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Ecotoxicological assessment of the effects of fluoxetine on *Daphnia magna* based on acute toxicity, multigenerational reproduction effects, and attraction-repellence responses

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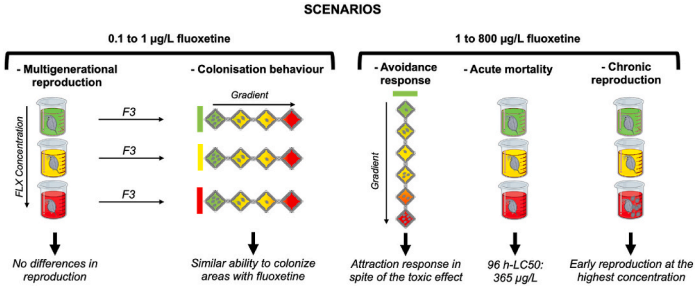
HIGHLIGHTS

- Effects of fluoxetine on *D. magna* were studied: survival, behaviour, and reproduction.
- Fluoxetine had no clear impact on reproduction in multigenerational experiments.
- Previous exposure to fluoxetine did not alter the colonisation response.
- Fluoxetine triggered early production of neonates in the 21-day chronic test.
- An attraction at higher concentrations was observed in the avoidance experiments.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluoxetine, a common pharmaceutical used as an antidepressant, is already considered potentially hazardous to biota due to its increasing use and detection in European, North American, and Asian rivers. We studied the effects of fluoxetine on *Daphnia magna*, as we hypothesized that fluoxetine might have harmful effects, short and long-term, at different levels: survival, behaviour, and reproduction (offspring production). We applied two different approaches: (i) a scenario at environmentally relevant concentrations (0.1–1.0 µg/L) and (ii) a scenario simulating a future worsening of contamination (1–800 µg/L) until the reach of lethal concentrations. In the former, we examined whether there are multigenerational effects on reproduction and on the avoidance/colonisation behaviour in previously exposed populations. In the latter, three responses were assessed: survival, avoidance behaviour and reproduction. We did not detect differences in the reproduction output of *D. magna* among the treatments over the three generations examined. Irrespective of the multigenerational treatment, *D. magna* colonised the environments with fluoxetine in a similar way. In the second scenario, we determined the lethal concentration for 50% of the population (96 h-LC_{50} = 365 µg/L), which, in spite of the toxic effect, was

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attractive to organisms during the avoidance tests (24 h); in fact, *D. magna* were attracted (no repellence) even to the highest concentrations of fluoxetine tested (800 μg/L). Lastly, in a 21-day chronic toxicity test the reproduction output of *D. magna* increased with higher concentrations of fluoxetine. This effect might be related to the fact that the organisms in the contaminated treatment began their first reproduction earlier, when compared to that in the control treatments. In conclusion, this study discusses an identified hazard for aquatic biota due to the fluoxetine attraction effect and a predictive assessment of the consequences expected if its indiscriminate use increases.

1. Introduction

Fluoxetine (trademark Prozac®) is a pharmaceutical compound, administered as a racemic mixture, which belongs to the group of Selective-Serotonin-Reuptake Inhibitors (SSRIs) and is widely prescribed in its water soluble chemical form as a hydrochloride salt (fluoxetine hydrochloride) (Wenthur et al., 2013; Risley and Bopp, 1990). Fluoxetine is a neuroactive substance that inhibits the reuptake of serotonin, which leads to an increasing extracellular serotonin level (Blardi et al., 2002; Bymaster et al., 2002); therefore, it is widely used as a treatment for depressions, eating-disorders, compulsive behaviour and panic disorder among others (U.S. FDA, 2021). In the year 2018, fluoxetine ranked 23 in the USA on the list of the most prescribed drugs (Kane, 2021). Its use has increased over the years, as documented by the U.S. Department of Health and Human Services (2021), and there is a growing concern regarding the presence of fluoxetine in some aquatic ecosystems. In fact, fluoxetine has been detected in untreated wastewaters and effluents of wastewater treatment plants (WWTP) at concentrations that vary between 0.012 and 3.5 μg/L (Brooks et al., 2003; Schultz et al., 2010; Writer et al., 2013; Ribeiro et al., 2014). Fluoxetine cannot be eliminated completely by WWTPs as shown by Yuan et al. (2013) and Cao et al. (2020). In fact, Ma et al. (2018) reported that the effluents of WWTPs had an even higher concentration; up to 300%. A gradient from treated wastewaters, freshwater and saltwater systems has shown maximum concentrations of 2.7 μg/L, 0.3 μg/L and 0.03 μg/L, respectively (Mole and Brooks, 2019). To cite some examples, the presence of fluoxetine has been recorded in some US rivers, streams and wastewaters (Ericson et al., 2002; Writer et al., 2013), in Spanish rives (Fernández et al., 2010; López-Roldán et al., 2010), in rivers and tap water in Poland (Giebułtowicz et al., 2014), in wells that are a water supply for the population in India (Fick et al., 2009) and in sewage and in receiving waters in Norway (Vasskog et al., 2008) and China (Wu et al., 2017; Ma et al., 2018). The Global Burden of Disease Study has shown an increase of 50% of diagnosed depressions worldwide from 1990 to 2017 (Liu et al., 2020), so it is to be expected that concentrations of antidepressants such as fluoxetine will increase further in aquatic systems, which requires more studies regarding its environmental effects. In spite of fluoxetine being a globally used drug for almost 50 years, the data available about the concentration of fluoxetine, its fate in the environment and the impact it has on the aquatic ecosystems are rather sparse.

