vulnerable (V) populations over time (days). Data points represent observed values, and logistic regression curves illustrate the survival trends for each population.

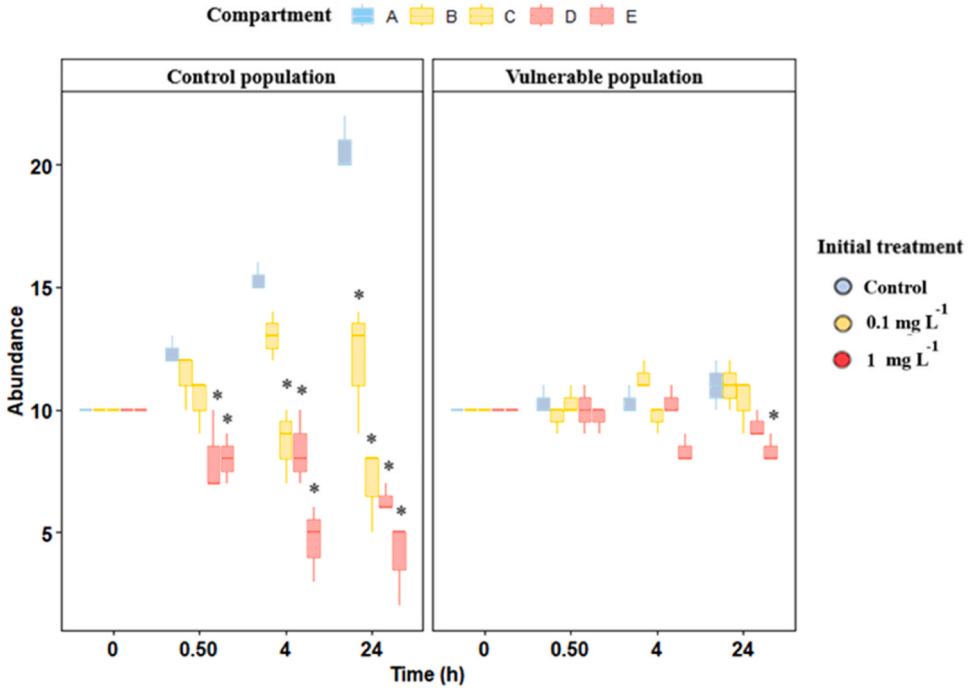

Fig. 3. Changes over time of *D. magna* abundance in the compartments during the avoidance tests for both Control (left) and Vulnerable (right) populations exposed to a glyphosate gradient. The selected times for this analysis were: Initial time (0 h), 30 min, and 4 h, and 24 h from the start of the assay. Each colour corresponds to the specific compartment initial concentration: Blue for Control: water without Glyphosate; Yellow for Glyphosate: 0.1 mg L^{-1}; Red for Glyphosate: 1 mg L^{-1}. Asterisks represent statistically significant differences observed to for the control compartments in each time. (*): $p < 0.05$.

44.5, df = 12, $p < 0.001$), and the significant results of Tukey's post hoc test, were: A-C ($p < 0.001$), A-D ($p < 0.001$), A-E ($p < 0.001$), B-C ($p = 0.01$), B-D ($p = 0.08$), B-E ($p < 0.001$), C-E($p = 0.02$), and D-E ($p = 0.03$) (Fig. 3). After 24 h of exposure, the differences among the compartments were even higher (Fig. 3; $\chi 2 = 44.5$, df = 12, $p < 0.001$). The significance results of Tukey's post hoc test were as follows: A-B ($p < 0.001$), A-C ($p < 0.001$), A-D ($p < 0.001$), A-E ($p < 0.001$), B-C ($p < 0.001$), B-D ($p < 0.001$), B-E ($p < 0.001$), and C-E ($p = 0.01$) (Fig. 3). These results showed that individuals exposed to the two different concentrations of glyphosate flee towards the clean water compartments throughout the experimental period. The average percentage of organisms obtained at 0.5, 4, and 24 h in the avoidance test conducted on individuals belonging to the control population (C) is shown in Fig. 4.

