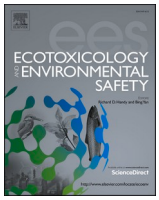
Ecotoxicology and Environmental Safety 289 (2025) 117633

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Multi-generational exposure of *Daphnia magna* to pharmaceuticals: Effects on colonization, reproduction, and habitat selection behavior

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ARTICLE INFO

Edited by Professor Bing Yan

Keywords:
Caffeine
Ibuprofen
Fluoxetine
Habitat selection
Multi-generational test

ABSTRACT

The presence of pharmaceuticals in the aquatic environment is increasing due to their growing use for human health. Although most studies are based on short exposures to these contaminants, the present study has emerged from the need to study pharmaceuticals in aquatic organisms over a long-term exposure to understand any multi-generational chronic effects and alterations regarding habitat selection. Therefore, this study shows: (1) the ability of *Daphnia magna* to colonize environments contaminated with caffeine, ibuprofen and fluoxetine, and (2) the effect of these pharmaceuticals on reproduction and habitat selection (under two scenarios: with and without food) after a long-term exposure period of three generations. It was observed that caffeine shortened the time between generations and caused an increase in the number of neonates per female. The opposite was observed with ibuprofen: the time to reach the third F3 generation was double when compared to those exposed to caffeine. Fluoxetine did not alter the reproduction, nor was repellent/attractive for daphnids. In the habitat selection tests, organisms cultivated in clean water preferred the compartment with caffeine, highlighting its attractive effect. Caffeine was also attractive for daphnids in the colonization test. Apart from this, no chemical showed any attractive or repulsive effect in the absence of food during the habitat selection tests. Our findings show that the presence of some pharmaceuticals could cause alterations in distribution and habitat selection patterns, and a significant effect on the reproduction of this species. underlining the importance of studying the effects of contamination by long-term exposure.

1. Introduction

The production of new chemical compounds and the consumption of pharmaceuticals have increased in recent years (González Peña et al., 2021; OECD Data Explorer, n.d.) with the aim of improving the quality of human health. From 2019 to 2021, this increase has been estimated at around 10 % on average in OECD countries (Bogowicz et al., 2021). Within this group of chemicals, we find a high variety of compounds with different compositions and modes of action in organisms, among which are: anti-inflammatory products, antidepressants, analgesics, anti-infectives, and antihistamines (WHO Model List of Essential Medicines – 23rd List, 2023). As a consequence of this increasing production and consumption, many of them have been detected in different compartments of the aquatic environment (e.g., water, sediments, and biota) in the range of ng/L to μg/L in increasing number of studies (see review by Ebele et al., 2017; Wilkinson et al., 2022). The main route of these products into the aquatic environment is from wastewater treatment plants, as most of them are ineffective in removing pharmaceuticals (Osuoha et al., 2023), representing a serious environmental problem.

Within this group of contaminants, caffeine stands out for its significant increase in consumption, indeed, it is considered a ubiquitous substance and an effective indicator of anthropogenic contamination (Buerge et al., 2003). Caffeine is a stimulant which can be considered also as a pharmacological product since it is incorporated in some medicines, such as anti-flu drugs, and it is also used for the treatment of

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https://doi.org/10.1016/j.ecoenv.2024.117633
Received 25 October 2024; Received in revised form 27 December 2024; Accepted 27 December 2024
Available online 3 January 2025
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some respiratory disorders in newborns (DrugBank Online, n.d.). Although it is possible to reach an efficient elimination of more than 80 % at wastewater treatment plants (Buerge et al., 2003; Paíga et al., 2019), caffeine has been detected at concentrations in the range of μg/L in surface water (Spongberg et al., 2011), estuaries (Benotti and Brownawell, 2007), seawater (French et al., 2015), and even in drinking water (Ayman and Işik, 2015). Another large group consumed and quite ubiquitous in the aquatic environment is the anti-inflammatory drugs, such as ibuprofen, which is also detected in surface water in concentrations from ng/L to μg/L (see review by Ortúzar et al., 2022). Finally, one group that has had an increase in consumption of more than 200 % according to the OECD is antidepressants. Fluoxetine is an antidepressant of special interest due to its efficacy and high consumption in the treatment of various mental disorders as a selective serotonin reuptake inhibitor (SSRI). In addition, fluoxetine has been detected in many aquatic ecosystems at the range of ng/L (Bringolf et al., 2010; de Souza et al., 2021; Mole and Brooks, 2019).

The adverse impacts of these pharmaceuticals on aquatic species have been observed with endpoints such as lethality, oxidative stress, as well as neurotoxic effects, on reproduction, and development (Brodin et al., 2014; Li et al., 2020; Srain et al., 2021). To understand how these compounds affect the populations of organisms present in the environment, we must consider that they are frequently present in nature, which could lead to constant and long-term exposures. To this end, multigenerational tests with *Daphnia magna* are a crucial tool for understanding the long-term ecological risks they may have on aquatic ecosystems (see review by Padilla Suarez et al., 2023). In these studies, it is possible to observe delayed toxic effects that are not evident in single-generation studies, such as increased mortality and reduced fertility in subsequent generations (Dalla Bona et al., 2016; Jeong et al., 2016; Olkova, 2022), as well as to identify cumulative toxicity effects that may be accentuated in subsequent generations (Barata et al., 2017; Brennan et al., 2006; Campos et al., 2016).

Behavioral parameters are early and sensitive indicators of toxicity and stress that can be used to study the effects of pharmaceuticals over a long-term exposure (Chevalier et al., 2015). In particular, effects have been observed in *D. magna* at different levels, such as swimming speed, motility, feeding activity and reproduction (Adamczuk, 2022; Dalla Bona et al., 2015; Dietrich et al., 2010; Heckmann et al., 2007). Furthermore, Silva et al. (2017a) found cumulative DNA damage over generations. All these changes suggest that the life cycle and population dynamics of *D. magna* may be affected by a wide range of concentrations of environmentally relevant contaminants.