Although there are few studies on the effects of fluoxetine on non-target organisms, the results show hazards at different levels. For instance, Grzesiuk and Pawelec (2021) found a delay in the response time to a possible dangerous situation (e.g. a predator) in *Neogobius fluviatilis* (monkey goby) and Duarte et al. (2020) showed that fluoxetine was accumulated in fish tissue (*Argyrosomus regius;* Salmon-bass) causing DNA damage in liver cells, affecting growth, and triggering antioxidant defence responses. In the clam *Tegillarca granosa* (blood clam), fluoxetine reduces the haemocyte viability and imposes physiological stress, which leads to a suppressed immune response (Shi et al., 2019). The accumulation of fluoxetine in the brain tissue of the fish *Catostomus commersonii* (white sucker) was reported by Schultz et al. (2010). Fong (1998) showed an effect of induced spawning in *Dreissena polymorpha* (zebra mussels) by fluoxetine. Effects studied on *Ceriodaphnia dubia* have shown a reduction in the number of neonates and broods produced (Henry et al., 2004). In *D. magna*, fluoxetine interferes with sexual reproduction by increasing the clutch size, and leading to smaller-sized offspring (Campos et al., 2012, 2016). It was reported by Campos et al. (2012) that the effect that fluoxetine has on *D. magna* reproduction is similar to those produced by low food quality and availability. Aulsebrook et al. (2022) showed that the effects of fluoxetine on fecundity, body size and the intrinsic growth rate of *D. magna* are dependent on genotypes and that in combination with different temperatures the effects may differ markedly. Fluoxetine accumulated in *D. magna* within 48 h and increased the filtration rate as reported by Ding et al. (2017).

Considering the evidence of the environmental risks of fluoxetine concerning non-target organisms, we hypothesized that fluoxetine might have negative effects, in the short and even long term, on different life-history traits (survival and reproduction) and on different behavioural responses (colonisation and avoidance). Therefore, two scenarios were simulated: (i) a real scenario at environmentally relevant concentrations and (ii) a future worse scenario at higher concentrations simulating a severe contamination event until the reach of lethal concentrations. The goals in the first scenario were to assess how fluoxetine affects *D. magna* reproduction and colonisation behaviour after multigenerational exposure to low and high levels of fluoxetine. To achieve this, we analysed the effects of fluoxetine on offspring production, ephippia production (resting eggs) and mortality rates over three generations (F1, F2 and F3). The behavioural effect on the F3 generation was analysed by assessing the capacity of organisms, previously exposed to fluoxetine, to colonise habitats contaminated with different concentrations of fluoxetine. In the second scenario, three approaches were employed: (a) acute exposure to obtain the lethal concentration (LC_{50}) of fluoxetine over a period of 96 h, (b) behavioural effects using avoidance assays to determine any repellent effect of fluoxetine and (c) sub-lethal consequences to assess how fluoxetine affects the reproduction of *D. magna* using a 21-day chronic toxicity test (OECD, 2004; OECD, 2012).

2. Material and methods

2.1. Fluoxetine

Fluoxetine-Hydrochloride (CAS-No 56296-78-7, LOT-No LRAC3045, of analytical standard, 100% purity purchased from Sigma-Aldrich, made in the USA) was employed in the experiments. We prepared a 100 mL solution of 20 mg/L (nominal concentration) with Milli-Q water and preserved it in a glass bottle, at 4 °C. Concentrations of fluoxetine were determined following the methods described by Baena-Nogueras et al. (2016). The extraction of aqueous samples was carried out by using on-line extraction (OLE), directly coupled to Ultra Performance Liquid Chromatography tandem mass spectrometry analysis (UPLC-MS/MS) using a Bruker EVOQ Elite system (Bruker). The samples were filtered using 0.22 μm polytetrafluoroethylene (PTFE) filters (Teknochroma, Barcelona, Spain) to prevent the possible entry of particles through the LC system. The online-SPE program consisted of five phases including conditioning, equilibration, loading, washing, and extracting. Water and methanol were used as washing solvents (Scharlab, Barcelona, Spain). After washing, the fluoxetine captured on the cartridge (C18 Trap, 30 mm × 2.1 mm i. d., Bruker, USA) was eluted in back flush mode using

the chromatographic gradient (overall run time 8 min). After the elution, the valve was switched back to the loading position for the next injection. The chromatographic separation was obtained using an ACQUITY UPLC BEH C18 Column (2.0 μm, 2.1 mm × 100 mm, Waters, Milford, MA). The limit of detection was of 0.075 μg/L. Further information on the UPLC analysis can be found in Baena-Nogueras et al. (2016).

2.2. Test species and stock culture conditions

The test organism was the commonly used *D. magna*. We used an uniclonal stock culture of *D. magna* provided by the University of Jaén (Spain), sampled from Laguna Grande (Jaén, Spain). The culture was maintained for six months in our laboratory under a temperature of 23 ± 1 °C and a photoperiod of 12:12 h (light:dark). The medium (renewed weekly) used for the stock culture of *D. magna* consisted of bottled spring water (Font Natura®, Sierra de Loja, Granada, Spain) enriched with biotin (0,75 μg/L; CAS-58-85-5), thiamine (75 μg/L; CAS-67-03-8), sodium selenite (2 μg/L; CAS-10102-18-8) and vitamin B12 (2 μg/L; CAS-68-19-9) as suggested by Castillo Morales (2004). The organisms were fed three times a week with the green unicellular algae *Scenedesmus* sp. (concentration of 10^6 cells/organism), maintained in a non-marine optimal *Haematoccus* medium (Fábregas et al., 2000).

