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Presence, variation, and potential ecological impact of microplastics in the largest shallow lake of Central Europe

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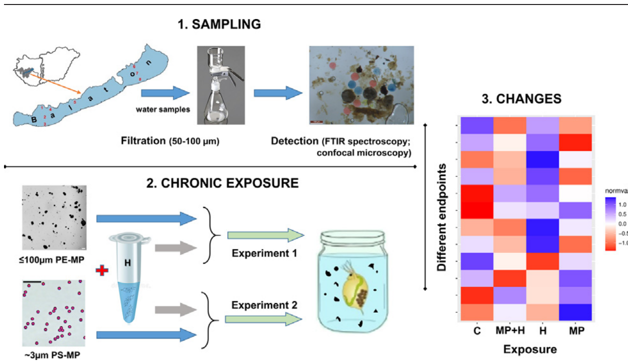
HIGHLIGHTS

- The presence of 7 polymer types of microplastics in the size range of 50–100 μm was detected in Lake Balaton.
- Microplastics and progestogens can affect *Daphnia magna* at the behavioral and biochemical levels.
- The presence of microplastics may lead to reduced fitness in the aquatic biota in freshwaters.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of microplastics (MPs) in the global ecosystem has generated a rapidly growing concern worldwide. Although their presence in the marine environment has been well-studied, much less data are available on their abundance in freshwaters. MPs alone and in combination with different chemicals has been shown to cause acute and chronic effects on algae and aquatic invertebrate and vertebrate species at different biological levels. However, the combined ecotoxicological effects of MPs with different chemicals on aquatic organisms are still understudied in many species and the reported data are often controversial. In the present study, we investigated, for the first time, the presence of MPs in Lake Balaton, which is the largest shallow lake of Central Europe and an important summer holiday destination. Moreover, we exposed neonates of the well-established ecotoxicological model organism *Daphnia magna* to different MPs (polystyrene [3 μm] or polyethylene [≤ 100 μm]) alone and in combination with three progestogen compounds (progesterone, drospirenone, levonorgestrel) at an environmentally relevant concentration (10 ng L^{-1}) for 21 days. The presence of 7 polymer types of MPs in the size range of 50–100 μm was detected in Lake Balaton. Similarly to the global trends, polypropylene and polyethylene MPs were the most common types of polymer. The calculated polymer-independent average particle number was 5.5 particles m^{-3} (size range: 50 μm – 100 μm) which represents the values detected in other European lakes. Our ecotoxicological experiments confirmed that MPs and progestogens can affect *D. magna* at the behavioral (body size and reproduction) and biochemical

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(detoxification-related enzyme activity) levels. The joint effects were negligible. The presence of MPs may lead to reduced fitness in the aquatic biota in freshwaters such as Lake Balaton, however, the potential threat of MPs as vectors for progestogens may be limited.

1. Introduction

Nowadays, the widespread presence of microplastics (MPs) in the marine and freshwater ecosystems has been a hot topic in environmental sciences and generated a rapidly growing concern worldwide (Li et al., 2018; Sana et al., 2020). The main sources of MPs to aquatic systems are the industrial, agricultural, domestic, and coastal activities with the wastewater treatment plants (WWTPs) being the most dominant sources (Auta et al., 2017; Li et al., 2018; Murphy et al., 2016; Sana et al., 2020; Wu et al., 2022). Although the presence of MPs has been investigated for almost 50 years in the marine environment, research has only started to focus on freshwaters in recent years (Scherer et al., 2020). The abundance of MPs show high variability in different surface waters ranging from <1 piece 100 m^{-3} to >1 million pieces m^{-3} (Auta et al., 2017; Desforges et al., 2014; Li et al., 2018). For example, a comprehensive survey investigated the surface waters of 67 lakes across Europe (from Croatia to Norway; between April and September 2019) and revealed that the microparticle concentrations varied between 0 and 7.3 particles m^{-3} (Tanentzap et al., 2021). Moreover, MP concentrations in European river waters were reported to range from 0.03 (Mani et al., 2019) to 187,000 particles m^{-3} (Leslie et al., 2017). However, still much less data are available about the abundance of MPs in freshwaters than in the marine environment.

