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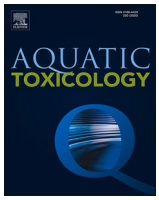

Fate and effects of an environmentally relevant mixture of microplastics in simple freshwater microcosms

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ABSTRACT

Most studies assessing the effects of microplastics (MPs) on freshwater ecosystems use reference materials of a certain size, shape, and polymer type. However, in the environment, aquatic organisms are exposed to a mixture of different polymers with different sizes and shapes, resulting in different bioaccessible fractions and effects. This study assesses the fate and effects of an environmentally relevant mixture of high-density polyethylene (HDPE) fragments, polypropylene (PP) fragments, and polyester (PES) fibres in indoor freshwater microcosms over 28 days. The MP mixture contained common polymers found in freshwater ecosystems, had a size range between 50 and 3887 μm, and was artificially aged using a mercury lamp. The invertebrate species included in the microcosms, *Lymnea stagnalis* (snail) and *Lumbriculus variegatus* (worm), were exposed to four MP concentrations: 0.01, 0.05, 0.1 and 1 % of sediment dry weight. MPs fate was assessed by performing a balance of the MPs in the surface water, water column, and sediment after a stabilization period and at the end of the experiment. Sedimentation rates per day were calculated (2.13 % for PES, 1.46 % for HDPE, 1.87 % for PP). The maximum size of MPs taken up by the two species was determined and compared to the added mixture and their mouth size. The size range taken up by *L. variegatus* was smaller than *L. stagnalis* and significantly different from the size range in the added mixture. The No Observed Effect Concentrations (NOECs) for the reproduction factor of *L. variegatus* and the number of egg clutches produced by *L. stagnalis* were 0.01 % and 0.1 % sediment dry weight, respectively. The EC10 and EC50 for the same endpoint for *L. stagnalis* were 0.25 % and 0.52 %, respectively. This study shows that current MP exposure levels in freshwater sediments can result in sub-lethal effects on aquatic organisms, highlighting the importance of testing MP mixtures.

1. Introduction

Microplastics (MPs) are found in freshwater ecosystems as a product of macro- and meso-plastic breakdown, the discharge of treated and untreated wastewater, atmospheric deposition, and agricultural runoff (Liu et al., 2019; Schell et al., 2020, 2021; Sarijan et al., 2021). Several reviews and meta-analyses investigating the risks of MPs in freshwater ecosystems point at the sediment compartment as a major sink for MPs, with sediment concentrations that are orders of magnitude higher than in the water phase (Scherer et al., 2020). Although some studies have modelled the distribution of MPs in freshwater and marine sediments (Besseling et al., 2017; Murawski et al., 2022; Uzun et al., 2022), ecotoxicity studies assessing the effects of MPs on freshwater organisms have rarely assessed water-sediment exposure dynamics (Yıldız et al., 2022). In addition, most experiments have used pristine MPs, while in nature these particles go through weathering (Alimi et al., 2022; Liu et al., 2020) and biofouling processes (Hariharan et al., 2021; Vroom et al., 2017), which can change their transport dynamics as well as their bioavailability and impact on aquatic organisms.

A major drawback of most toxicity studies assessing the effects of MPs on aquatic organisms is that they have been conducted using only one MP type, while in the environment aquatic organisms are exposed to complex mixtures of MPs with different size, shape, and polymer composition. Some studies have demonstrated that MP shape and polymer type significantly influence bioavailability and uptake of MPs depending on the species traits, thus yielding to different effect

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mechanisms and threshold concentrations (Porter et al., 2023; Prata et al., 2023; Renzi et al., 2019; Schell et al., 2022; Scherer et al., 2017; Ziajahromi et al., 2017). Kooi and Koelmans (2019) proposed a novel approach to overcome the misalignment between measured environmental concentrations and the outcomes of toxicity studies conducted with a particular MP size and shape ranges, which is based on the application of correction factors and the alpha values obtained from fitted power law distributions. By doing so, they could characterize the shape of the power law distribution of environmental concentrations of MPs in different compartments as well as the distribution of MPs taken up by freshwater organisms. These methods have been used to provide fully aligned risk assessments for MPs in aquatic and sediment compartments, as well as in soil, for measured concentrations at a global scale (Koelmans et al., 2020; Redondo-Hasselerharm et al., 2024, 2023). However, several uncertainties arouse in these assessments such as the maximum size of MPs that could be taken up by different organisms, and how this varies among polymer types and shapes when exposed through different environmental compartments (*e.g.*, water column and sediment). Therefore, further studies are needed to understand the actual uptake distribution of MPs by aquatic invertebrates and their toxic effects when exposed to mixtures formed by different size, shape, and polymer density. A recent study by de Ruijter et al., (2023) assessed the effects of environmentally relevant mixtures of aged MPs to benthic organisms using laboratory single-species tests and showed that the growth and reproduction of some species (*e.g.*, *Lumbriculus variegatus, Gammarus pulex*) may be affected at environmentally relevant concentrations of MPs. Therefore, more studies are needed to characterize the effects of aged MP mixtures with a wider range of benthic species to prevent uncertainties and biases in the extrapolation of the observed outcomes to field conditions.

The aim of this study is to assess the fate and effects of a realistic mixture of MPs on two freshwater invertebrate species, the snail *Lymnea stagnalis* and the blackworm *Lumbriculus variegatus,* using simple freshwater microcosms. The MP mixture was prepared using aged MPs with relative proportions of particle size and shape that resemble monitoring data taken in aquatic ecosystems next to urban and industrial areas. The specific objectives of this study are (1) to determine the fate and water-sediment fluxes of the tested MP types in a model ecosystem, (2) to characterize the distribution of MPs taken up by two invertebrate species with different biological traits, and (3) to assess the chronic effects of an environmental realistic mixture of MPs on lethal and sub-lethal endpoints. We hypothesize that the sediment exposure profile will differ temporally depending on the polymer type and shape, and that different feeding traits and physiological characteristics of the tested species will contribute to different uptake distributions of MPs and effects.