2.3. Experimental design

The experimental approach was divided in two groups: (i) environmentally relevant concentrations simulating a real scenario and (ii) a future worst scenario of severe contamination (see scheme in Fig. 1). In the realistic scenario, the concentrations of fluoxetine varied from 0.1 to 1 μg/L and two endpoints were measured: the multigenerational reproduction response and colonisation behaviour. In the worst scenario, the concentrations of fluoxetine were in the range of 1–800 μg/L and the endpoints studied were: acute mortality, avoidance behaviour and chronic toxicity reproduction. For the experiments in which two treatments of fluoxetine were used, the levels of fluoxetine are named as FLX_L and FLX_H for low and high levels, respectively. As reported concentrations vary between 0.012 and 3.5 μg/L (Brooks et al., 2003; Schultz et al., 2010; Writer et al., 2013; Ribeiro et al., 2014) we decided to perform the avoidance tests with concentrations (0, 1, 5, 10, 50 and 100 μg/L) and to use higher concentrations (800 μg/L) to find the threshold for mortality and then to calculate the LC50 value.

2.3.1. Multigenerational reproduction assessment

For the multigenerational experiments, three treatments of fluoxetine [control, 0.1 (FLX_L) and 1 μg/L (FLX_H)] were tested (see scheme in Fig. S1). For each treatment, 25 individuals (not older than 48 h) from the stock culture were introduced into a 1 L glass container. Each treatment was tested with five replicates. The parental generation (P0) was exposed for 14 days, and the offspring were counted after 9 and 14 days. The solutions of fluoxetine and vitamins of the culture medium were completely renewed every week. After two weeks, another 25 individuals (<48 h) of each replicate were again transferred to a new 1 L glass container to start the next generation. To make sure that neonates selected were <48 h, we did count and removed any neonates present on day 12. This procedure (counting of offspring and the renewal of the medium) was similarly repeated for the neonates produced in F1 and F2. During the experiment with P0, the organisms were fed *Scenedesmus* (~6 × 10^4 cells/ml) three times a week (Monday, Wednesday, and Friday); however, due to a delay in reproduction, individuals from F1 and F2 were fed with ~5 × 10 cells/mL three times a week and ~3 × 10^4 cells/mL twice a week (Tuesday and Thursday). The exposure conditions were similar to those described for the culture conditions and the replicates were randomly distributed at least two times a week.

2.3.2. Colonisation behaviour assay after multigenerational exposure

Organisms (10 days old) from the last generation (F3) of every treatment [control, 0.1 (FLX_L) and 1 μg/L (FLX_H)] of the multigenerational experiment were tested for the colonisation response using the HeMHAS version 3 (Figs. S2 and S3). HeMHAS is a system characterised

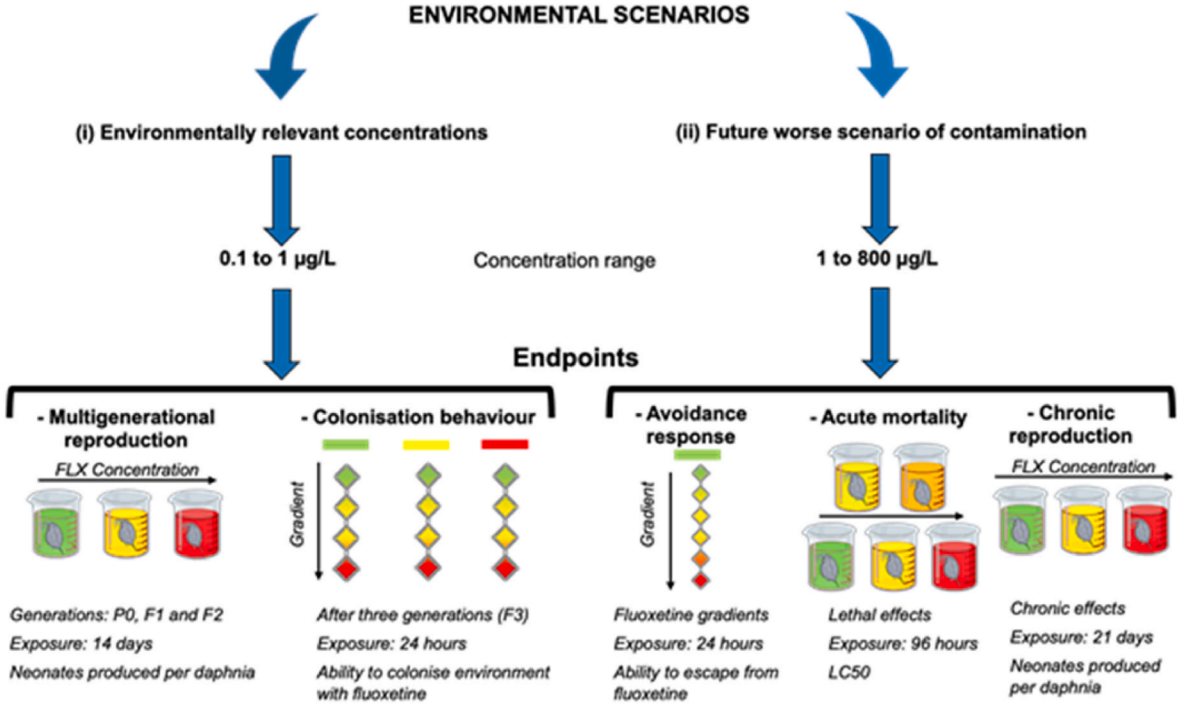

Fig. 1. Schematic display of the risk assessment approaches used in the study: (i) environmentally relevant concentrations and (ii) concentrations simulating a worse contamination scenario. The colours green, shades of yellow, and red in the test recipients represent different and increasing levels of contamination by fluoxetine. The bars with different colours (▬, ▬, and ▬) indicate *D. magna* populations previously exposed to the control, 0.1 μg/L and 1 μg/L of fluoxetine, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

by multiple, interconnected compartments, that contain different contamination scenarios. The system was constructed using acetal (polyoxymethylene), an engineering thermoplastic with a high density and a low chemical reactivity, to prevent adsorption of contaminants. The HeMHAS has a dimension of 110 × 74 × 12.5 cm (length, width, and height) and is formed by 24 compartments (4 × 6) with 6 cm of height and capacity of 320 mL, which are connected to the adjacent compartments by gyratory doors. The opening and closing of the doors among the compartments are controlled electronically through the HeMHAS Software. Each compartment has a lid to reduce evaporation.