Based on the existing data, MPs alone can affect algae (Cao et al., 2022; Guschina et al., 2020; Liu et al., 2020a; Wu et al., 2019; Zhang et al., 2017; Zheng et al., 2021). Moreover, it has been shown that MPs alone can induce toxic effects on aquatic invertebrate species including cnidarians (Beraud et al., 2022; Chen et al., 2022b; Lim et al., 2022; Marangoni et al., 2022), mollusks (Detree and Gallardo-Escarate, 2018; Green et al., 2019; Magni et al., 2019; Pedersen et al., 2020; Uguen et al., 2022; Weber et al., 2021; Zheng et al., 2022), cladocerans (Chenxi et al., 2022; Eltemsah and Bohn, 2019; Kokalj et al., 2018; Mondellini et al., 2022; Wang et al., 2022b; Wang et al., 2022c; Zebrowski et al., 2022), and echinoderms (Martinez-Gomez et al., 2017; Murano et al., 2020; Ng et al., 2022; Nobre et al., 2015; Rendell-Bhatti et al., 2021; Richardson et al., 2021; Trifuoggi et al., 2019). Also, fish species have been shown to be sensitive to the single effects of MPs (Banaei et al., 2022; Chen et al., 2017; Chen et al., 2020; Cormier et al., 2022; Felix et al., 2023; Jabeen et al., 2018; Kim et al., 2022; LeMoine et al., 2018; Rabezanahary et al., 2023; Yu et al., 2022a; Zhang et al., 2022). Besides, given their high surface hydrophobicity, MPs have a very high sorption capacity for hydrophobic pollutants (e.g., different pharmacologically active compounds [PhACs] or polycyclic aromatic hydrocarbons); hence they can increase the toxic effects of chemicals and help their bioaccumulation and biomagnification (Chen et al., 2022a; Gonzalez-Soto et al., 2019; Lee et al., 2014; Li et al., 2022; Na et al., 2021; Rainieri et al., 2018; Stollberg et al., 2021; Wang et al., 2022a; Wu et al., 2016; Yu et al., 2022b; Zhou et al., 2020). However, the combined ecotoxicological effects of MPs with different chemicals on aquatic organisms are still understudied in many species and there is a debate about the extent of the potential role of MPs as vectors for different chemicals.

Lake Balaton (Hungary) is the largest shallow lake of Central Europe and an important summer holiday destination visited by millions of tourist every year (Maasz et al., 2019; Molnar et al., 2021). Hence, the preservation of its good water quality and biodiversity is of strategic importance from an ecological and economic point of view. Previous environmental analytical studies revealed the presence of many PhACs in Lake Balaton and its catchment area (Avar et al., 2016a; Avar et al., 2016b; Maasz et al., 2019; Maasz et al., 2021; Molnar et al., 2021). Moreover, of the detected PhACs, previous studies investigated the impact of progestogens (progestogen and its synthetic analogues) on invertebrate (water flea [*Daphnia magna*] and the great pond snail [*Lymnaea stagnalis*]) and vertebrate (roach [*Rutilus rutilus*]) aquatic species representing the ecosystem of Lake Balaton and demonstrated several effects from the behavioral to the molecular level (Maasz et al., 2017; Svigruha et al., 2021b; Svigruha et al., 2021a; Zrinyi et al., 2017). However, similar surveys regarding MPs have not yet been carried out.

Keeping this in mind, the present study was conducted to investigate the presence and potential ecological impact of MPs in Lake Balaton. To accomplish our aim, we first investigated the presence and variance of MPs on an average summer week. Also, we studied the potential effects of polystyrene and polyethylene MPs alone and, given their sorption capacity for hydrophobic pollutants, in combination with three progestogen compounds (progesterone [PRG], drospirenone [DRO], and levonorgestrel [LNG]) on *D. magna*. This species has been a widely used ecotoxicological model organism for decades (reviewed by (Stollewerk, 2010; Tkaczyk et al., 2021)) and is highly suitable for such investigations. Moreover, *D. magna* has been shown to be sensitive to MP pollutions (reviewed by (Samadi et al., 2022; Yin et al., 2023)) and to steroid hormones (Luna et al., 2015; Martins et al., 2007; Svigruha et al., 2021b; Torres et al., 2015; Zheng et al., 2020). At the behavioral level, survival, growth, and reproduction were examined. At the biochemical level, the activity of three enzymes, glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD), involved in detoxification pathways was investigated.