2. Material and methods

2.1. Test materials

We used a mixture of three polymers: high density polyethylene (HDPE), polypropylene (PP) and polyester (PES), which are the most common MPs in environmental samples (Burns and Boxall, 2018). HDPE and PP fragments were obtained by cryo-milling industrial pellets (3 - 5 mm maximum length) with a ball mill (Retsch MM400) using stainless-steel jars. Milling was performed separately for each polymer type at a frequency of 30 Hz. While milling, the jars containing the plastic pellets were cooled down every 2 min by submerging them into liquid nitrogen. The total milling time was decided based on the stiffness of each polymer. To obtain PP fragments, a total milling time of 12 min was used, while 30 min were applied in order to obtain the HDPE fragments. After the MP fragment generation process, these particles underwent artificial aging. MPs were exposed to UVA and UVB radiation for 3 days in milli-Q water (4-5 % w/w) using a mercury lamp (Novalight TQ 150) equipped with cladding tube in a borosilicate glass that removes wave lengths < 300 nm following the protocol described by Sorasan et al. (2021), which assumes an aging process equivalent to the UVA + UVB dose received from solar irradiation during one year. After aging, MPs were filtered using a 20 μm stainless-steel sieve to remove the smallest particles and the milli-Q water. Then, to eliminate any potential chemical attached to the MP surface, the particles were washed 3 times using methanol and a shaking table according to Redondo-Hasselerharm et al., (2018).

PES fibres were obtained by manually cutting a synthetic blanket (IKEA, Polarvide) with scissors into small pieces (2-3 mm), and later using an electric coffee grinder (Cecotec TitanMill 300 DuoClean). The cutting by scissors and the grinder helped to detach the fibres, and both processes were repeated several times to maximize fibre separation. Then, this material was sieved using a stainless-steel sieve of 1 mm pore size (203 mm Ø and 50 mm height) to remove big fibre clumps.

Each of the three polymers used in this study had a different colour (PP was green, HDPE blue, and PES red), which allowed the visual determination of the polymer type. The size distribution (based on the longest axis) of the MPs produced was assessed by measuring the length of 500 randomly selected particles of each polymer type using a stereomicroscope (Olympus SZX7) connected to a camera (Olympus DP 21).

Following the equations described by Kooi and Koelmans, (2019) to simplify the size range of environmental samples (Eq.1), the alfa values (α) corresponding to the exponent of the power law distribution fitted to the different MP polymer size distributions were calculated.

$$y = bx^{-\alpha} \quad (1)$$

In this equation, b is the slope, y is the relative abundance, and x is the particle size (based on the longest axis). Finally, the alpha values were compared with the alpha values calculated by Kooi et al., (2021) for environmental samples of sediment to confirm that the generated MP mixture can be considered environmentally realistic. Additionally, alpha values were calculated for the internal MP distributions in the tested invertebrate species to assess differences with the exposure MP distribution and with previous studies assessing internal MP distributions in other aquatic organisms (Kooi et al., 2021). Further details regarding the characterization and counting of MPs in the tested invertebrates are provided in *Section 2.5*.

2.2. Test organisms

The test organisms included in this study were *Lymnea stagnalis,* a pelagic/benthic species, and *Lumbriculus variegatus* an endobenthic species. *L. stagnalis* is a pulmonated gastropod, it is hermaphroditic and has mostly herbivore feeding behaviour on algal biofilms and aquatic vegetation. It inhabits lakes, ponds, or rivers with very slow flow, usually in proximity to plants or vegetation, and is distributed throughout the northern hemisphere (Kuroda and Abe, 2020). *L. variegatus* is a deposit feeding oligochaete and lives burrowed inside the sediment of lakes, shallow ponds, and marshes (Seeley et al., 2021). It is found in North America and Europe and its reproduction is mainly asexual (Dermott and Munawar, 1992).

Both species were cultured at the IMDEA Water institute (Spain) using water from an artificial lagoon (filtered through a 20 μm net) at 20 ± 1 °C, with a light:dark cycle of 16:8 h, and an aeration system. *L. stagnalis* individuals were fed using lettuce leaves following the OECD Guideline No. 243 (OECD, 2016). *L. variegatus* were cultured using silica sand and fed with fish food (Tetramin, Tetra GMB, Germany). Ten days prior to start of the test, the reproduction of *L. variegatus* was synchronized by cutting adult individuals of similar size in halves as described in the OECD Guideline No. 225 (OECD, 2010).

Ten organisms of each species from the laboratory cultures were examined under a binocular (*L. stagnalis*) or microscope (*L. variegatus*) to take an approximate measurement of their mouth size as shown in Figure S1.

Table 1
Concentration of MPs in dry sediment by polymer type (g/kg dw).

	Concentrations (g/kg dw)				
	Control	0.01 %	0.05 %	0.1 %	1 %
All polymers	0	0.1	0.5	1	10
PP fragments	0	0.02	0.11	0.22	2.20
HDPE fragments	0	0.05	0.23	0.45	4.50
PES fibres	0	0.03	0.17	0.33	3.30

2.3. Experimental design

Twenty- eight microcosms were set-up in the Ecotoxicology Laboratory of the IMDEA Water Institute (Spain). The experimental design consisted of four MP treatments: 0.01, 0.05, 0.1 and 1 % of MPs in sediment dry weight (dw), with 4 replicates each (n=4). The first three concentrations aimed to cover the range of concentrations found in different aquatic ecosystems (Klein et al., 2015; Schell et al., 2021; Scherer et al., 2020), while the highest concentration was one order of magnitude larger. The MP mixtures were formed by PP and HDPE fragments, and PES fibres according to the quantities shown in Table 1. The percentage of each polymer type selected (22 % PP, 45 % HDPE and 33 % PES in mass) in the MP mixture was based on the relative percentages provided by Scherer et al. (2020), which are based on water and sediment samples taken in the Elbe River next to urban and industrial areas.