A gradient with four concentrations of fluoxetine (0, 0.1, 0.5 and 1.0 μg/L) was created in these experiments, to provide a heterogeneous environment through which the organisms could move. Before placing the concentrations into the compartments of the HeMHAS, the doors connecting the adjacent compartments were closed. Solutions (320 mL) of fluoxetine were put into the compartments forming a gradient of contamination. Afterwards, 40 organisms were introduced into the control (no contaminant) compartment and the connecting doors were opened using the electronical interface; then, the organisms were able to move freely. The position of the organisms was recorded during 24 h; even though we have data throughout 24 h we considered the results after 24 h the most important as this is the time required for organisms to colonise all the compartments in the system. The experiments were performed (with F3-organisms from the preceding multigenerational experiment after it was ended) twice with two replicates each (n = 4 in total). The exposure occurred in darkness. No food was provided during the experiments.

2.3.3. Avoidance-repellence response assay

Three independent avoidance experiments (each N = 4) were performed to assess the potential of fluoxetine to trigger avoidance in *D. magna*. The experiments differed regarding the gradient of fluoxetine: assay #1 (0, 1, 5, 10, 50 and 100 μg/L), assay #2 (0, 5, 20, 50, 100 and 200 μg/L) and assay #3 (0, 100, 200, 400, 600 and 800 μg/L) (Fig. S4). As in the avoidance assay #1 a displacement of organisms towards the highest concentration (100 μg/L) was observed (see results), we decided to increase the concentration to 200 μg/L and then to 800 μg/L to check if the attraction effect of fluoxetine would be observed even at lethal concentrations. After placing the concentrations into the compartments in HeMHAS, individuals (10 days old) from a control treatment (no fluoxetine) were distributed in every compartment of the HeMHAS (n = 10 organisms per concentration) while the connections were closed. This differs from colonisation, in which all the organisms are put in the first uncontaminated compartment to see if they are able to colonise all the system. After opening the connections, the organisms were able to move freely along the gradient. The three experiments were performed in quadruplicate. During the 24 h period, the spatial distribution of the organisms along the gradient was periodically (1, 2, 3, 4 and 24 h) assessed. The exposure occurred in darkness. No food was provided during the experiments.

2.3.4. Acute mortality/immobility test

Lethality tests were performed following the standard procedure according to OECD 202 guidelines (OECD, 2004), except regarding the age of the individuals. Five individuals (8–10 days old) were introduced in 50 mL containers, which contained a distinct fluoxetine concentration (0, 100, 200, 400, 600 and 800 μg/L). Checks for any dead organisms were performed at each 24 h during 96 h. Each treatment was tested using five replicates. The laboratory conditions were similar to the culture conditions. No food was provided during the experiments.

2.3.5. Chronic toxicity test

The chronic toxicity test on reproduction was carried out following the OECD 211 guidelines (OECD, 2012). Organisms (between 6 and <24 h) were exposed individually in 100 mL flasks containing different fluoxetine treatments: 0, 10 (FLX_L) and 100 μg/L (FLX_H). Five replicates were performed for each treatment. The organisms were fed ~3 × 10^6 cells/*D. magna* (~3 × 10^4 cells/mL) three times a week (Monday, Wednesday, and Friday) and ~1.5 × 10^6 cells/*D. magna* (~1.5 × 10^4 cells/mL) were provided two times a week (Tuesday and Thursday). Neonates were counted daily. The test lasted 21 days at a temperature of 20 ± 1 °C and a photoperiod of 12:12 (light:dark). The medium was renewed after 7 and 14 days. The replicates were distributed randomly on a daily basis.

2.4. Data analysis

For statistical analysis we tested for normal distribution, when our data followed a parametric data set we used a one-way ANOVA, using RStudio® version 1.4.17 with R version 4.1.0 (R Core Team, 2021) and to determine LC_{50} we did a Probit analysis (Sakuma, 1998) by PriProbit® Software version 1.63.

2.4.1. Multigenerational reproduction assessment

We used a One-Way-Analysis of Variance (ANOVA) to compare the means (of the same generation and the same period (9 or 14 days)) among the three treatments. If the ANOVA showed a statistically significant difference a post-hoc Tukey test was performed. The significance level was set at 5%.

2.4.2. Colonisation

The potential of the organisms from the last generation of the multigenerational experiment to colonise environments (% of colonisers: N_C) with fluoxetine was calculated following the formula (Islam et al., 2019):

$$N_c = (N_O \ / \ N_E) \text{ x } 100$$

where the number of organisms observed (N_O) is the number of organisms in a given compartment including the organisms observed in the following compartments with higher concentration, whereas N_E (number of expected organisms) was calculated as follows: we introduced 40 organisms in the first compartment (control) and we had four compartments, we divided those organisms through 4 compartments, which resulted in 10, as a hypothetical uniform distribution. Then, N_E considers the organisms expected in a given compartment plus the organisms expected in the compartments with higher concentrations: for the first compartment (control without fluoxetine) N_E was 40, and for the following compartments it was 30 and 20, and in the last compartment it was 10 (see Islam et al., 2019).

We calculated a one-way ANOVA after 24 h for each concentration and each population. If the ANOVA showed a statistically significant difference, a post-hoc test was performed. The significance level was set at 5%.