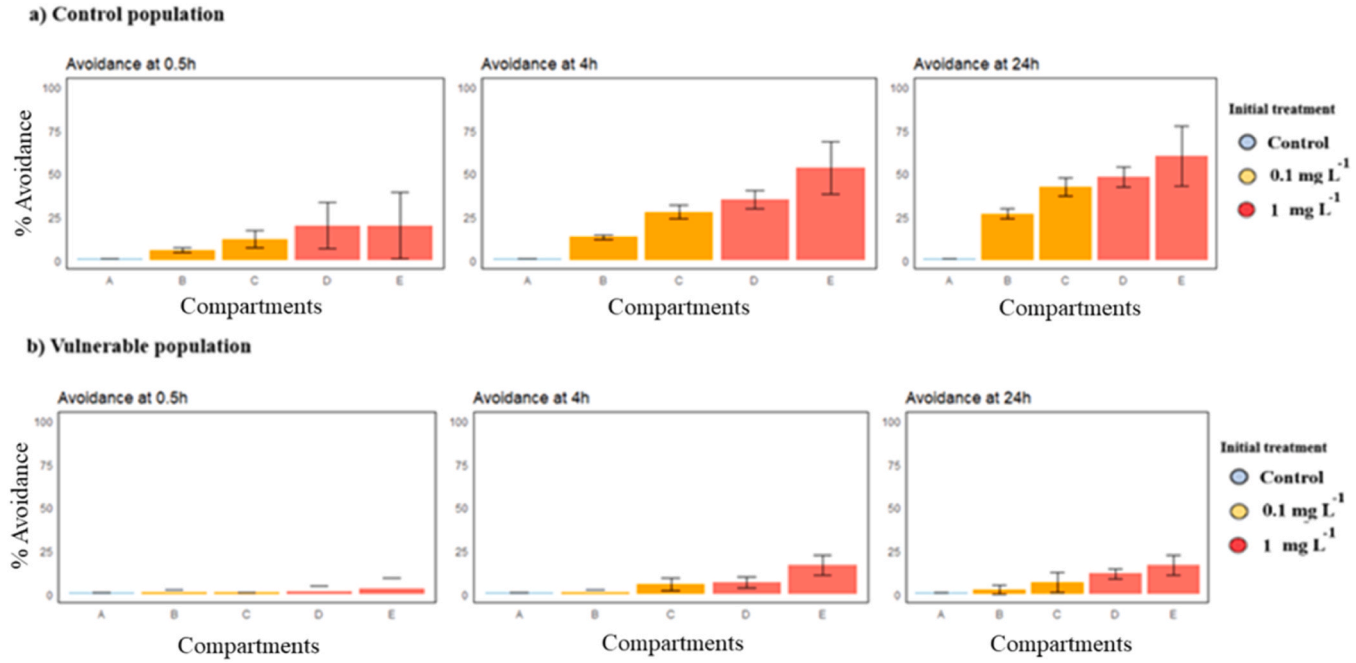

Fig. 4. Avoidance (in %) of *D. magna* Control (a) and Vulnerable (b) populations in the avoidance test after 0.50, 4, and 24 h of exposure to a glyphosate gradient. Each colour corresponds to the specific initial concentrations in the compartments: Blue for Control: water without Glyphosate; Yellow for Glyphosate 0.1 mg L^{-1}; Red for Glyphosate: 1 mg L^{-1}.

3.3.1.2. Avoidance test in Vulnerable populations. At 0.5 h from the start of the test, the number of organisms in the compartment with different glyphosate concentrations were similar ($\chi2 = 44.5$, df = 12, $p = 0.77$) (Fig. 3), as well as after 4 h of exposure ($\chi2 = 44.5$, df = 12, $p = 0.15$) (Fig. 3). However, after 24 h, the number of organisms in each compartment varied, with those exposed to higher glyphosate concentrations showing a slightly lower count, although significant ($\chi2 = 44.5$, df = 12, $p = 0.03$). The significant results of Tukey's post hoc test were as follows: A-E ($p = 0.02$) and B-E (0.02) (Fig. 3). These results show a greater inability to avoid glyphosate concentrations, with a few individuals avoiding compartment E after only 24 h of exposure. The average percentages of organisms obtained at 0.5, 4, and 24 h in the avoidance test conducted on individuals belonging to vulnerable populations (V) are shown in Fig. 4.