Although the effects of chemicals on organisms can occur at several biological levels, the reproductive response is of great interest in environmental risk assessments (Ankley et al., 2010; Savitz and Harlow, 1991). This endpoint of physiology has received special attention due to its direct effects on population decline by chronic exposure to environmental concentrations that could normally be considered of low risk. However, in the last few years, a novel trend to assess the contamination-driven population decline has also focused on how contamination triggers the avoidance response in organisms, leading to changes in the habitat selection behavior (Araújo et al., 2016a; Rosa et al., 2012; Silva et al., 2017b; Umeokeke et al., 2022). In this new approach, populations can be reduced not as a result of a direct effect on survival or reproduction, but one concerning the spatial distribution of the species (Araújo et al., 2016b; Moreira-Santos et al., 2019). Thus, if organisms move away from a habitat due to contamination, it is possible to detect a partial or complete biodiversity loss at the local scale. The combination of these approaches (reproduction, avoidance and changes in habitat selection) may lead to significant consequences concerning population decline in an ecosystem.

Specifically for some pharmaceuticals and stimulant chemicals, a serious threat to be considered is their attractive effect on organisms, which acts as a trap instead of repelling them (Abreu et al., 2016; Jacob et al., 2021; Stremmel et al., 2023). Although this preference may seem, at least initially, not to produce a population decline, after many generations, populations could be affected in their potential to reproduce and interact with their surrounding environment. This preference assumes a previous colonization (the opposite approach of avoidance) of the disturbed habitat, where the species are able to expand to new areas. Specifically in *D. magna*, it has been shown that its ability to colonize can be altered as a consequence of the presence of contaminants at environmentally relevant concentrations (Moreira et al., 2023; Stremmel et al., 2023; Vera-Herrera et al., 2022).

This study focused on the ability of *D. magna* to colonize environments contaminated with caffeine, ibuprofen, and fluoxetine. In addition, emphasis was given on the traditional response related to population loss such as reproduction over different generations. Finally, the study examined whether this continued exposure could affect the ability to: (i) select habitats avoiding the exposure to contaminants and (ii) identify how the presence of food might modify habitat selection in *D. magna*.

2. Materials and methods

2.1. Culture of daphnids

A stock culture of *D. magna* sampled from *Laguna Grande* (Jaén, Spain) was cultured at the Institute of Marine Sciences of Andalusia (ICMAN – CSIC, Spain). The culture consisted of ca. 40 daphnids in glass recipients of 1 L of commercial mineral water (Font Natura ®, Sierra de Loja, Spain), enriched with the followed vitamins: thiamine (CAS number 67–03–8 at 75 μg/L), sodium selenite (CAS number 10102–18–8 at 2 μg/L), vitamin B12 selenite (CAS number 68–19–9 at 2 μg/L), biotin (CAS number 58–85–5 at 0.75 μg/L) according to Vera-Herrera et al. (2022). The culture was maintained in a cultivation chamber at 20 ± 2 °C with a photoperiod of 16:8 h light/darkness. The medium was renewed weekly, and the neonates were removed twice a week. The organisms were fed with a concentration of 1.5×10^6 cells/mL of the microalgae *Scenedesmus* sp. three times a week. This algal species was obtained from the ICMAN – CSIC collection and cultured under aseptic conditions in an enriched non-marine medium, as detailed in Fábregas et al. (2000) and under continuous white light at 20 ± 2 ºC.

2.2. Chemicals

Caffeine (CAS number 58–08–2), fluoxetine hydrochloride (CAS number 56296–78–7), and ibuprofen (CAS number 15687–27–1) were provided by Sigma-Aldrich (Steinheim, Germany). We prepared 100 mL of stock solution of each contaminant at 20 mg/L (nominal concentration) with Milli-Q water. The concentration measured in the stocks of the different contaminants ranged from 15.16 to 23.45 mg/L (Table S2). All the stock solutions were kept in a glass bottle in darkness at 4 ºC. Test concentrations (10 μg/L of contaminant) for the multi-generational exposure were prepared from this stock solution and were selected because in the case of caffeine and ibuprofen they are concentrations found in aquatic ecosystems (see reviews by Ortúzar et al., 2022 and Rodríguez-Gil et al., 2018). Fluoxetine was selected based on results of Stremmel et al. (2023), where at 10 μg/L and above, a certain attractiveness of the pharmaceutical was observed, as well as changes in reproduction. Samples from each stock solution and treatment with chemicals were analyzed for chemical concentrations during all the experiments (Table S2, S3, S4 and S5). All the samples were stored at −8 °C in a freezer until the analyses. Chemical concentrations were determined using the high-pressure liquid chromatography system (HPLC-MS). The limit of detection (LOD) was 0.1 μg/L for caffeine, 0.5 μg/L for fluoxetine, and 0.25 μg/L for ibuprofen.

2.3. Colonization test

A colonization assay was performed for each pharmaceutical product

(caffeine, ibuprofen, and fluoxetine). This assay consisted of exposing *D. magna* to a linear gradient of 5 concentrations of each contaminant (0, 1, 5, 10, and 50 μg/L) using version #3 of the HeMHAS - Heterogeneous Multi-Habitat Assay System (Stremmel et al., 2023) without the electronic control to open and close the doors. Firstly, the different concentrations were introduced into the system with the doors closed to avoid mixing the concentrations. Secondly, 50 juveniles of 10–11 day old of *D. magna* taken from the stock culture were introduced into the first compartment, which had a concentration of 0 μg/L (non-contaminated compartment) (Fig. 1). Finally, the doors were opened carefully in order to allow the displacement of organisms, colonizing the different compartments. A total of 3 replicates were performed for each contaminant, therefore, a total of 150 organisms were used per contaminant. During the first 4 h of the experiment, the number of organisms in each compartment was recorded every hour; with an additional recording at 24 h and at 48 h. A control experiment, with 3 replicates, was performed to confirm a random distribution of organisms throughout the system. All the experiments were performed in the dark at 20 ± 2 ºC, and the organisms were starved for 24 h before the assays.