2.4.3. Avoidance

For the avoidance tests, the number of avoiders (N_A) was calculated following the formula described by Araújo et al. (2018):

$$N_A = N_E - N_O$$

Where N_E (number of organisms expected) is the number of organisms released into a given compartment plus the organisms introduced into the compartments with higher concentrations, whereas N_O (the number of organisms observed) is the number of organisms recorded in a compartment plus the organisms observed in the compartments with higher concentrations. For instance, as we introduced 10 organisms in every compartment and we had a gradient with six compartments, N_E for the first compartment (control without fluoxetine) was 60, and for the following compartments it was 50, 40, 30 and 20, and in the last compartment it was 10. Then, the number of avoiders was divided by the number of organisms expected, according to the following formula, to calculate the percentage of avoidance:

Avoidance (%) = (N_A / N_E) x 100

We calculated a one-way ANOVA after 24 h for each concentration and each population. If the ANOVA showed a statistically significant difference, a post-hoc Tukey test was performed. The significance level was set at 5%.

2.4.4. Acute mortality

The percentage of dead organisms was calculated for each concentration after 96 h of exposure and the lethal concentration for 50% of the *D. magna* population, $LC_{50,}$ and the 95% confidence intervals, were determined using PriProbit®. We also determined the LC_{20} as this value could be considered the threshold for an initial risk to the population exposed.

2.4.5. Chronic toxicity test 21 d

The sum of neonates for every organism accumulated each day along the 21 days of exposure was calculated for each organism. The treatments were compared using a one-way ANOVA. If the ANOVA showed a statistically significant difference, a post-hoc Tukey test was performed. The significance level was set at 5%.

3. Results

3.1. Multigenerational reproduction assessment

There was no mortality or ephippia observed in any experimental treatment during the multigenerational tests. The results showed that the multigenerational reproduction was not affected by fluoxetine (Fig. 2). The comparisons of the neonates produced per *D. magna* among the different treatments showed no significant differences, regardless of the days of exposure (9 or 14) or generation (P0, F1 or F2); except for the 14-day exposure of the F1, when a significant difference between the control and the high exposure treatment was observed ($F_{2,12} = 4.3$ ($F_{Crit} = 3.89$) and $p < 0.05$) (Fig. 2).

3.2. Colonisation behaviour after multigenerational exposure

D. magna exposed to low and high fluoxetine levels showed a similar ability to colonise the fluoxetine-contaminated environments, as observed in the control population during a 24 h experiment (Fig. 3). In general, the colonisation of the lowest concentration (0.1 μg/L) was achieved by 40% of the organisms expected to colonise it, while only around 20% of the expected populations colonised the highest concentration (1.0 μg/L).

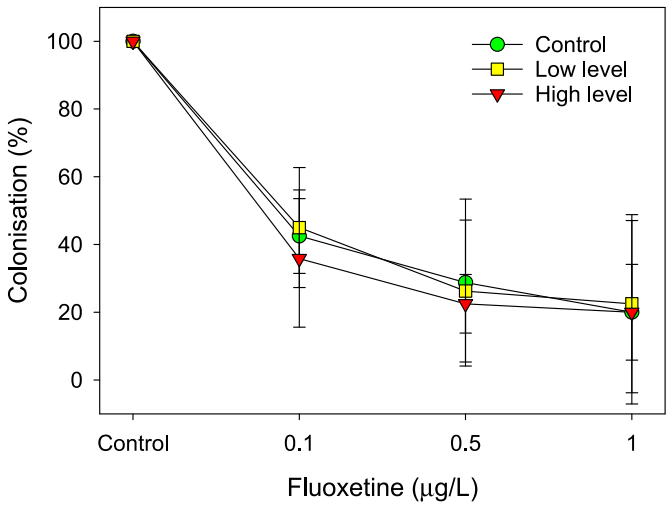

Fig. 3. The mean (and standard deviation) of the colonisation of the expected population (%) of environments contaminated with fluoxetine by three different populations of *D. magna*, which had been previously exposed to fluoxetine for three generations: control (no previous exposure to fluoxetine) and exposure to fluoxetine: FLX_L (0.1 μg/L) and FLX_H (1 μg/L).

3.3. Avoidance-repellence response

In all three avoidance assays, the organisms were shown to be attracted to fluoxetine and, in two assays (#1 and #3), the organisms were significantly ($p < 0.05$) attracted to the highest concentration of fluoxetine (Fig. 4). Assay #1 shows around 15% of the organisms in the control compartment, with a slight trend of avoiding fluoxetine until the concentration of 10 μg/L. However, that trend changed into attraction to the highest concentrations, with around 50% of the organism preferring the last two compartments (50 and 100 μg/L). In assay #2, the organisms showed a significant ($p < 0.05$) attraction to 20 μg/L (30% of organisms), while the highest concentrations were not preferred (around only 16% of organisms moved to the compartment with the highest concentration, which was almost equal to the control compartment). In assay #3, organisms clearly showed a significant ($p < 0.05$) preference to the highest concentration of fluoxetine; the last two compartments (600 and 800 μg/L) attracted around 22% of organisms each, while only 10% of the organisms were found in the control compartment.