2. Materials and methods

2.1. Study area and sample collection

Our study was carried out on Lake Balaton, Hungary (Supplementary Fig. 1), which is the largest (A: 594 km^2, mean depth: 3.2 m, *V*: ~1.8 km^3) freshwater shallow lake in Central Europe (Istvanovics et al., 2007). The lake and its catchment area can be characterized by diverse flora and fauna (Istvanovics et al., 2007; Palffy et al., 2013; Sipkay et al., 2007; Specziar et al., 2009). Nowadays, >40 WWTPs can be found in its catchment area (Maasz et al., 2019). Lake Balaton is an internationally important tourist attraction and recreation center visited by about 2,000,000 tourists a year (Maasz et al., 2019; Molnar et al., 2021). The number of guest-nights is unevenly distributed and weighted to two summer months (July and August) mostly to the southern shoreline of the eastern basin of the lake (Horváth, 2011; Maasz et al., 2019).

To reveal the presence and variance of MPs in Lake Balaton, 8 sampling points were assigned (Supplementary Fig. 1). The sampling was conducted in 2022 from the 27th July to the 29th July with a previously described filtration system (Bordos et al., 2021). Briefly, for each sample, 2000 L of water were pumped through an in situ fractionated filtration device. The device contained a 1 mm mesh size pre-filter in order to avoid larger particle entering in the sample and stainless-steel filters with the smallest mesh size of 50 μm. Hence, the collected particles are considered between 50 μm and 1 mm (Supplementary Fig. 2). The reported MP particles fall between 50 and 100 μm (Fig. 1; Supplementary Table 1), because this is the relevant size range from the point of view of the study.

2.2. Sample preparation of MPs

Solid particles were washed down from the filter, and the samples water content were reduced using a stainless-steel 50 μm mesh size sieve. A pre-oxidation step was conducted on the samples by adding 100 mL of 30 % hydrogen peroxide (H_2O_2) and incubating them overnight. The samples were then filtered again with the mesh size of 50 μm. To remove the biogenic organic matter, an advanced oxidation process, Fenton reaction, was