The microcosms had a diameter of 16.5 cm, a total height of 19 cm, and were filled with 3 cm of sediment. The sediment consisted of 95 % silica sand and 5 % sphagnum peat. The sand was previously sieved at 2 mm and washed to reduce turbidity in water, and the peat was previously dried at 50 °C and grinded to obtain particle sizes below 0.5 mm. The pH of the sediment mixture was adjusted with $CaCO_3$ to reach a final pH between 6.5 and 7.5. The sediment was mixed with the MP mixture using a stainless-steel spoon. Then, 2 L of water were carefully added to each system using a pipette against the microcosm wall. The water used in the experiment was collected from an artificial lagoon and filtered through a 20 μm plankton net. The microcosms were left to stabilize for two weeks prior to the addition of the test organisms and the experiment was run for 28 days after the start of the exposure period. At the start of the exposure period, 5 individuals of *L. stagnalis* (2-3 cm length) and 20 individuals of *L. variegatus* were introduced into each microcosm. Small lettuce pieces and fish flocks were added once per week to supplement *L. stagnalis* and *L. variegatus,* respectively. According to the habitat and feeding preferences of the test species, it was confirmed that *L. stagnalis* was distributed through the water column, crawling, and grazing along the microcosm walls and the top sediment, while *L. variegatus* was mostly buried into sediments feeding on organic matter detritus during the experimental period. During the experimental period, the water temperature of the microcosms was kept at approximately 20 °C, and the light followed a light/dark regime of 16:8 h. Aeration was set inside each microcosm with a small aquarium pump. Physicochemical water quality parameters (temperature, pH, dissolved oxygen, and conductivity) were measured weekly in the controls and in all microcosms at the end of the experimental period using a YSI Pro DSS multiparameter meter (Table S1).

2.4. MP exposure and fate

Six microcosms were set up to investigate MP exposure and fate. Three of those six microcosms were sampled after the stabilization period and three at the end of the experiment. These microcosms contained the second highest MP exposure concentration used in the tests (0.1 %) and were set up exactly in the same way as the above-mentioned systems including the same number of individuals of the test species. Samples were taken from the top layer of the water column, the rest of the water column, and the sediment to determine MP concentrations in the different compartments. The top water layer (0-3 cm depth) of the microcosms was sampled by passing a 25 μm net to determine the concentration of MPs in the surface water. Afterwards, the rest of the water column was collected using a syringe with an opening size of 3 mm to avoid resuspension of the sediment. Finally, all sediment was collected and dried at 50 °C.

The volume of the surface water samples was first standardized with milli-Q water to have a consistent volume of 600 mL across all samples. Then, they were stirred using a magnetic stirrer and two drops of the surfactant TX45 was added to achieve a homogeneous mixture. Finally, three subsamples of 50 mL were taken using a glass beaker and filtered individually onto cellulose nitrate membrane filters (Scharlau, diameter 47 mm and pore size 0.45 μm) using a glass filtration unit with a vacuum pump. Filters were analysed using a stereo microscope (Olympus SZX10) at 4x, and the colour and shape of the MPs were recorded. The size of the longest axis of each MP was measured using a digital microscope camara (Olympus DP21) and the corresponding software (DP2-TWAIN).

Water column samples were first filtered through a stainless-steel sieve of 20 μm (Ø 50 mm, 25 mm high). Then, the content of the sieve was transferred to a glass beaker by rinsing the sieve thoroughly with H_2O_2. The quantity of H_2O_2 used depended on the amount needed to thoroughly rinse the sieve. The samples were then kept at 45 °C for 48h at 120 rpm in an orbital shaker (ThermoScientific MaxQ 4000) to reduce the organic matter content. Following this, the samples were vacuum-filtered onto the cellulose nitrate membrane filters and analysed as described above.

MP extraction from sediment was done by taking three replicates of 2 g of dry sediment from each sample. Then, 25-50 mL of H_2O_2 (30 %), depending on the amount of organic material, were gradually added in steps of 1 mL to reduce the organic matter content. Next, the samples were incubated in an orbital shaker (ThermoScientific MaxQ 4000) at 45 °C for 48 hours at 120 rpm. Following this, the samples were placed into an oven at 50 °C to evaporate the H_2O_2. Once evaporated, the beakers were topped off with a $ZnCl_2$ solution (ρ>1.6 g/cm^3). Subsequently, the samples were agitated for 20 min on a magnetic stirrer and allowed to settle for 48-72 h to separate the microplastics from the sediment matrix. The density separation step was carried out twice to maximize extraction. The supernatant was filtered onto cellulose nitrate membrane filters (Scharlau, diameter 47 mm and pore size 0.45 μm) using vacuum filtration. Filters were then analysed as described above for water samples.

With the data of the size and polymer found in each compartment, first a particle balance analysis was performed to calculate the percentage of each polymer in each compartment with respect to the total MP amount, and the difference between the end of the stabilization period (T0) and the end of the experiment (T28). Then, to calculate sedimentation rates, the number of particles in the water compartments (water surface and column water) at T0 was subtracted from the number at T28 and divided by the number of days to have a mean daily sedimentation rate.

2.5. MP uptake by invertebrates

To assess the uptake of MPs by the test organisms, surviving individuals were rinsed carefully with milli-Q water. After that, *L. variegatus* were dried at 50 °C onto aluminium foil to assess their dry weight, and later they were transferred into a glass beaker. *L. stagnalis* were first frozen at -20 °C to relax their muscles and remove their shell using tweezers, and then they were dried into glass beakers at 50°C. All *L. variegatus* of one replicate were grouped together, while *L. stagnalis* were analysed individually. Then, 10-20 mL of H_2O_2 (30 %) were added to digest the organic matter at 45 °C for 48 hours at 120 rpm in an orbital shaker (ThermoScientific MaxQ 4000). After that, samples were vacuum filtered onto cellulose nitrate membrane filters (Scharlau, diameter 47 mm and pore size 0.45 μm) and analysed as outlined in Section 2.4 for the water samples.