3.4. Acute mortality/immobility test

The mortality response of *D. magna* in the 96 h-acute toxicity test

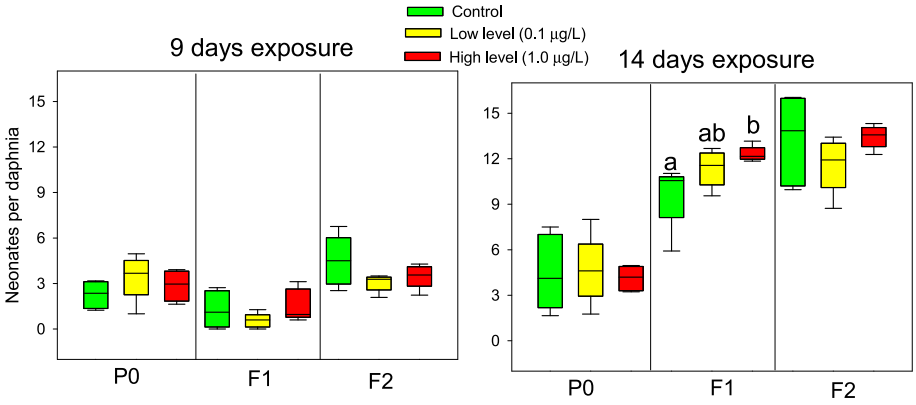

Fig. 2. Box plot (medium, 1st and 3rd quartiles and maximum and minimal values) of neonates per *D. magna* for every treatment and every generation after the 9th and 14th day. Statistically significant differences among the fluoxetine treatments, within each generation at each exposure period, are represented by different letters.

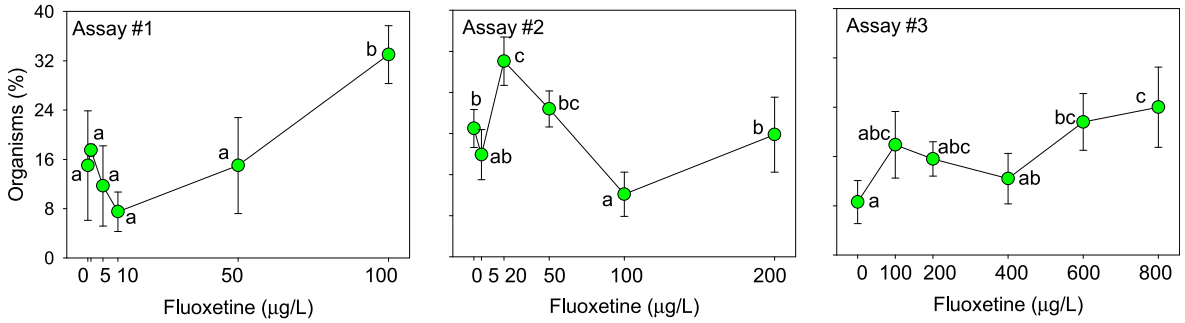

Fig. 4. The mean (and standard deviation) of the percentage of organisms found in different compartments with a gradient of fluoxetine concentrations. The organisms used in the experiments had not been previously exposed to fluoxetine. Statistically significant differences (Tukey test; $p < 0.05$) of each day are represented by different letters.

showed a clear and increasing concentration-response pattern (Fig. 5). The mortality rate in the control group was 8% after 96 h, while at the highest concentrations (600 µg/L) the mortality rate reached 71%. In the previous exposure periods (24, 48 and 72 h), the mortality was always lower than 50%. The concentration-response curve of the sigmoid model for a 96 h exposure period showed a r^2-value of 0.9948 and the LC_{50} and LC_{20} values (and confidence intervals) were determined as 365 µg/L (216 and 570 µg/L) and 154 (29–246) µg/L, respectively.

3.5. Chronic toxicity test

The results of the number of neonates produced per *D. magna* during the 21-day reproduction test are shown in Fig. 6. Our results show that fluoxetine stimulated an earlier production of neonates, as the first offspring occurred after 7 days in the organisms exposed to fluoxetine, while the first neonates were born after 9 days in the control treatment. In addition, the number of neonates was higher in the highest concentration of fluoxetine (100 µg/L) and statistically different from the control in almost all the exposure period, reaching a mean value of 58.8 ± 4.6, while in the control and low treatments the mean neonate production was 46.4 ± 3.4 and 50.6 ± 5.9, respectively.

4. Discussion

The aim of this study was to assess the risks that the antidepressant fluoxetine might pose to aquatic ecosystems, in particular to *D. magna*. We focused on two different approaches, considering (i) environmentally relevant concentrations and (ii) levels of fluoxetine expected to occur if its use continues to rise until lethal levels. A discussion about the main findings of the current study is presented in the following subsections.

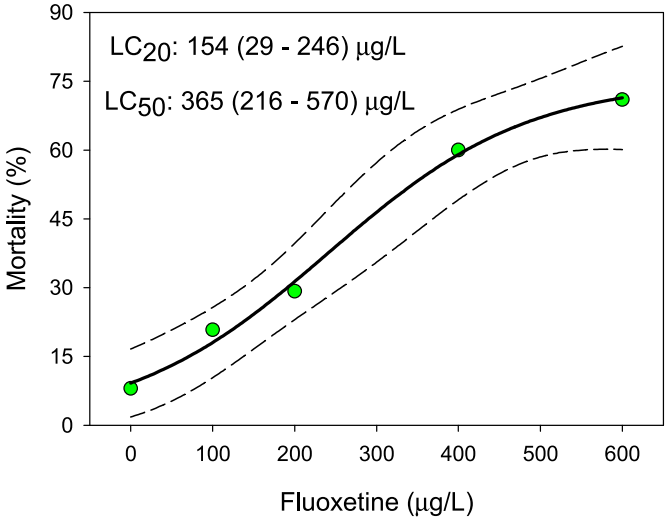

Fig. 5. Curve of concentration-mortality (and the confidence interval) in the acute test with *D. magna* exposed to fluoxetine during 96 h. Values of LC_{20} and LC_{50} (lethal concentration for 20 and 50% of populations, respectively) are also presented.