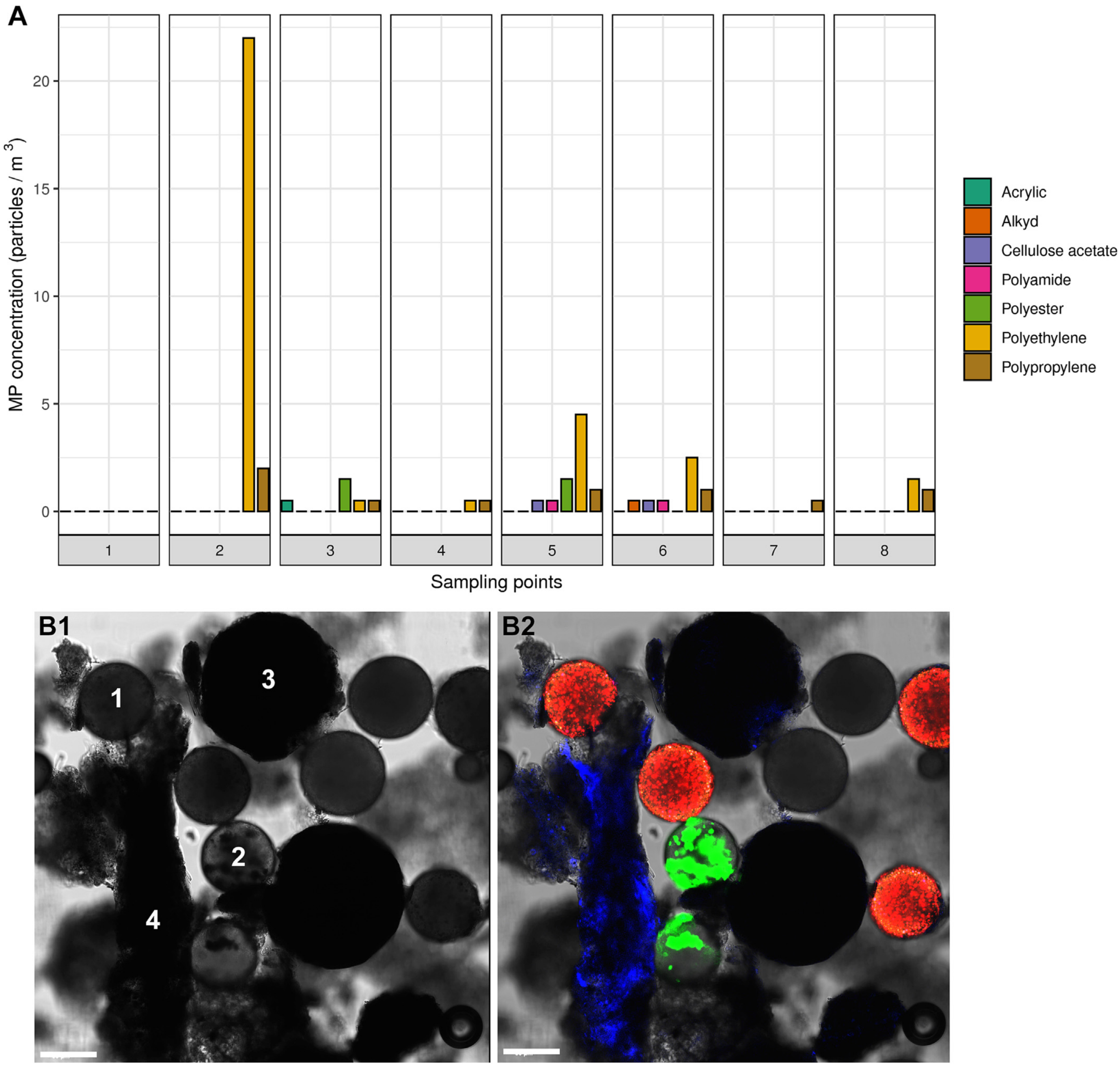

Fig. 1. Presence and variation of MPs (50–100 μm) in Lake Balaton. (A) Particle number of different polymer types of MPs at the individual sampling points (1–8). The numbers are the result of a single indication (without repetitions) to prove that MPs in the 50–100 μm size range are present in the lake at all. The presence of MPs was confirmed by the LOD and LOQ values. (B1-B2) Confocal microscopic images of MPs collected from a 50 μm filter paper. The micrographs show the same section without (B1) and with (B2) fluorescence detection. 1–4 numbers represent the different type of MPs (based on fluorescent emission). Scale bar = 50 μm.

performed in which ferrous ion initiates and catalyze the decomposition of H_2O_2 leading to the in-situ generation of hydroxyl and hydroperoxyl radical. First, 200 mL of MP free deionized water (deionized water was filtered through a 5 μm metal filter and a 0.7 μm glass fiber filter), then 200 mL 30 % H_2O_2 and 62 mL iron sulphate catalyst were added to the samples. The samples were incubated in the reaction overnight, then filtered again, and placed in a small volume glass separator (SVGS) which was previously described by (Mari et al., 2021). The SVGS was filled with zinc-chloride brine solution (1000 g of zinc-chloride salt with a density of 1.7 g cm^{-3} dissolved in 800 mL of MP free water) and a glass-coated magnetic stirring rod was added to the SVGS. The separation device was put onto a magnetic stirrer (#F20500162. ARE Hot Plate, Velp Scientific Inc., Italy) and stirred for 10 min at 700 rpm. After stirring, the density separation was performed for 120 min. The device was then placed in an ultrasonic bath (Realsonic Cleaner, KLN Ultraschall GmbH., Germany), where it was sonicated (on nominal 37 kHz) for 5 min. To note, the sonication and the stirring were done to ensure that the particles did not stick to the neck or the bottom of the equipment. After the sonication, the solution was left to settle for another 2 h. Finally, the particles were washed down from the upper part of the SVGS with MP free water and were filtered on a Whatman Anodisc (Ø = 25 mm; pore = 0.2 μm, GE Healthcare, UK).

2.3. Identification and quantification of MPs

The anodisc filters were placed on a calcium fluoride crystal (EdmundOptics, Germany) to avoid filter from bending. Chemical mapping of these samples was done in transmission mode for pre-defined large filter areas (25 mm in diameter). The window was scanned at a spatial resolution of 25 μm (micro Fourier-Transform Infrared [μFTIR] imaging) with a Nicolet™ iN10 MX Imaging Infrared Microscope (Thermo-Fisher) using a Mercurye Cadmium-Telluride, focal plane array detector. The following measurement parameters were specified: the spectral resolution 8 cm^{-1} and 4 scan per pixel was conducted covering the wavenumber range 4000–1250 cm^{-1}. The generated FTIR data were analyzed with siMPle freeware, which can be downloaded via https://simple-plastics.eu/. In the software, the spectral data were compared with reference spectra library and particles with >70 % correlation was considered as MPs between 50 and 100 μm.