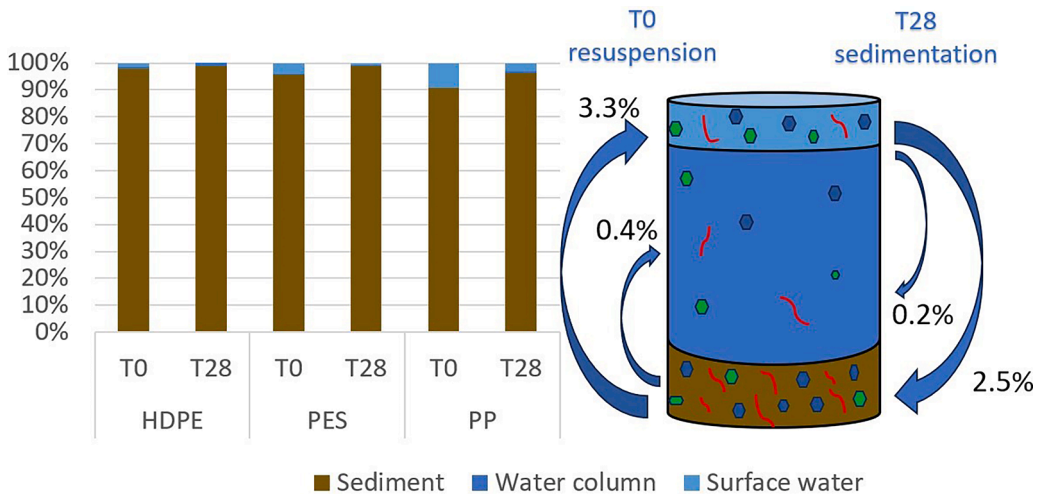

Fig. 1. Average percentage of each polymer type contained in the added MP mixture at the start (T0) and at the end of the experiment (T28) in the three microcosm compartments (i.e., surface water, water column and sediment).

2.6. Quality assurance and quality control

Throughout the experiment and during the processing of samples, cotton lab coats were worn. Three blanks were included for each batch of samples processed for MP analysis. Recovery tests were conducted in triplicates for each sample type (sediment, water, *L. variegatus*, and *L. stagnalis*), with the addition of 20 particles of each type (*i.e.*, PES, HDPE, and PP). The size ranges of the added particles and the results of the recovery tests are shown in Table S2.

2.7. Microplastic effects

We tested the effects of the MP mixture on the survival, production of egg clutches, and reproduction of *L. stagnalis*, and on the reproduction and growth of *L. variegatus*. *L. stagnalis* individuals were considered alive when they responded to a tactile stimulus. Reproduction of *L. stagnalis* was defined as the number of egg clutches found in each microcosm divided by the number of alive adults at the end of the experimental period. *L. variegatus* reproduction was assessed using a reproduction factor. The reproduction factor was calculated as the number of living organisms at the end of the experiment divided by the number of organisms introduced at the start of the experiment to each microcosm (n=20). Growth of *L. variegatus* was assessed as the increase in dry biomass (after desiccation at 50°C for 24 h). The initial weight was determined at T=0 using the average weight of 3 subsamples, each consisting of 20 organisms.

2.8. Data analyses

The number of MPs taken up by the test organisms in the different treatments was compared using the ANOVA test followed by a Tukey's post-hoc test when data was normally distributed (according to the Kolmogorov-Smirnov test) and there was homogeneity of variance (according to the Levennés test). When data did not meet these assumptions, the Kruskal Wallis test followed by the post-hoc Conover test was used.

We assessed whether the test organisms will uptake only a certain MP size range, thus showing a kind of uptake selective behaviour. For this, we compared the size distribution of the MPs that were taken up by the test species with the size distribution of the MPs added to the microcosms, using the non-parametric Wilcoxon Signed Rank test. Furthermore, we determined the maximum MP particle size that was up taken by the test species as the 90th percentile of the MP uptake distribution for each of the three polymers included in the MP mixture.

The No Observed Effect Concentration (NOEC) was calculated for each of the evaluated ecotoxicological endpoints described in *Section 2.7* using the Williamś test. Moreover, the adverse effect thresholds EC10 and EC50 were calculated for reproduction data when the magnitude of the effects was sufficiently large to fit a dose-response curve.

To assess the risks of MPs in freshwater sediments using the effect thresholds obtained in the present study, we compared the rescaled (1 – 5000 μm) NOECs with the rescaled (1 – 5000 μm) Measured Environmental Concentrations (MECs) for freshwater sediments reported by Redondo-Hasselerharm et al. (2023). For this purpose, we first converted the calculated NOEC values into number of particles per kg, using the average number of MPs measured at the start of the experiment in the microcosms with a nominal concentration of 0.1 %, and then extrapolated to the rest of the concentrations. Afterwards, we rescaled de NOECs following the methodology described in Redondo-Hasselerharm *et al.* (2023), which accounts for the polydispersity of environmental MPs and the bioaccessible MP fraction for different species. As in Redondo-Hasselerharm *et al.* (2023), the ecologically relevant dose metrics (ERMs) of particle volume and area were selected for the mechanisms driving effects of food dilution and tissue translocation, respectively.

All analyses were performed using R (version 4.3.1; R Core Team, 2023) in Rstudio (Posit team, 2023) with the packages PMCMRplus, DescTools, Lattice, and Car (Fox & Weisberg, 2019; Pohlert, 2023; Signorell, 2023; Sarkar 2008), except for the EC10 and EC50 values that were estimated using MOSAIC (Charles et al., 2018). Differences were considered statistically significant when the calculated p-value was lower than 0.05.

3. Results and discussion

3.1. MPs fate in the microcosms

When setting up the experiment we observed that although MPs were directly mixed with the sediment, a significant part of them were resuspended during the addition of water into the systems. Two weeks after the stabilization period (T0), 3.3 ± 0.64 % and 0.45 ± 0.26 % of the applied amount was still available in the surface water and in the water column, respectively (Fig. 1). The majority of those were PP fragments, followed by PES fibres and HDPE fragments (Table S3). The higher resuspension of PP might be related to its lower density (0.87 g/cm^3, measured with the material used in the experiment) compared to the other polymers (PES: 1.3-1.4 g/cm^3; HDPE: 0.93 g/cm^3).