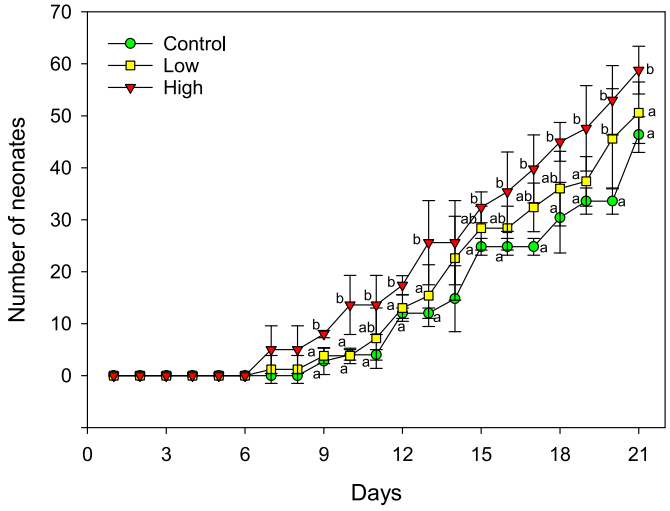

Fig. 6. Number of neonates per *D. magna* accumulated along a 21-day exposure to different concentrations of fluoxetine (control, low and high: 0, 10 and 100 µg/L). Statistically significant differences (Tukey test; $p < 0.05$) on each day are represented by different letters. No letter was included when there were no statistical differences.

4.1. Multigenerational response

Our results concerning the multigenerational reproduction assessment have not shown any effect on the organisms that could be considered of concern. We did not observe any effect on survival, or other signs of stress such as the production of ephippia or a change in fecundity during the experiment. Other authors have shown that fluoxetine at higher concentrations, influenced the reproduction of *D. magna,* similarly to food quality, in a range of fluoxetine concentrations between 10 and 80 µg/L (Barata et al., 1998; Campos et al., 2012; Campos et al., 2016). Heyland et al. (2020) showed that fluoxetine at 0.054 and 0.54 µg/L caused significant changes such as higher mortality, decreasing growth rate, reduction in average offspring size and higher production of ephippia among the generations and treatments with fluoxetine. Another multigenerational study (Barbosa et al., 2017) showed significant differences between the control and fluoxetine concentrations of 0.012 and 0.54 µg/L. The reproduction success

throughout three generations was studied and, later, the fourth generation was tested in relation to reproduction combining fluoxetine and different temperatures. Their results detected a decrease in neonates in the treatments with fluoxetine (17% and 29% in the low and high concentrations, respectively); in addition, those authors observed that fluoxetine in combination with different temperatures (treatments of 20 °C or 25 °C or a fluctuation between 15 °C and 25 °C) affected the reproduction of the fourth generation (Barbosa et al., 2017). However, they were using another clone lineage (Clone F) and the food quantity per *D. magna* was 10 times lower compared to our food conditions. Henry et al. (2022) showed in a 3-year multigenerational study with the freshwater snail *Physa acuta* that amounts as low as 0.03 μg/L affected the egg mass produced; fluoxetine exposed groups had a decrease in egg mass, although it did not affect the number of embryos nor the average embryos produced per egg mass.

4.2. Colonisation response

To our knowledge, our study is the first to assess the effects of multigenerational exposure to fluoxetine on the colonisation behaviour of *D. magna*. We could not find a statistically significant difference in the ability to colonise environments with fluoxetine among any previously fluoxetine-exposed populations. Within 24 h there was only a significant difference among all treatments between the control compartment and the compartments containing fluoxetine, but this result is biased because the organisms were initially introduced into the control compartment (see details in Fig. 3). Organisms colonised the complete system within 24 h in the control experiment. Therefore, we can only suspect that the exposure time in the multigenerational experiment was too short to see any effect on the colonisation response (fluoxetine exposure to more generations might be required).

4.3. Avoidance-repellence response

We expected that *D. magna* would eventually avoid fluoxetine, but the results of the three avoidance assays showed that fluoxetine proved to be attractive to organisms instead. In assays #1 and #3, *D. magna* was drawn to the highest concentrations of 100 and 800 μg/L. Some chemicals seem to exert an attractive effect on organisms despite their known toxic effects. For instance, the attraction response was observed for *Orconectes virilis* (crayfish) exposed to sertraline (Woodman et al., 2016), for*Cortunix japonica* (Japanese quails) exposed to glyphosate (Ruuskanen et al., 2019), the fish *Cyprinus carpio* (common carp) exposed to diazepam (Jacob et al., 2021). It is known that some toxic chemicals might have the ability to attract rather than repel, such as reviewed and discussed by Araújo et al. (2020). Abreu et al. (2016) showed that *Danio rerio* (zebra fish) were attracted to concentrations of 25 and 50 μg/L of fluoxetine, but not at 1 μg/L; they assumed that the attraction was probably via olfaction as anosmic fish did not show this behaviour. Hence, the attraction effect produced by potentially toxic chemicals could be related to damage to chemoreceptors (Tierney et al., 2007; Cherkashin and Blinova, 2011). Another possible explanation might be that the reuptake of serotonin in the nervous system leads to a 'positive' effect on the organisms' behaviour, triggering an adverse reproduction response (Campos et al., 2016). Therefore, it is important to study whether the attraction produced by fluoxetine is not related to a false benefit, but instead to possible damage caused to the organism's ability to recognise risks chemically; although in this latter situation, a random distribution would be more expected than an attraction response.