The percentage of colonization in each compartment was calculated accorded to Islam et al. (2019) and Vera-Herrera et al. (2022):

$$\%Colonization = \frac{NE - NA}{NE} \times 100$$

where NE is the number of expected organisms in each compartment, considering the number of organisms introduced and the number of compartments, and NA is the number of avoiders.

2.4. Reproduction during the multi-generational exposure

To study the effect of pharmaceuticals on *D. magna* reproduction, four populations were cultured under the same conditions as described for the culture of the organisms (see Section 2.1) until obtaining the F3 (third generation): [1] control population (animals exposed to clean water, without contaminant), [2] caffeine population (animals exposed to 10 μg/L of caffeine), [3] fluoxetine population (animals exposed to 10 μg/L of fluoxetine), and [4] ibuprofen population (animals exposed to 10 μg/L of ibuprofen). 120 neonates (< 24 h) were randomly selected as the F0 generation and then divided into three glass bottles (40 daphnids/L) for each treatment. 40 neonates (< 24 h) of the second clutch of the F0 generation (n2) of each population were the founders of the next generation (F1), and so on consecutively until the third generation (F3; Fig. 2). The culture medium of all the treatments was renewed weekly to avoid any degradation of the contaminant over time, and the unused neonates (first brood) were counted and removed daily. The following endpoints were measured to know the effects of these contaminants on reproduction throughout the exposed generations: size of first brood (number of neonates born in the first clutch), cumulative offspring per female, and time of the first brood. In addition, the time taken to attain the third generation (F3) was noted.

2.5. Habitat selection behavior after multi-generational exposure

F3 individuals between 10 and 13 days old and already acclimated to the treatments (different pharmaceuticals) were selected for the habitat selection behavior experiments. All the experiments were performed in version #1 of the HeMHAS – Heterogeneous Multi-Habitat Assay System (Araújo et al., 2018). This experimental setup was designed in the shape of a "flower" (Fig. 3 and Table S1), where the pre-exposure medium (clean water, caffeine, fluoxetine, or ibuprofen) was placed in the central compartment at a concentration of 5 μg/L. The two other remaining contaminants were placed in the adjacent compartments, as well as a clean water compartment (no contamination), and a compartment with the mixture of the three chemicals (3 μg/L per contaminant). In addition, these tests were repeated with control populations which was not pre-exposed to any contaminant. From each treatment, 80 daphnids were selected, starved for 24 h, and placed in the central compartment (20 ind/replicate) with a Pasteur pipette. A total of 4 replicates per treatment were made, in which the spatial arrangement of contaminants in each replicate was randomized. Before adding the organisms, the test solutions were introduced into the test system with the access doors closed, to avoid mixing the concentrations; then, when the organisms were put in, the doors were opened carefully. The experiments were performed without food in the system for 2 h, which was considered enough time for at least 25 % of the organisms to leave the central compartment. The number of organisms in each compartment was recorded at each 30 min. After this, a recovery time of 2 h was provided (same time as the experiment). The recovery period consisted in putting the organisms (20 organisms per replicate) in clean control medium for a few minutes and then they were transferred to their pre-exposure medium (always coinciding with the water of the central compartment of the experimental setup). After this period, the same organisms were exposed to the same experimental conditions as before but, in this case, around 5×10^4 cells of *Scenedesmus* sp. were placed in the surrounding compartments (not in the initial central compartment), providing an attractive stimulus to leave the central compartment. In this second phase for habitat selection response, the organisms were similarly exposed for 2 h and the positions of the organisms were recorded at each 30 min. A control experiment containing clean water in all compartments was also performed to confirm a random distribution of organisms throughout the system and no preference for a particular compartment. All the experiments were performed in a dark room at 20 ± 2 ºC.

2.6. Statistical analysis

Statistical analyses of the colonization control were performed at the final time of 48 h, as this was the time at which an 80 % colonization response was achieved in the last compartment, and also to avoid any bias due to the fact that the experiment starts with all organisms in the initial compartment. For the different treatments, a statistical analysis was performed at 24 and 48 h, times for which there was a colonization response in all compartments. A normality and homoscedasticity study of the data was performed using the Shapiro-Wilk test and Levene's

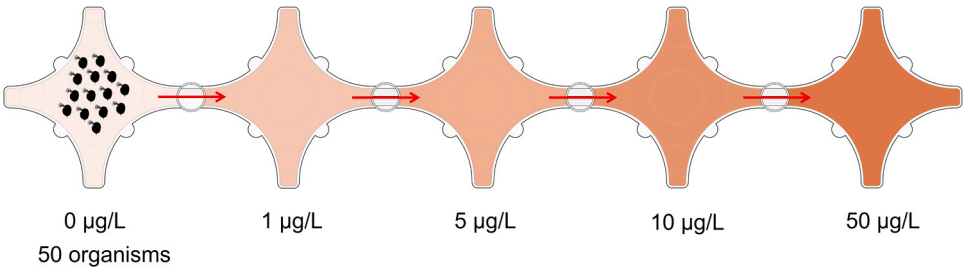

Fig. 1. Schematic setup of a replicate in the colonization test. The five nominal concentrations of the tested pharmaceuticals are represented by a color gradient. 50 organisms of *D. magna* were introduced into the first compartment.