2.4. Contamination prevention and quality control

Previous reviews and critical evaluations highlighted the importance of (a) contamination prevention and (b) quality control in MP research (Miller et al., 2021; Pérez-Guevara et al., 2021). Considering these requirements, the following approaches were made in the present study:

(a) During the sample preparation, many efforts have been made to prevent contaminations: 1) the sample holding beakers were covered with aluminum foils, 2) cotton lab coats were worn, 3) the filtration steps were conducted under a laminar flow cabinet, and 4) the oxidation steps were took place under a fume hood. Although the fume hood does not protect fully the samples from contamination, it had to be used for personal protection.
(b) Laboratory blank samples were also investigated alongside samples in order to quantify the contamination level (background measurement). Three parallel sample were taken to measure the contamination level of the procedure. In order to reliably quantify MPs in samples, it is recommended to calculate the limit of detection (LOD) and limit of quantification (LOQ) values. Following a previous paper (Liu et al., 2023), the LOD was defined as 3.3 x the standard deviation of the blank plus their mean, while the LOQ was defined as 10 x the standard deviation of the blank plus their mean.

2.5. MPs and chemicals for chronic exposures

Two types of MP solution were used for the treatments. *Experiment 1*: Given that most of the relevant ecotoxicological studies apply polystyrene MP particles with their "original" structure (i.e. regular sphere), we first used a commercially available polystyrene suspension (5 % *w/v*; particle size of 3 μm; #42922; Merck) without any further home-made modification (referred to as PS-MPs). A representative image of the PS-MP solution is shown in Fig. 2B1. During the treatments, the animals were exposed to a PS-MP concentration of 1.25 mg L^{-1}. *Experiment 2*: we also used a commercially available polyethylene powder (particle size of 125 μm; #434264; Merck) with home-made fragmentation (referred to as PE-MPs). The fragmentation was carried out by measuring 700 μg particles into 20 μL DMSO, completing the solution to 1 mL artificial water, then shaking it in a TissueLyser LT (QIAGEN) device (4 min; 45 Hz; magnetic beads). A representative image of the PE-MP solution is presented in Fig. 2C1. Particle distribution of the home-made solution is shown in Supplementary Fig. 3. During the treatments, the animals were exposed to a PE-MP concentration of 2.8 mg L^{-1}.

In both Experiment 1 and 2, PRG (CAS No.: 57-83-0, Merck), DRO (CAS No.: 67392-87-4, Merck), and LNG (CAS No.: 797-63-7, Merck) were used for the treatments as progestogen agents that had previously been detected in Lake Balaton and its catchment area (Avar et al., 2016b; Maasz et al., 2019). From these, 1 mg mL^{-1} stock solutions were prepared in ethanol (ACS reagent, ≥ 99.5 %; CAS No.: 64-17-5; VWR, Hungary). From these stock solutions, 100 ng mL^{-1} working solutions were prepared in ethanol and then added in the appropriate volume to the experimental glass beakers to reach the desired nominal equi-concentration of 10 ng L^{-1} (mixture of progestogens) (≤ 0.01 % final solvent concentration). The 10 ng L^{-1} concentration value represents the detectable concentration level of the three compounds in Lake Balaton and its catchment area (Avar et al., 2016b; Maasz et al., 2019) as well as reflects to the average global concentration values (Svigruha et al., 2021a).

2.6. Daphnia magna culture and chronic treatments

D. magna specimens have been maintained at a constant temperature of 23 ± 1 °C on a light:dark regime of 16:8 h with light of natural wavelength (400–700 nm) and an intensity of 500–700 lx at the Balaton Limnological Institute (Tihany, Hungary) for over 6 years. They were cultured in 500 mL glass beakers containing 450 mL artificial water (OECD, 2012) and were fed daily on *Scenedesmus obliquus* and/or *Raphidocelis subcapitata* (0.5 × 10^{-6} cells mL^{-1}). The medium was renewed two times a week.