4.4. Lethal effect

Data of acute toxicity of fluoxetine on *D. magna* showed that our population was highly sensitive, with an LC_{50} of 365 μg/L after 96 h. These data are much lower than those described in other studies, although the comparison should be made with caution due to the differences in the exposure time; for instance, in a study with *D. magna* (6–24 h neonates) the 48 h-LC_{50} was determined at 820 μg/L by Brooks et al. (2003), Minguez et al. (2014) reported values of around 6 mg/L for a 48 h-LC_{50} and Schlussel et al. (2019) found a 24 h-LC_{50} at around 4 mg/L. As in our study, the organisms were six-day-old juveniles, it is not easily comparable to the above-mentioned studies. The exposure time was 96 h, because at previous exposure periods (24, 48 and 72 h) the mortality observed was lower than 50%. Besides the differences in exposure time and age of organisms, other factors like the culture medium and the clones used might also affect the consequences the organisms experience as a result of chemicals. These results evidence that the variability of an LC_{50} and, therefore, a low or high risk will depend not only on the concentrations tested, but also on the differences in the environmental conditions of the exposure. In fact, Aulsebrook et al. (2022) concluded that not only the genotype of *D. magna* but also the combination with environmental temperature might determine the effects of fluoxetine on the life history traits of *D. magna* at concentrations as low as 0.03, 0.3 and 3 μg/L, and which showed a non-monotonic manner as well.

For *C. dubia* a 48 h-LC_{50} of fluoxetine was determined at 234 μg/L (Brooks et al., 2003) and 510 μg/L (Henry et al., 2004). The LC_{50} value reported for fluoxetine concerning *Pseudokirchneriella subcapitata* (microalgae) was 24 μg/L (Brooks et al., 2003). A recent study by Rezende et al. (2021) showed LC_{50} values of fluoxetine after 24, 48, 72 and 96 h of 40, 36, 28 and 26 μg/L, respectively, for *Palaemon pandaliformis* (phantom shrimp). This shows that other organisms are clearly more sensitive to fluoxetine than *D. magna*. It seems that the environmentally relevant concentrations of fluoxetine as a single substance do not imply a truly acute risk for some aquatic organisms (under laboratory conditions), yet it is important to consider that the continuous increase in the levels of fluoxetine in the environment is indicative of an even higher imminent risk.

4.5. Chronic toxicity test

Our data shows that reproduction under a high concentration of fluoxetine (100 μg/L) started two days earlier and the number of neonates in the first brood was higher than in the control and the FLX_L (10 μg/L). Over the 21 days, it seems that there is a higher number of offspring in the FLX_H, but this might be attributed to the first brood, which took place two days earlier than in the control and FLX_L. A reproduction study with *D. magna* was also carried out by Péry et al. (2008) with concentrations from 3 to 300 μg/L; they found a difference in reproduction of 32% and mortality of 40% (day 21) at the highest fluoxetine concentration (241 μg/L). Moreover, in a 21-day test with neonates from the fifth brood, 70% of the neonates exposed to 102 μg/L died and reproduction was reduced by 18% at 31 μg/L. Such as observed in our study, Péry et al. (2008) did not find differences in reproduction at concentrations below 241 μg/L for the parental generation. On the other hand, in a 30 day acute toxicity test, *D. magna* exposed to 36 μg/L of fluoxetine showed no significant difference in survival, growth or sex ratio, but did in the number of neonates, with a reproduction rate almost three times higher than in the control group (Flaherty and Dodson, 2005). These results must also be treated with caution when compared with ours, as the temperature they used was 25 ± 1 °C, that might significantly affect the response to fluoxetine.

4.6. Environmental consequences of contamination by fluoxetine

The damage that fluoxetine can potentially cause is very diverse and complex. Al Shuraiqi et al. (2021) used concentrations of 0.005–5 μg/L and found that the swimming behaviour and reaction to a conspecific alarm substance measured at day 7, day 14 and 28 differs not only with time but was also non-monotonic (a non-linear dose-response pattern). Aulsebrook et al. (2022) observed that the effects of fluoxetine on *D. magna* might vary according to genotypes and the temperatures of

exposure.

In our study, even very low concentrations of fluoxetine (20 μg/L) attracted the organisms, while a very similar concentration (36 μg/L) was shown to induce an overproduction of offspring (Flaherty and Dodson, 2005). Although these responses could be considered a benefit to *D. magna*, an integrated analysis could lead us to understand the real risk of fluoxetine. As shown by Campos et al. (2016), even under conditions of food deprivation, the presence of fluoxetine stimulates reproduction, but in a maladaptive manner (more and smaller offspring even though this is not necessary to survive as it would be under predation pressure). Therefore, the increase in offspring and attraction to fluoxetine could lead to serious consequences for *D. magna* populations.

5. Conclusion

The current study observed that environmentally relevant concentrations of fluoxetine did not affect the reproduction output in *D. magna* after exposure over three generations. It was also observed that individuals of the last generation did not improve their ability to colonise fluoxetine-contaminated environments. Therefore, the possibility of an adaptation/acclimation could not be considered. In a second approach based on scenarios with very high environmental concentrations (worse scenarios), effects were found at different levels: survival, avoidance behaviour and reproduction. Regarding avoidance behaviour, *D. magna* was attracted by fluoxetine, even at concentrations considered dangerous, much higher than the median lethal concentration. Fluoxetine also stimulated the reproduction of daphnids. Both effects, the attraction to fluoxetine and the stimulation of reproduction should be analysed with caution, because instead of bringing benefits to organisms, they might indicate a serious behavioural, sensorial, and physiological imbalance caused by fluoxetine. More studies are required to understand the cellular, biochemical, and genetic mechanisms involved in the interaction of fluoxetine with aquatic organisms, in line with other natural and anthropogenic stressors, better.

Credit author statement

Helmut Stremmel: Conceptualization; Methodology; Formal Analysis; Investigation; Writing – original draft; Visualization, Linda Weiss: Resources; Writing – review and editing, Gema Parra: Conceptualization; Methodology; Writing – review and editing, Eloísa Ramos-Rodriguez Conceptualization; Methodology; Writing – review and editing, Cristiano V.M. Araújo: Conceptualization; Methodology; Formal Analysis; Investigation; Resources; Writing – review and editing; Visualization; 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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