After 28 days of experiment, the concentration of MPs in the water

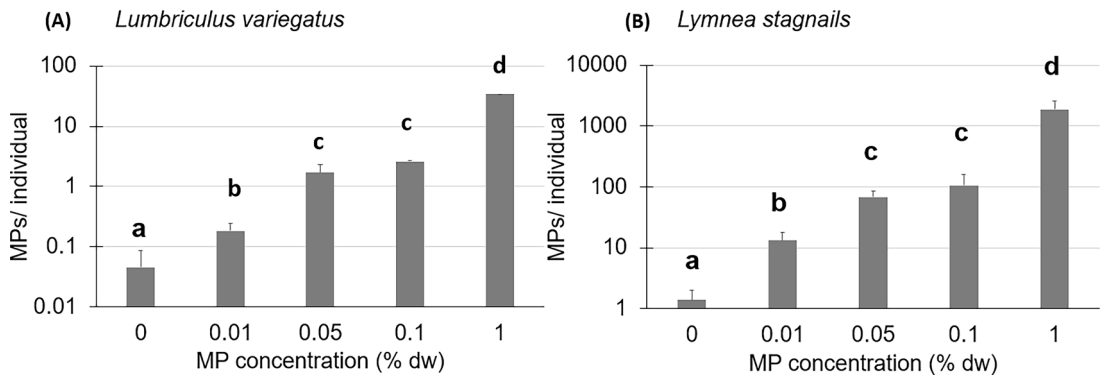

Fig. 2. Bar plot showing the mean ± standard deviation of MPs taken up per individual of *L. variegatus* (A) and *L. stagnalis* (B) at the end of the 28-day exposure period. Concentrations that do not share the same letter are significantly different according to the Tukey test for *L. variegatus* and the Conover test for *L. stagnalis*. Note that the y-axis is shown in a logarithmic scale.

surface was reduced by 2.67 %, and the MP concentration in the sediment increased by 2.44 %, while the concentration in the water column remained almost the same (increasing only 0.23 %). Regarding polymer type, the highest migration from the water surface to the sediment compartment between T0 and T28 was recorded for PP (Table S4). Sinking of polymers with lower density than water has been reported in different studies and attributed to the growth of different microorganisms (e.g. bacteria, diatoms) or the interaction with other particles such as organic matter or clay (He et al., 2021;Kaiser et al., 2017; Martínez-Campos et al., 2023).

Sedimentation rates showed great variability between replicates (Table S5), but on average the daily sedimentation rate of all polymers was 745 MPs/ day. In percentage, this was 1.88 % of MPs suspended in the water column sinking per day since the start of the experiment (T0), which amounted to 53 % during the exposure period (T0-T28). Regarding polymer type, the calculated sedimentation rate for PES was 60 %, for HDPE 41 %, and for PP 52 %, for the whole exposure period (T0-T28). The vertical movement of MPs in freshwater systems has been studied by Range et al., (2023), who pointed at size, shape and biofilm growth as the main factors controlling it. Therefore, the higher sinking rate of PES can be attributed to its greater density compared to water (1.37 g/cm^3), but also their capacity to be better entangled with the sediment particles once it touches this compartment (Kowalski et al., 2016). Comparing the sedimentation rates of HDPE and PP, the lower sedimentation of HDPE might be related to its smaller size as Ahmadi et al. (2024) found that there is an inverse relationship between the size of MPs and the time they remain in the epilimnion.

3.2. MPs uptake by freshwater invertebrates

The test organisms sampled from the control microcosms had, in general, very low MP contamination levels. For example, we quantified

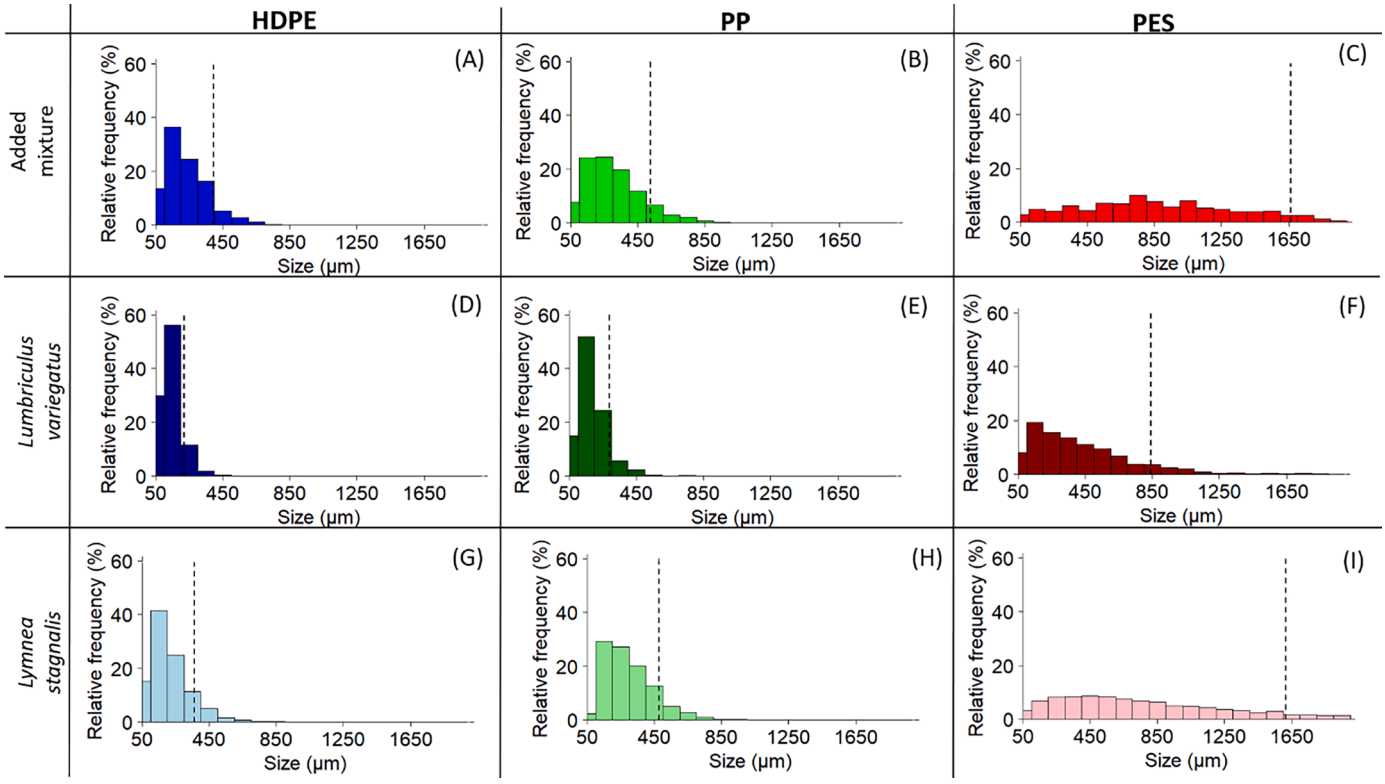

Fig. 3. Histograms of the three different polymers (HDPE, PP and PES) found in the added MP mixture (graphs A-C), in *L. variegatus* (D-F), and in *L. stagnalis* (G-I). Dashed lines mark the maximum uptake size, estimated as the 90th percentile of the MP size distribution.