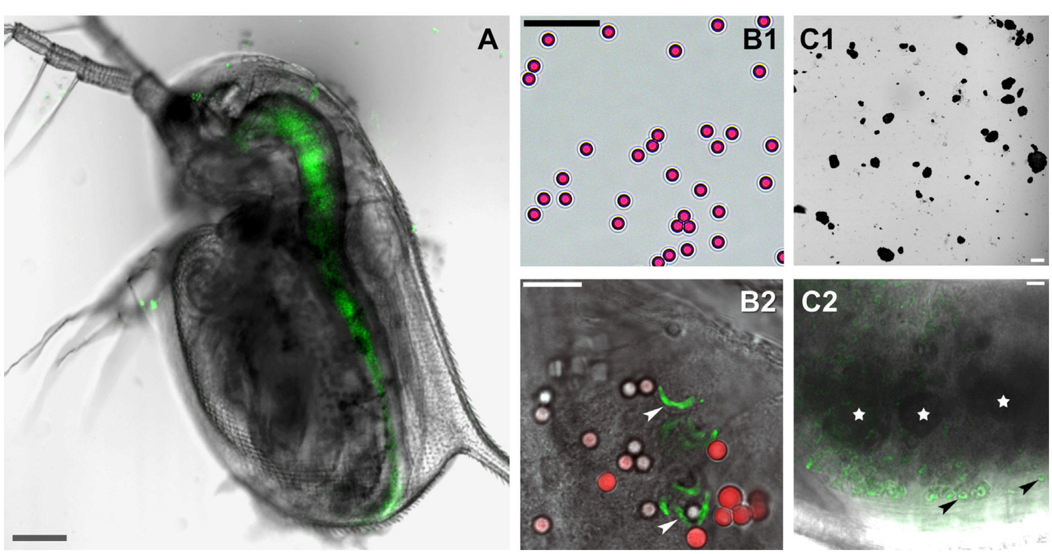

Fig. 2. Representative micrographs on the applied MP particles and the biodistribution of MPs in *D. magna* specimens during the 21-day exposures. (A) Laser confocal microscopic image of a control animal fed on algae (*R. subcapitata*; marked by green color; excitation: 638 nm; emission: 650–680) and cultured in MP-free artificial water. (B1-B2) A representative white light image about the red-colored 3 μm PS-MP particles (B1) and a confocal microscopic image about their presence in a *D. magna* specimen (B2). Arrowheads represent the algae, while red color indicates the PS-MPs in the intestine tract. (C1-C2) Representative confocal microscopic images about the ≤100 μm PE-MP particles (C1) and their presence in a *D. magna* specimen (C2). Arrowheads represent the algae, while stars indicate the PE-MPs in the intestine tract. Scale bars = 10 μm (B1, B2, C2) 100 μm (A, C1).

From our healthy parent stock, neonates (<24 h) were selected for investigating the adaptive behavioral and enzymatic responses. The animals were divided into 4 experimental groups for both Experiment 1 and 2: 1) control, 2) MP-treated, 3) progestogen-treated, and 4) MP + progestogen-treated ($n = 10$ neonates/each group). Hence, the 4 experimental groups contained $n = 40$ total animals per replicates for both Experiment 1 and 2. The neonates were individually placed into 100 mL media in 150 mL glass beakers and hence individually exposed to the MPs and/or progestogens for 21 days (chronic exposure). Animals in the control group were kept in 100 mL media. Before the experiments, neonates were always acclimatized for 2 h in glass beakers in artificial water. During the 21-day exposures, the specimens were fed three times a week. During the treatments, the light intensity was also set to 500–700 lx. After the exposures, the animals were pooled in each group and prepared for biochemical measurements (see below). Three replicates were set up for each experimental group.

As we utilized a static exposure system, based on our preliminary recovery data for the used progestogens (Supplementary information), the water was totally refreshed every second day and MPs and/or progestogens were re-added to continuously maintain the nominal concentrations.

2.7. Mortality, growth, and reproduction

Observations of *D. magna* survival, growth, and reproduction were based on a previously published method (Svigruha et al., 2021b). The investigations were obtained using LEICA M205C stereomicroscope (BioMarker Ltd., Hungary) and evaluated with LAS software (version: 4.12). Body size was calculated using the length and width parameter of the specimens.

2.8. Enzyme activity measurements

The measurement of the total protein content and different enzymatic activity of the specimens was performed based on our previous works (Gyori et al., 2017; Svigruha et al., 2021b; Vehovszky et al., 2010). After the 21-day exposures, the pooled samples from each group were and homogenized in 500 μL 0.5 M phosphate buffer saline (pH = 7.4) using a TissueLyser LT (QIAGEN) device (4 min, 45 Hz). After centrifugation (10,000 *g* for 15 min at 4 °C), the supernatants were aliquoted and used for the biochemical measurements.