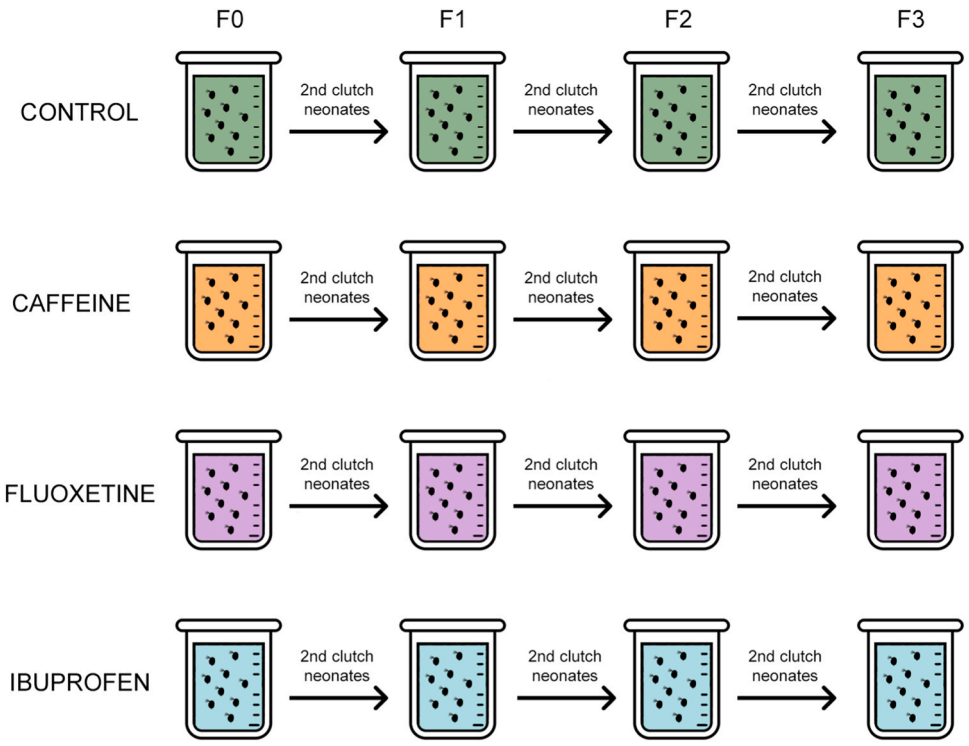

Fig. 2. Schematic diagram of the long-term exposure of *D. magna* to the three pharmaceuticals (caffeine, fluoxetine, and ibuprofen) over three generations (F3). F0, F1, F2, and F3 represent the different generations.

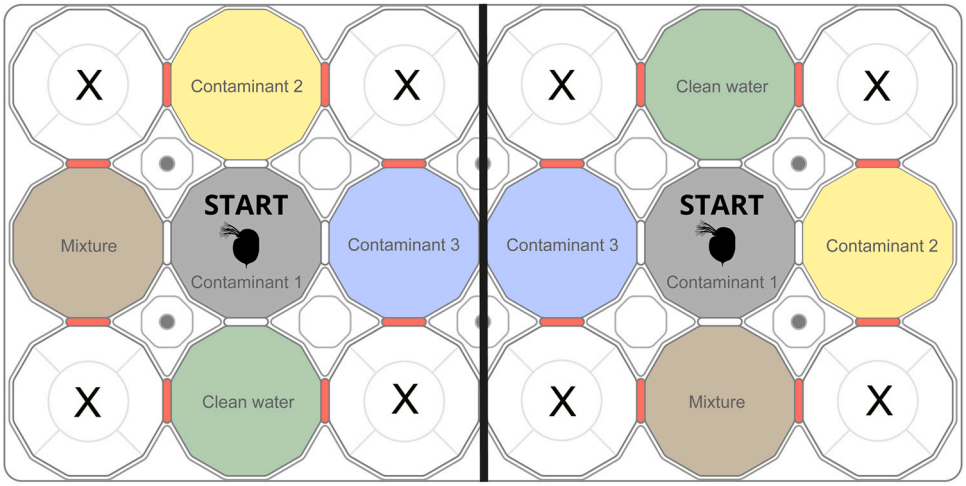

Fig. 3. Schematic setup of the HeMHAS (Heterogeneous Multi-Habitat Assay System) version #1 used in the habitat selection tests. Two blocks of experiments are shown, with the main contaminant in the central compartment (contaminant 1) and the other four treatments in the adjacent compartments (clean water, contaminant 2, contaminant 3 and the mix of all the contaminants). The red lines represent doors that were closed during the experiments. The colored parts represent the exposure compartments (320 mL each), and the noncolored compartments with an "X" represent the compartments not used in these experiments.

statistical test, respectively. In addition, one-way ANOVA analyses were performed to see differences between the treatments. When statistically significant differences were found ($p \leq 0.05$), the post hoc HSD Tukey test was also performed for differences among concentrations of the same contaminant when the assumption of homogeneity of variances was satisfied, and the post hoc T3 Dunnett test for differences among the concentrations if the variances were unequal. All these analyses were performed using IBM SPSS Statistics software (v. 25).

Statistical analyses for the reproduction test were performed using Sigmaplot (v.14.0). Normal distribution of data and the equality in variances were checked using the Shapiro–Wilk test and Brown-Forysthe tests, respectively. Significantly different treatments ($p \leq 0.05$) were identified by one-way ANOVA, when the two previous tests were $p > 0.05$, and Kruskal-Wallis's test (nonparametric test) when at least one of the two previous tests was $p \leq 0.05$. When statistically significant differences were observed among groups, a multiple comparison was made between the different contaminants and the control: Dunnett's method (parametric test) or Dunn's method (non-parametric test).

Finally, Sigmaplot software (v.14.0) was also used for the statistical analysis of the habitat selection tests. Here we analyzed the distribution of organisms (%) for each compartment at the end of the experiment (at 2 h) to avoid any bias, as all organisms started from the same compartment and 30 min is not enough time to consider a preference for a specific treatment. The assumptions of normality and

homoscedasticity were studied using the Shapiro–Wilk test and Brown-Forysthe tests, respectively. When these two assumptions were satisfied, a comparison of means among groups was performed using the one-way ANOVA test. When one of the two assumptions was not satisfied, a Kruskal-Wallis's test (nonparametric test) was performed. When significant differences were observed ($p \leq 0.05$) among treatments, an all pair-wise multiple comparison procedure (Tukey Test) was performed. In addition, a two-way ANOVA was performed with the different contaminants and the presence of food as factors using IBM SPSS statistics (v.25).

3. Results

3.1. Colonization test

The mortality of the organisms in all the colonization experiments was lower than 10 %. In addition, the pharmaceutical concentrations measured in the HeMHAS system after 48 h in the colonization tests are reported in Table S3.