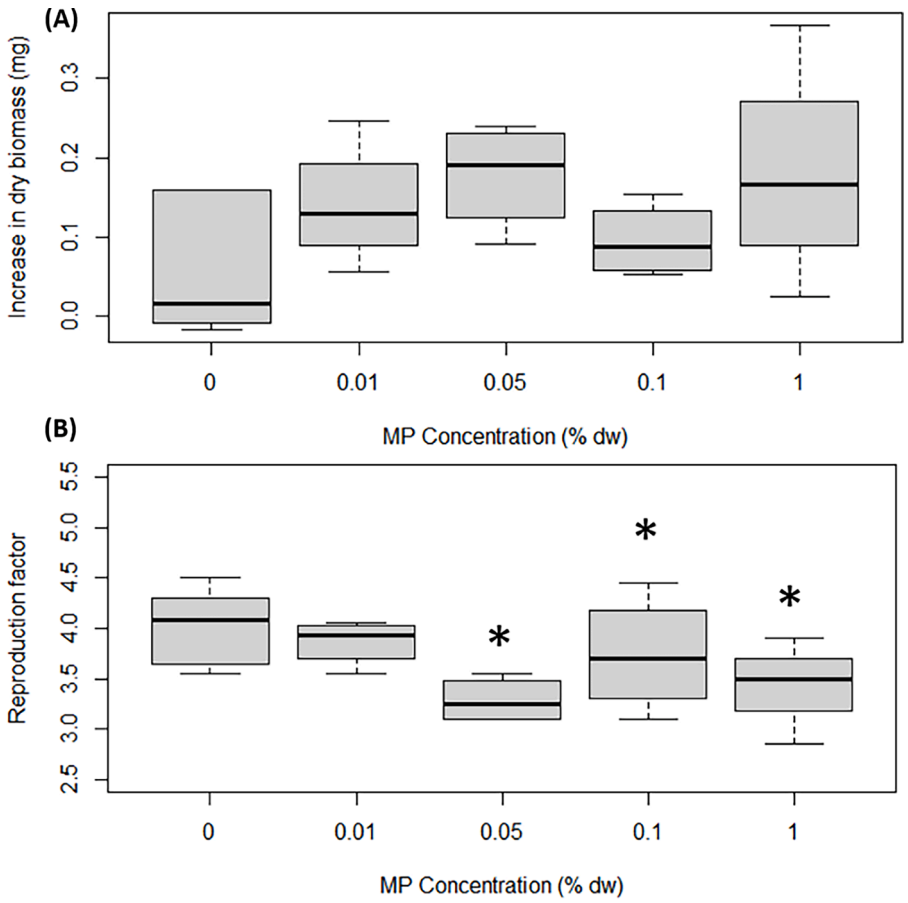

Fig. 4. Boxplots showing the effects of the MP mixture on *L. variegatus*: (A) growth, (B) reproduction factor. The asterisk indicates a significant difference between the test concentration and the control based on the Williamś test.

0.05 ± 0.04 MPs per organism for *Lumbriculus variegatus*, while *Lymnea stagnalis* had 1.4 ± 0.6 MPs per organism (Table S6). It is not clear the origin of such pollution levels, neither whether such MPs were already present in the test organisms before the start of the experiment. In any case, the statistical analysis showed that the number of particles taken up by individuals of both test species exposed to MPs in the experimental treatments was significantly higher than the background contamination levels found in the control (Fig. 2). For instance, at the highest test concentration, the mean uptake by *L. variegatus* was 34 ± 17 MPs per individual, and the mean uptake by *L. stagnalis* was 1889 ± 744 MPs per individual (Table S6).

In our study, the average uptake of *L. variegatus* exposed to 1 and 10 g/kg sediment dry weight (0.1 and 1 %) were 2.6 ± 0.8 and 34 ± 17 MPs per individual, respectively, while Schell et al. (2022) reported values of 15 ± 10 MPs for organisms exposed to 2 g/kg (0.2 %). However, the size of the particles used in the experiment by Schell et al. (2022) was considerably smaller (25-75 μm). The mean uptake by *L. stagnalis* at the concentration of 10 g/kg sediment dry weight (1 %) was 1889 ±744 MPs per individual. In a previous study with *L. stagnalis*, a maximum ingestion of 2235 MPs/individual for organisms exposed to a concentration of 100,000 MPs/mL has been observed (Weber et al., 2021), while we found a maximum ingestion per individual of 2529 MPs at the highest test concentration (1 %).

When comparing the size distribution of the MPs taken up by the test species and the size distribution of the MPs added to the microcosms, it was observed that *L. variegatus* took up a significantly smaller size range than the size range in the exposure mixture (Wilcoxon Signed Rank test p-value <0.001; Table S7), while for *L. stagnalis* the differences were not statistically significant (Table S7; Fig. 3). The maximum MP size taken up by *L. variegatus* (90th percentile of the uptake distribution) was 221 μm for HDPE fragments, 289 μm for PP fragments, and 840 μm for PES fibres. As for *L. stagnalis,* the maximum size was 263 μm for HDPE fragments, 479 μm for PP fragments, and 1616 μm for PES fibres (Table S8).

We compared the maximum uptake size of fragments (HDPE and PP) with the mouth size of the organisms and found that for *L. variegatus* the mouth size (162, 100 - 200 μm; mean, min-max) was slightly lower than the maximum particle size found in its body (232 μm). Silva et al. (2021a) reported a maximum particle size for *L. variegatus* of 250 μm, which is close to the value reported in our study. The difference with the measured mouth size can be related to the difficulty to measure the maximum mouth size of these organisms. As for *L. stagnalis*, we found that the mouth size for organisms of approximately the same size (2-3 cm of shell length) was 1155 μm, 804 -1612 μm (mean, min-max). The large difference between the measured mouth size and the maximum size of the ingested MPs (399 μm) can be related to the presence of the radula inside their mouth, which is used for scraping and makes the maximum ingestible size much smaller than the actual mouth opening (Romaine Carriker, 1946).