4. Discussion

A heterogeneously contaminated system was simulated using (HeMHAS) to verify the avoidance capacity of *D. magna* populations with induced vulnerability. Vulnerability was initially induced in *D. magna* populations by creating a disturbance history through exposure to sub-lethal concentrations of glyphosate. The purpose of this procedure was to study the avoidance capacity of vulnerable populations towards contaminated habitats compared with the avoidance capacity of control populations. Our results show that control populations have the ability to escape from contaminated compartments, whereas vulnerable populations take longer to escape from them, and therefore remain occupying contaminated habitats much longer. Although the tests were carried out with the glyphosate formulation Roundup, we used the term glyphosate to simplify the text, as it is the main active ingredient of the Roundup formulation.

4.1. Vulnerability induction and verification

Exposure to sublethal concentrations of glyphosate did not produce differences in the abundance of *D. magna*, microalgal abundance, or algal colony formation. It is also important to highlight the verification of vulnerability induction through the starvation test, in which significant differences were observed between the control and vulnerable populations, which is consistent with our previous studies (López-Valcárcel et al., 2021, 2023, 2024). However, vulnerability was confirmed, as individuals were less resistant to starvation, raising concerns about the risk that sublethal agrochemical concentrations might impose on natural communities. It is important to note the lack of studies investigating these low concentrations during chronic exposure periods. For instance, the study by Cuhra et al. (2013) conducted various chronic toxicity tests with Roundup on *D. magna* at glyphosate concentrations ranging from 0.05 to 4.05 mg L^{-1} . Although this range of concentrations does not entirely cover the concentrations used in this study nor the typical concentrations found in the environment, which are usually lower (generally in the μg L^{-1} range), it provides relevant data on the toxic effects at higher levels. In this study, a significant reduction in juvenile size was observed even at the lowest concentration of 0.05 mg L^{-1} , along with negative effects on reproductive parameters due to an increase in abortion rates. This indicates that although the evaluated concentrations are not fully representative of environmental conditions, the study serves as a relevant starting point for understanding the potential impacts of glyphosate formulations and provides important references for future research.

4.2. Avoidance test

4.2.1. Comparison of the avoidance test in control and vulnerable populations

We analysed avoidance behaviour in *D. magna* populations with and without a history of disturbance. It was observed that control populations showed an early reaction to escape from compartments with concentrations of glyphosate, avoiding the highest concentrations at 0.5 h from the start of the experiment and increasing this response as the experimental time progressed up to 24 h (Figs. 2 and 3). This is consistent with the study by Lopes et al. (2004), in which populations of *D. longispina* showed early reactivity to avoid exposure to copper concentrations, and the study by Rosa et al. (2012), in which *D. magna* significantly avoided atrazine after a 12-h exposure. Therefore, glyphosate could be considered a repellent contaminant for *D. magna* as areas with lower glyphosate concentrations may act as a refuge. This could lead to alternative habitat selection, with individuals moving from contaminated to cleaner or less-disturbed habitats (Araújo et al., 2020).

Although there are cases in which the level of toxicity of a contaminant and its repellence level are not related (e.g. a contaminant may be highly toxic and simultaneously attract the study organisms) (Costantini et al., 2010), glyphosate exhibits both high levels of toxicity and repellence in *D. magna*, making this information relevant for the protection of aquatic systems. It is crucial not only to understand the toxic effects of contaminants, but also their repellence levels and whether they induce fleeing behaviour, as these factors directly affect the spatial distribution of zooplankton populations within aquatic systems. In this context, it is important to highlight the potential interactions of glyphosate with non-active components, as mentioned by Peixoto (2005) and Marc et al. (2002). According to those authors, alterations in mitochondrial bioenergetics caused by Roundup® may involve other chemicals, like POEA, and possible synergies between glyphosate and formulation products. This reinforces the necessity for further investigation of these interactions and their potential effects on *D. magna*, including their effects on survival, reproduction, and behaviour. This information should be studied and considered in ecological risk assessments, as it would allow for the creation of better ecotoxicological assessments with high ecological realism, and could reduce uncertainty by contributing to the inclusion of greater ecological complexity in studies (Raimondo et al., 2008; Alcívar et al., 2021). Concerning *D. magna* Vulnerable populations, the individuals showed avoidance behaviour towards glyphosate after 24 h, which directly contrasts with the early reaction of the control populations (Figs. 2 and 3). Studies such as those by Araújo et al. (2018) and Moreira et al. (2023) explained that in these types of assays, daphnids are in direct contact with the contaminant from the start of the experiment, which could impair the population escape capacity, leading to lethargy in organisms. In our study, after observing the early reaction of the control population, we assumed that glyphosate was a repellent contaminant for *D. magna* (Costantini et al., 2010). However, *D. magna* Vulnerable populations showed avoidance behaviour towards glyphosate after 24 h, which directly contrasts with the early reaction of the control populations (Figs. 2 and 3). It is important to highlight that the escape capacity of *D. magna* is diminished in vulnerable populations (exposed to sublethal glyphosate concentrations for three generations) and acclimation could be considered an option as organisms remained in contaminated compartments for most of the exposure period. In a study by Poulsen et al. (2021), the acclimation capacity of *D. magna* to the pesticide prochloraz was studied and confirmed after continuous exposure for three generations. Furthermore, Saro et al. (2012) discussed the association between the acclimation process and fitness cost after a long-term sublethal exposure period involving *Daphnia longispina*. No evidence of acclimation was observed among the resistant and sensitive clonal lineages, suggesting a lack of physiological adjustment. Although fitness costs linked to resistance to lethal metals were not detected, resistance to sublethal levels of Cu correlated with a lower intrinsic growth rate under uncontaminated conditions. This rationale may be associated with the vulnerability induced in the present study. However, we suggest that the vulnerability of the vulnerable population may impair their pollutant detection capacity as a consequence of exposure damage (e.g., sensory, biochemical, and/or natatory disruption). Sensory disruptions may result from neurotoxic effects of glyphosate-based herbicides, which have been