In the control experiment, without the presence of any contaminant, a homogeneous distribution for the percentage of organisms at 48 h was observed throughout the system, with no statistically significant differences among compartments ($p = 0.42$), so there was no preference for any particular compartment (Figure S1).

Regarding the percentage of colonization among concentrations of the same contaminant after 24 h of exposure, no statistically significant difference was observed neither with the presence of caffeine, ibuprofen, nor fluoxetine ($p = 0.87$, $p = 0.09$, $p = 0.78$, respectively). However, after 48 h of exposure, differences in the colonization response were found between the caffeine and ibuprofen treatments ($p = 0.03$, $p = 0.00$, respectively). In particular, there was a higher percentage of colonization at 50 μg/L caffeine than at 5 μg/L ($p = 0.02$), which were 117.1 % and 95.5 % respectively, when compared to the expected colonization. Referring to the percentage of colonization with the presence of ibuprofen, there were differences among treatments. The 10 μg/L ibuprofen treatment stood out, as it has a significantly lower percentage of colonization (59.4 %) compared to all other concentrations ($p < 0.05$). In the presence of fluoxetine, no difference was observed among the different treatments ($p = 0.82$).

Finally, comparing the colonization results for each concentration of each contaminant after 48 h of exposure, statistically significant differences were observed in the 5 μg/L treatment between the control and ibuprofen ($p = 0.04$), which was 1.29 times lower in the presence of ibuprofen; differences in the 10 μg/L concentration between caffeine and ibuprofen ($p = 0.03$), with 99.9 % colonization in caffeine and 59.4 % in ibuprofen; and differences in the 50 μg/L concentration between caffeine and ibuprofen ($p = 0.04$), with 117.1 % colonization in caffeine and 81.4 % in ibuprofen (Fig. 4).

3.2. Reproduction in a multi-generational exposure

During multi-generational exposure there was less than 10 % mortality in the control population and no mortality in the caffeine treatment. In the case of fluoxetine and ibuprofen exposure, mortality of less than 10 % was observed except in F2 which had a mortality of 17 % and 15 %, respectively. In addition, the concentration of caffeine, ibuprofen, and fluoxetine in the culture medium varied from 2.88 to 9.13 μg/L, 2.87–10.57 μg/L, and 7.25–10.11, respectively (Table S2) throughout the long-exposure of *D. magna*.

This multi-generational exposure to pharmaceuticals had a different effect on the reproductive traits of *D. magna* (Table 1). In F1, significant differences were observed in the total number of neonates in the first brood ($p = 0.03$), and in the time to reach this brood ($p < 0.001$). Specifically, an advance of the first brood in organisms exposed to 10 μg/L caffeine ($p = 0.007$), as well as a production of neonates two times higher than the control treatment ($p = 0.04$) was observed. On the contrary, exposure to ibuprofen caused a delay of three days to attain the first brood ($p = 0.02$). In the case of the fluoxetine exposure, no statistically significant differences were observed in any of the responses measured.

Regarding the time to reach the F3, there were statistically significant differences among treatments ($p < 0.001$). While the F3-*Daphnia*

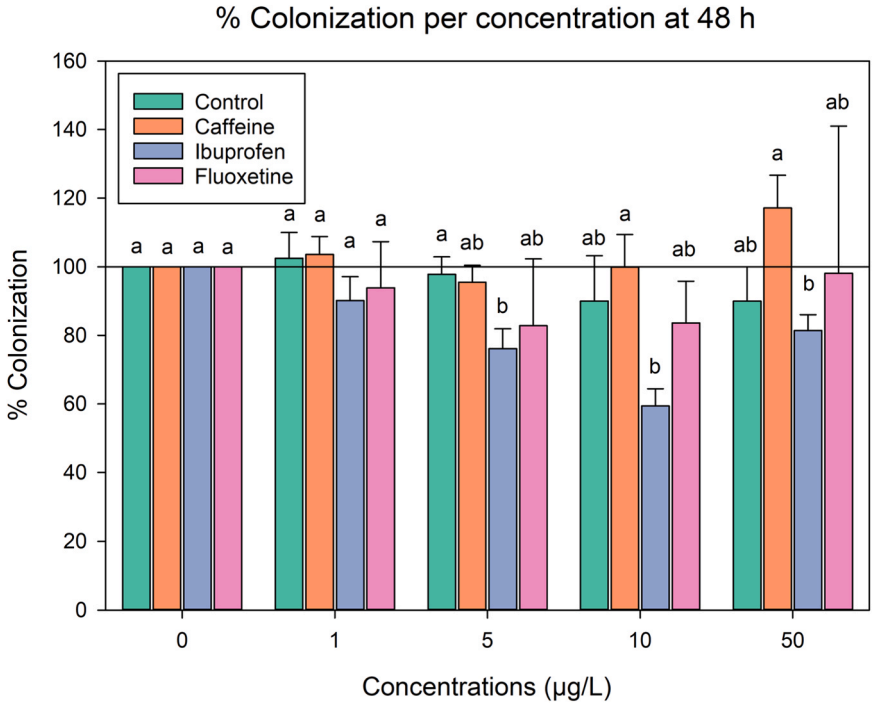

Fig. 4. Colonization response (%) of the different treatments (0, 1, 5, 10, 50 μg/L) of caffeine, ibuprofen and fluoxetine (represented by different colors) after a 48 h exposure for *D. magna*. Colonization results of the control test (compartments with no chemical) are also shown. Different letters indicate statistically significant differences at 48 h among the contaminants and control for a same compartment/concentration. a = no statistical difference.

Table 1
Data (Mean ± SD) and statistical analysis of reproduction response in the first, second, and third generation of *D. magna* to the different treatments (control, caffeine, fluoxetine, and ibuprofen). The last column represents the time to attain the F3 generation in each of the treatments.