Power law distributions for the MPs added to the microcosms and for the MPs taken up by the test species are shown in Figure S2. The alfa value for the MP mixture added to the microcosms was 3.87 ± 1.25 (Table S9), which is similar to that reported by Kooi et al. (2021), for natural sediments (3.25 ± 0.19), indicating that the size distribution of the tested mixture can be considered environmentally relevant. In the same study, Kooi et al. (2021) provided alfa values for MPs taken up by biota of 2.59 ± 0.04, while in our study the alfa value for *L. variegatus* was 2.70 ± 0.05, and for *L. stagnalis* was 2.58 ± 0.79 (Table S9).

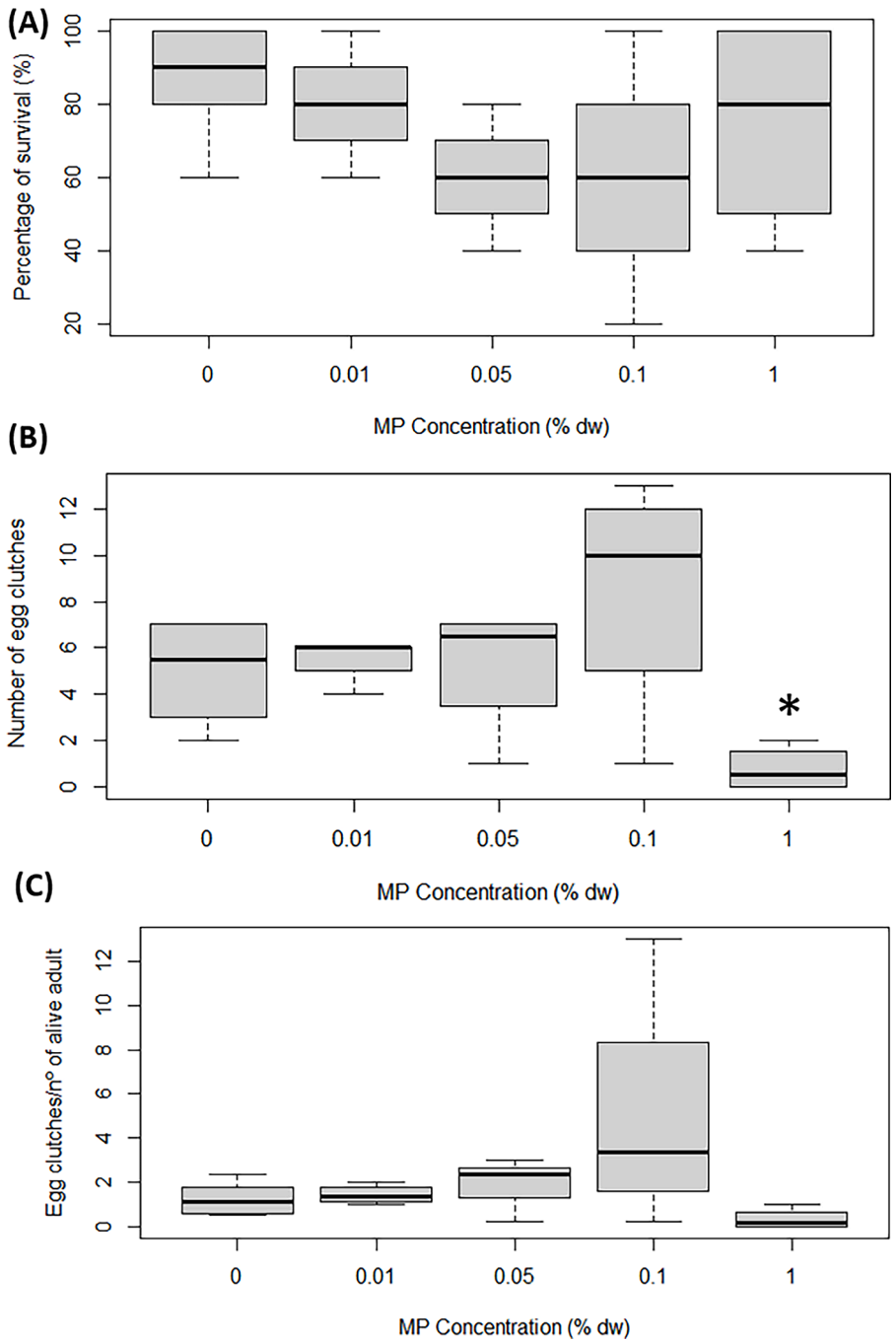

Fig. 5. Boxplots showing the effects of the MP mixture on *L. stagnalis*: (A) survival, (B) total number of egg clutches, (C) number of egg clutches per individual. The asterisk indicates a significant difference between the test concentration and the control based on the Williamś test.

3.3. MP effects

No significant effects of MPs were found on the growth (increase in dry biomass) of *L. variegatus*. However, the tested MP mixture significantly reduced the reproduction capacity of this species at the three highest test concentrations (NOEC = 0.01 %) with an approximate reduction of 20 %, although there was not a very clear dose-response relationship (Fig. 4). This prevented the fitting of the dose-response model and the calculation of the EC10 and EC50 for this species. In a previous study, no significant effects were found on biomass and reproduction of *L. variegatus* after a 28-day exposure to irregular PE fragments (32 to 500 μm) at concentrations up to 20 g/kg (Silva et al., 2021b). Carrasco-Navarro et al. (2022) exposed *L. variegatus* to tire debris of two size classes (82.3 ± 40 μm and 3,724 ± 775 μm) at concentrations up to 10 % dry weight and did not find significant long-term effects in growth, survival, or reproduction. These findings suggest that mixtures of different polymers and shapes can have greater effects on this species than exposure to single size/shape particles. Similar conclusions have been found by other authors (de Ruijter et al. 2023; Redondo-Hasselerharm et al., 2023), however further investigations are needed to explain the mechanisms that produce such change in toxic potential and the often-observed lack of clear dose-response relationships. As for the latter, recent studies suggest that factors such as the heteroaggregation with other particles can affect bioavailability as well as the toxic mechanisms responsible for the observed effects (e.g., blockage of the intestinal tract and food dilution, oxidative stress,

Table 2
Calculated EC10, EC50, and NOEC values in mass percentage, g/kg of sediment dry weight, and number of MPs per kg of sediment dry weight, and re-scaled NOECs for the size range 1-5000 μm following Redondo-Hasselerharm et al. (2023). CI: 95 % confidence intervals. [a] A dose-response model could not be properly fitted due to the low magnitude of effects in the highest test concentration.