shown to interfere with physiological functions critical for behavioral responses, including pollutant detection and avoidance (Duan et al., 2019). Biochemical alterations, such as oxidative stress and damage to cellular structures, are well-documented effects of glyphosate exposure and its co-formulants, further compromising the physiological integrity of aquatic organisms (Klátyik et al., 2024). Additionally, natatory disruption has been observed in *D. magna* following glyphosate exposure, potentially linked to neuromuscular dysfunction and reduced locomotor activity (Gustinasari et al., 2021). These combined impairments could lead to slowed avoidance responses and alter the capacity of vulnerable populations to respond effectively to a range of other stressors, such as starvation, changes in temperature, salinization, and pharmaceutical products (López-Valcárcel et al., 2021, 2023).

This assumption is supported by Seuront (2010), who demonstrated differences in contaminant detection capacity among different zooplankton species. This implies that some zooplankton species are more sensitive or effective at detecting and responding to contaminants. Furthermore, if we consider that these species are continuously exposed to sublethal concentrations of agrochemicals, this could have significant consequences on the dynamics and interactions among species within a community.

Chemical habitat discontinuity is becoming a significant issue in aquatic systems because of functional changes, such as alterations in species interactions and nutrient cycling, and structural changes, including the loss of habitat complexity and substrate diversity caused by the reduction in connectivity in ecosystems (Alcívar et al., 2021). Although zooplankton organisms can detect contaminated patches (Seuront, 2010), the effects of previous stressors can alter these abilities, as we have shown. Consequently, this study highlights the need to reevaluate, on the one hand, the safe concentrations within aquatic systems considering the use of behavioural endpoints and, on the other hand, how changes in avoidance behaviour could impact zooplankton spatial distribution and its effect at the ecosystem level.

5. Conclusions

The vulnerability induced by exposure to sublethal glyphosate concentrations impaired the avoidance response of *D. magna*.

Environmentally relevant concentrations of glyphosate affected the ability of *D. magna* to cope with starvation and altered their avoidance behaviour. The energetic cost of sublethal exposure may negatively affect zooplankton by affecting their regular behaviour. This raises concerns that despite scientific and regulatory efforts to achieve legally safe concentrations of agrochemicals, levels below legal thresholds may pose risks to zooplankton. Contamination by agrochemicals in aquatic systems can create discontinuities in habitats by creating aversive areas that affect the spatial distribution of species and make the population more vulnerable, subsequently impacting ecosystem structure and functioning. Therefore, we argue that incorporating the evaluation of vulnerability and spatial avoidance behaviour into ecotoxicological risk assessments would enhance ecological realism.

CRediT authorship contribution statement

Araújo Cristiano: Writing – review & editing, Writing – original draft, Validation, Supervision. **Parra Gema:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology. **del Arco Ana:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis. **López Valcárcel María Eugenia:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.117673.

Data availability

Data will be made available on request.

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