Treatment	Generation F0				Generation F1				Generation F2				
	Total neonates in the first brood	Cumulative neonates/female[a]	Time of the first brood (week)	Mortality (%)	Total neonates in the first brood	Cumulative neonates/female[a]	Time of the first brood (week)	Mortality (%)	Total neonates in the first brood	Cumulative neonates/female[a]	Time of the first brood (week)	Mortality (%)	Time to reach the F3 generations (week)
Control	64.3 ± 37.9	2.9 ± 1.1	2 ± 0	3.3	49.0 ± 19.9	5.4 ± 0.97	7.3 ± 1.5	0	79.7 ± 30.9	8.6 ± 0.37	11 ± 1	0	13 ± 1.4
Caffeine	139.3 ± 61.6	4.2 ± 2.6	2 ± 0	0	122.0 ± 0.0*	10.1 ± 0.0	4 ± 0.0**	0	129.7 ± 24.0	10.2 ± 4.1	6 ± 0	0	7 ± 0**
Fluoxetine	35.3 ± 16.2	2.03 ± 0.33	4 ± 0	6.7	43.7 ± 32.6	5.1 ± 3.6	7.5 ± 0.71	0	41.3 ± 29.9	8.7 ± 3.7	11 ± 0	16.9	15.5 ± 0.71
Ibuprofen	64.5 ± 0.71	3.48 ± 0.43	3 ± 0	1.3	51.5 ± 50.2	6.9 ± 0.11	10.5 ± 0.71*	7.14	72.0 ± 66.5	12.4 ± 1.2	15.5 ± 0.71	15.2	19 ± 2.83**

[a] Neonates accumulated per day since the beginning of the experiment (day 0)
* Significantly different from the control ($p < 0.05$)
** Significantly different from the control ($p < 0.01$)

was obtained in clean water after 13 weeks, the organisms exposed to ibuprofen needed 19 weeks, and the organisms exposed to caffeine needed only 7 weeks (Fig. 5). In relation to fluoxetine, there was no statistically significant difference with respect to the control ($p = 0.09$), as it took an average of 15 weeks to reach the F3 generation.

3.3. Habitat selection behavior after multi-generational exposure

There was no mortality observed in daphnids during all the habitat selection experiments. Moreover, during this period there was a range of variation in the chemical concentrations (Table S4 and Table S5) after 4 h in the HeMHAS compartments, due to the mixing caused by the movement of the organisms throughout the system.

In the control experiment, without the presence of contaminants in the system, the organisms were randomly distributed throughout the system, with no specific preference for any compartment in the experiments without food ($p = 0.84$; Figure S2), and in the experiments with food ($p = 0.07$; Figure S2). The same occurred when the control population that had been in clean water for 3 generations was subjected to the presence of the different pharmaceuticals ($p = 0.78$; $p = 0.33$; Figure S3).

Daphnids cultured in clean water and following transferred to the compartment of caffeine selected to stay in this compartment, which was also observed in the presence of food (Figure S4). As for the organisms that were pre-exposed to caffeine, they were distributed throughout the system, without preference for any compartment in the experiments without food ($p = 0.99$; Figure S5), and with food ($p = 0.11$; Figure S5).

When the control population started the experiment in the ibuprofen compartment, there were significant differences in the percentage of organisms distributed throughout the system ($p = 0.01$; Figure S6), with a higher percentage of organisms present in the caffeine compartment with respect to the compartments with fluoxetine and clean water. This difference was not seen in the experiments with food ($p = 0.17$; Figure S6). On the other hand, the population pre-exposed to ibuprofen was distributed throughout the system without any statistical difference between the compartments ($p = 0.13$; Figure S7). In contrast, when food was introduced into adjacent compartments, there were differences at 2 h ($p = 0.04$; Figure S7) between the compartment with ibuprofen (initial compartment) and the compartment with fluoxetine (53.6 % and 3.7 % of the organisms, respectively).

During the fluoxetine experiment, without and with food, the control population was distributed throughout the system without any preference for any contaminants ($p = 0.16$; $p = 0.30$; Figure S8). The same happened with the experiment without food with the population that was pre-exposed to 10 μg/L fluoxetine for three generations ($p = 0.94$; Figure S9). However, when food was added to adjacent compartments, there were significant differences ($p < 0.001$; Figure S9), specifically between the compartment containing the mixture, which had more than 50 % of the organisms, when compared to all the other compartments.

4. Discussion

4.1. Behavioral responses: colonization and habitat selection

The study of sublethal effects, such as behavioral studies, is a rapid and sensitive tool for aquatic toxicity testing (Melvin and Wilson, 2013). These tests are crucial to understand the true magnitude of the impact of contaminants, more specifically, the pharmaceuticals (Brodin et al., 2014). Alterations in behavior, such as in colonization response or habitat selection response, involve a balance regarding the costs and benefits at a high ecological level as many other factors than contamination have implications for habitat selection (Araújo et al., 2020a; Araújo et al., 2020b; Salvatierra et al., 2022). This process can lead to changes in predator-prey dynamics (Huang et al., 2015; Zhou and Weis, 1999) and changes in reproductive success (Scott and Sloman, 2004),