Species	Endpoint	EC10 (%) (CI)	EC50 (%) (CI)	NOEC (%)	NOEC (g/kg dw)	NOEC (MPs/ kg dw)	Re-scaled NOEC (volume as ERM) (MPs/ kg dw)	Re-scaled NOEC (area as ERM) (MPs/ kg dw)
Lumbriculus variegatus	Dry weight	-	-	>1	>10	$>1.94 \times 10^7$	$>6.0 \times 10^8$	$>2.3 \times 10^9$
	Reprod.	- [a]	- [a]	0.01	0.1	2.00×10^5	4.8×10^6	2.4×10^7
Lymnea stagnalis	Survival	-	-	>1	>10	$>1.99 \times 10^7$	$>5.2 \times 10^8$	$>2.4 \times 10^9$
	N° of egg clutches	0.25 (0.07 - 0.86)	0.52 (0.26 - 0.94)	0.1	1	2.07×10^6	3.1×10^7	2.5×10^8
	Reprod.	-	-	>1	>10	$>2.09 \times 10^7$	$>9.9 \times 10^7$	$>2.5 \times 10^9$

metabolism; Jeong and Choi, 2019; Parrella et al. 2024).

For *L. stagnalis,* no significant effects were observed on survival. However, the total number of egg clutches was reduced by 80 % at the highest test concentration (NOEC = 0.1 %), and the number of egg clutches per adult showed a decline at the highest test concentration, although it was not statistically significant (Fig. 5). The 28-d EC10 and EC50 for the number of egg clutches of *L. stagnalis* were 0.25 % and 0.52 %, respectively. Horton et al., (2020) did not find significant effects of nylon particles (mean size: 13-19 μm) on *L. stagnalis* survival at a maximum sediment concentration of 1 % (10 g/kg) for 4 days. Similarly, Weber et al., (2021) did not find effects on energy reserves, oxidative stress, mortality, or reproduction after chronic exposure of irregular PS fragments below 63 μm at a high concentration (100,000 particles/mL). We hypothesize that the small particles used in these previous experiments could have been easily egested. Experiments performed with environmentally relevant mixtures of MPs similar to the ones used here (i.e., formed by fragments and fibres of PE, PP and PET) and other freshwater snails (*Potamopyrgus antipodarum*) have not found significant effects at concentrations up to 10 % sediment dw for 28 d (de Ruijter et al. 2023). Using the same species (*Potamopyrgus antipodarum*) and a mixture of different non-buoyant polymers (polyamide, polyethylene te rephthalate, polycarbonate, polystyrene, polyvinylchloride) added with their food, Imhof and Laforsch, (2016) neither observed any significant effect after adding up to 70 % of MPs in the food. *L. stagnalis* is larger than *Potamopyrgus antipodarum* and is an efficient scrapper, which could have contributed to the large accumulation of MPs in its body. Blockage of the digestive tract with plastic debris has been suggested to be one of the main mechanisms by which aquatic organisms can be chronically affected (Alexiadou et al., 2019). Thus, effects on nutrient assimilation and potential consequences for energy allocation to reproduction might explain the significant effects found at the highest test concentration on the production of egg clutches, although further studies are recommended to confirm this hypothesis.

The lowest NOEC values derived in this study were 2.0×10^5 MPs/kg dw and 2.1×10^6 MPs/kg dw for *L. variegatus* and *L. stagnalis,* respectively (Table 2). After rescaling them following the methodology described in Redondo-Hasselerharm *et al.* (2023), these values correspond to 4.8×10^6 (volume) and 2.4×10^7 (area) MPs/kg dw for *L. variegatus*, and 3.1×10^7 (volume) and 2.5×10^8 (area) MPs/kg dw for *L. stagnalis* (Table 2). The recent study by Redondo-Hasselerharm *et al.,* (2023) provides an exposure assessment of MPs in sediment samples collected from 103 freshwater bodies all over the world. After comparing the re-scaled MECs reported by Redondo-Hasselerharm *et al.,* (2023) with the lowest rescaled NOEC obtained for *L. variegatus* in this study, we found that 51 % of the mean MECs exceeded the NOEC for volume as ERM, while 24 % exceeded the NOEC for area as ERM, which correspond to food dilution and translocation-mediated effect mechanisms, respectively. In the case of *L. stagnalis*, 22 % of the mean MECs exceeded the NOEC for volume as ERM, while only the 1 % exceeded the NOEC for area as ERM. Therefore, we can expect MP mixtures to cause detrimental effects on the reproduction of these two species, principally by food dilution mechanisms, in freshwater ecosystems subjected to high MP contamination levels.

4. Conclusions

Our study shows that most MPs applied to lentic freshwater ecosystems tend to sink to the sediment compartment after a relatively short time, and that the sinking rate of MPs from the water phase to the sediment is influenced by the polymer type. Furthermore, we have demonstrated that the size distribution of the MPs taken up by freshwater species depend on their feeding habits and physiological characteristics, and have provided maximum ingestible sizes for different particle types for each test species. Our study also indicates that environmentally relevant mixtures of MPs could result in long-term effects on the reproductive output of aquatic invertebrates at concentration levels that have been found in freshwater ecosystems. Further work is recommended to disentangle the mechanisms that influence the bioavailability and toxicity of environmentally relevant mixtures of MPs to aquatic organisms using single-species laboratory tests and microcosms of higher ecological complexity.

CRediT authorship contribution statement

Sara Martínez-Pérez: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Theresa Schell:** Writing – review & editing, Methodology, Investigation. **Daniel Franco:** Writing – review & editing, Methodology, Investigation. **Roberto Rosal:** Writing – review & editing, Methodology, Investigation. **Paula E. Redondo-Hasselerharm:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Virtudes Martínez-Hernández:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Andreu Rico:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, 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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107104.

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