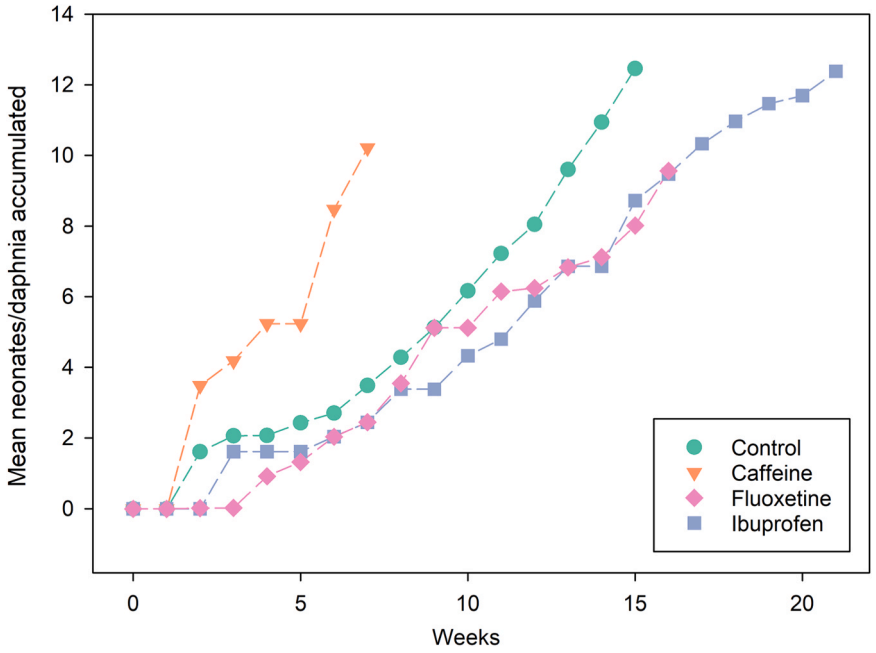

Fig. 5. Accumulated mean number of neonates of *D. magna* over time until the third offspring (weeks) per female during a long-exposure to 10 μg/L of caffeine, ibuprofen, fluoxetine and the control treatment (represented by different colors).

although taking this factor into consideration in ecotoxicology is relatively novel (Moreira et al., 2023; Vera-Herrera et al., 2022).

The results presented in this study show the effect on the ability of *D. magna* to colonize environments contaminated with some widely used pharmaceuticals such as caffeine, ibuprofen and fluoxetine. Furthermore, once the organisms reach these environments and, in some cases, do not avoid potentially toxic concentrations, the current study also suggests that a long-term exposure to environmental concentrations of these chemicals may alter habitat selection and reproduction success, which can directly affect the normal life cycle of *D. magna* populations.

Some concentrations of some pharmaceuticals such as diazepam, fluoxetine, risperidone and buspirone have proved to be attractive for aquatic organisms (Abreu et al., 2016; Jacob et al., 2021; Stremmel et al., 2023). According to our results, we can consider caffeine as such regarding both the colonization and habitat selection tests. This is because a colonization rate of more than 100 % was obtained at the highest caffeine concentration (50 μg/L) during the 48-h colonization test. Further, a preference for the caffeine compartment was observed in the control population in the habitat selection test after multi-generational exposure, regardless of the presence of food in the test, preferring to stay in the central compartment and not to forage in the compartments that have food. This preference was not seen in the population that had been previously exposed to this contaminant for three generations, as the population was distributed evenly throughout the system.

Concerning ibuprofen, the low percentage of colonization at the 10 μg/L concentration is notable, indicating some repulsiveness of this chemical to daphnids. Furthermore, after long exposure to this concentration, there was no significant effect on the selection of habitat from the population of daphnids as the percentage of organisms was homogeneous throughout the system. The avoidance of ibuprofen has already been observed at concentrations from 5 μg/L in other aquatic organisms, such as *Danio rerio* fish (Islam et al., 2023). Although there is no data about avoidance of ibuprofen in *D. magna*, some studies with daphnids have shown an avoidance response by *D. magna* to copper (Lopes et al., 2004), the herbicide atrazine (Rosa et al., 2012), the insecticide fipronil (Moreira et al., 2024), particulate material from metallurgical industries (González et al., 2023); however, sometimes this trend to avoid potentially toxic contaminants is not always observed (Moreira et al., 2023).

Finally, regarding fluoxetine, although a previous study has shown it to have some attraction on *D. magna* at concentrations over the LC50 (Stremmel et al., 2023), no such stimulation was observed in the current study. The ability to colonize environments contaminated from 1 to 50 μg/L fluoxetine was similar to the exposure to the control water. This was also found by Stremmel et al. (2023), when *D. magna* was exposed to low levels of fluoxetine. In other aquatic species, for example, in the fish *D. rerio*, an attraction to concentrations of 25 and 50 μg/L of fluoxetine was observed, but not at a concentration of 1 μg/L (Abreu et al., 2016). In our studied species, this attraction was not seen at these concentrations, even after a long-term exposure.

In the current study, the presence of food in the non-forced system, contrary to our expectations, was not a stimulus to leave the central compartment as, in most cases, the highest percentage of organisms was found in the central compartment with no food (in the environment in which daphnia had been exposed over a multi-generational period). This may have occurred because the exposure to some chemicals reduces the feeding of this species (Duan et al., 2022; Nkoom et al., 2019; Pan et al., 2017; Rocha et al., 2014). Specifically, this result has previously been seen with caffeine (Lu et al., 2013) and ibuprofen (Michalaki and Grintzalis, 2023). In contrast, although a decrease in feeding rate has been shown for other aquatic species (Grzesiuk et al., 2020; Ofoegbu et al., 2019), in *D. magna* it appears that exposure to fluoxetine does not alter its food intake rate (Ding et al., 2017).

4.2. Reproduction

Specifically, regarding the reproduction response, concentrations of 10 μg/L of caffeine shortened the period to reach reproductive maturity, resulting in a higher number of neonates, as well as a significant increase in the number of offspring. This reduction in time before the appearance of the first brood in *D. magna* exposed to caffeine has been previously reported by de Lima e Silva et al. (2022), which found an average of 2 days of anticipation between broods; significantly, this occurred for the third brood even at low concentrations, 5 μg/L. This increase in reproduction could be expected because caffeine is a substance considered a central nervous system (CNS) stimulant, as it blocks adenosine receptors, a CNS inhibitor (Fredholm et al., 1999; Moratalla, 2008).

Despite these results, this increase is not always observed, because at higher concentrations, from 0.12 mg/L, a reduction in the number of neonates by females has been observed (Lu et al., 2013), reaching an inhibition of 71.3 % of reproduction at 60 mg/L of caffeine (de Lima e Silva et al., 2022). Probably, there must be a threshold from which the stimulant effect of caffeine is surpassed by the toxic effect: hormesis ("beneficial") effect at low concentrations and toxic effects at high concentrations (Mushak, 2007).

On the other hand, a chronic exposure of 10 μg/L of ibuprofen lengthens the reproductive cycle, delaying broods. According to our results, the third generation was obtained approximately 6 weeks later compared to the control population and 12 weeks after the caffeine population. These negative effects in reproduction have also been observed in other studies (Du et al., 2016; Yang et al., 2013) even at concentrations from 0.5 to 50 μg/L (Wang et al., 2016) and at concentrations higher than those found environmentally (Han et al., 2010; Hayashi et al., 2008). Heckmann et al. (2007) consider that this substance has a strong negative concentration-dependent effect after 14 days of exposure. This may be because a long exposure to this contaminant generates a large number of embryos that present some deformation in their morphology, as Grzesiuk et al. (2020) found that 90 % of females had at least one deformed embryo at concentrations of 4 μg/L of ibuprofen.

In the case of fluoxetine, changes in reproduction are more complex, as exposure to low concentrations in the range of 0.1 and 1 μg/L, seems to increase the number of neonates in populations (Fuertes et al., 2020). This increase has not been observed when studying later generations. In fact, in a multigenerational approach, an increasing reduction of neonates has been detected (Barbosa et al., 2017). It also happens with concentrations higher than approximately 30 μg/L, whose chronic exposure increases the number of neonates in the first generation (Flaherty and Dodson, 2005; Varano et al., 2017), but this number tends to be reduced in the second generation (Péry et al., 2008). Stremmel et al. (2023) observed that fluoxetine stimulated earlier production of neonates even at concentrations of 100 μg/L during a 21-day exposure. Despite these effects on the reproduction of *D. magna*, these changes were not seen in the current study, as there are no differences in comparison with the control. We observed fewer neonates, but this difference was not significant. It is important to highlight that the number of neonates in our control was lower than previous reports, which suggests that caution is necessary when comparing these results.

Alterations in reproduction have been shown to occur at much lower concentrations than other endpoints such as immobilization, which has been widely studied in *D. magna*, for which an EC50 = 177.8 mg/L, 108 mg/L and 6.4 mg/L at 48 h is estimated for caffeine, ibuprofen and fluoxetine, respectively (Chevalier et al., 2015; Christensen et al., 2007; Cleuvers, 2003). This is relevant data given that, even at sublethal concentrations and if the exposure is extended in time, it is possible to observe a population loss at the local scale due to the impairments in the reproductive potential of the organisms.

4.3. Population decline at sub-lethal levels

Commonly, the loss of population at the local level is related to two important endpoints: mortality, when the concentrations are very high, and reproduction, when the concentrations of contaminant are lower but affect the number of potential descendants. This easy and direct relationship with the population decline allows the standardization of both mortality and reproduction tests (Ågerstrand et al., 2020). This current study brings an additional highlight how behavioral endpoints under the non-forced exposure approach can also contribute to assess any contamination-driven population decline. If organisms avoid contaminants or their presence prevents organisms from colonizing the area, then a direct relation with population decline can be established. This study integrates the traditional mortality and reproduction responses under forced exposure with avoidance/colonization and habitat selection responses in non-forced exposure systems to show that part of population might flee from the system, while the other part that was not able to detect the potential toxicity risk and avoid the exposure might suffer from sub-lethal effects. As a final remark, it is also important to highlight the importance of the multi-generation long-term study of contaminants as well as testing multiple contaminants simultaneously when behavioral responses based on habitat selection are studied. In addition, as we have seen, effects that go unnoticed in the first generation can occur in the next generations, causing an impact that has not been taken into account in short-term tests.

5. Conclusions

The presence of some pharmaceuticals could cause alterations to the habitat selection pattern and, therefore, on the distribution of daphnids, as well as having a significant effect on reproduction. In the current study, we observed an effect on the ability of *D. magna* to colonize environments contaminated by some pharmaceuticals. Specifically, an increase in colonization was observed in response to caffeine exposure, which might be an attractive stimulus for this species. This response was also observed in the selection of habitat, as the organisms preferred the area with caffeine to the area without the contaminant. In addition, this contaminant stimulated reproduction by producing larger broods with a higher number of neonates per female, as well as shorter intergeneration times. As for ibuprofen, the ability to colonize was decreased at higher concentrations and therefore the percentage of organisms was lower. Furthermore, ibuprofen decreased the reproductive capacity of the species given that fewer neonates per female were observed and the time between generations was longer. Finally, exposure to fluoxetine did not lead to any changes in the ability to colonize or any apparent changes in reproduction rates. Therefore, this study highlights the importance of considering different generations of the same species, by employing a multi-generation long-term exposure study. Applying a non-forced exposure approach makes it possible to understand more realistically the effects that pharmaceutical chemicals can have on the life cycle and population distribution of organisms.

CRediT authorship contribution statement

Cristiano Araújo: Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Eloísa Ramos-Rodríguez:** Writing – review & editing, Methodology, Conceptualization. **Gema Parra:** Writing – review & editing, Methodology, Conceptualization. **María Úbeda-Manzanaro:** Writing – review & editing, Methodology. **David Salvatierra:** Writing – review & editing, Methodology. **Ilaria Cecconi:** Methodology, Investigation, Data curation. **María Pilar González:** 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.

Acknowledgments

This study is part of the BeingHavior project (PID2022–137402OB-I00) funded by the Ministry of Science, Innovation and Universities of Spain (MCIN/AEI/10.13039/501100011033/FEDER, UE). M.P.G thanks the CSIC and the Youth Guarantee initiative for the contract (AND21_ICMAN_M2_115) financed by the European Union's Structural and Investment Funds. Thanks also go to the Spanish State Investigation Agency for the pre-doctoral contract (PREP2022–000459). D.S thanks the Andalusian Regional Government for the financing of the pre-

doctoral contract as part of the Andalusian Research, Development and Innovation Plan (PAIDI 2020). Finally, the authors would also like to thank Marina Pintado and Rubén Rios from the University of Cádiz for the analysis of the chemical samples, and Jon Nesbit for his revision of the English text.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117633.

Data availability

Data will be made available on request.

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