Enzymatic activity measurements for GST (GST Assay Kit, #CS0410, Merck), CAT (Catalase Assay Kit, # A22180, Invitrogen), and SOD (SOD Assay Kit, #19160, Merck) were also performed with commercially available kits following the manufacturers' instructions. All enzyme activities were normalized to the total protein content of the samples which was measured using the commercially available Bradford Assay Kit (#B6916, Merck) following the manufacturers' instructions. All measurements were carried out using a Victor 3 plate-reader (Perkin Elmer). Detailed methodology and calculations are presented in the Supplementary information.

2.9. Statistical analysis

Statistical analysis was performed using OriginPro 2018 software (OriginLab Corp., Northampton, Massachusetts, USA) and R v4.2.0 (R Core Team, 2022) programming environment. The normality of the datasets was investigated using the Shapiro-Wilk test, homogeneity of variances between groups was investigated using Levene's statistic.

In the case of body size, the nonparametric Scheirer-Ray-Hare test was performed to study the effect of time, treatment, and time x treatment interaction. This analysis was followed by Kruskal-Wallis test with Dunn's post hoc test to identify significant differences between control and treatment groups within a given time point (observation day). The time of first egg production data were analyzed using Kruskal-Wallis test with Dunn's post hoc test. The egg number in the first production data and the maximum egg number per individual data were analyzed using one-way ANOVA with Scheffe's post hoc test. Except GST activity data in Experiment 1, the enzyme activity data were analyzed using one-way ANOVA with Scheffe's post hoc test.

3. Results

3.1. MP content in the blank samples, LOD and LOQ values

MPs could be detected in two out of the three blank samples. On average, 1.33 polyethylene particles, 1.00 polypropylene particle, and 0.33 polystyrene particles could be identified in these samples. However, in the size range of 50–100 μm, only 0.66 (0-0-2 particle/blank samples) polyethylene and 0.33 (0-0-1 particle/blank samples) polypropylene were detected. There is still no consensus in the field of MP analysis to whether it is necessary to correct our data with blank results. Previous articles can be found in which values have been corrected (Liu et al., 2023) but one can also find articles where there was no correction (Miller et al., 2021). To note, we did not correct our result with the mean value of the blanks, since the level of the background contamination was insignificant. Given that the size range of 50–100 μm is the relevant one in the present study, the LOD and LOQ values were determined in this range. Although these values were different between the polymer types, we calculated them for the total number of MPs as recommended by (Liu et al., 2023). According to our calculations, the LOD and LOQ values were 3.7 and 9.2 MPs per sample, respectively.

3.2. Types and concentrations of MPs in Lake Balaton

The presence of 7 polymer types of MPs in the range of 50–100 μm was detected in Lake Balaton (Fig. 1; Supplementary Table 1). Except Sampling point 1 (Szigliget), MPs were present in all sampling points. The type of MPs and the number of detected particles showed considerable differences between the sampling points. Polypropylene (7 sampling points) and polyethylene (6 sampling points) had the highest frequency of occurrence, while the less frequented types were acrylic and alkyd (1–1 sampling point). The highest number (5 types) of MPs was detected in Sampling point 5 (Alsóörs) and Sampling point 6 (between Alsóörs and Siófok – middle of the lake). In most sampling points, the particle number was ≤10. The highest particle number (48 pieces) was found in Sampling point 2 (between Szigliget and Balatonfenyves – middle of the lake), with most of the particles being made of polyethylene.

3.3. Single and combined effects of MPs and progestogens on mortality, growth, and reproduction of D. magna

During the 21-day treatments, in contrast to the animals of the control groups (Fig. 2A), a high number of MP particles could be detected in the digestive tract of the specimens exposed to 1.25 mg L^{-1} of PS-MPs (Fig. 2B2) or 2.8 mg L^{-1} of PE-MPs (Fig. 2C2). No significant lethality was observed for *D. magna* in any experimental groups during the exposures (Supplementary Table 2). As a supplementary investigation, we tracked the circulation of MP particles through more generations of *D. magna* (Supplementary Fig. 4). The labeled particles entered the feeding cycle, did not decompose, and could appeared even in the third generation as they passed through the "closed" experimental system.

The average body size of the animals during the treatments is presented in Fig. 3. In the case of Experiment 1 (Fig. 3A), two-way repeated-measures Scheirer-Ray-Hare revealed significant effects of time (observation days) [$H = 239.276$, $P \leq 0.001$] and treatment [$H = 25.75$, $P \leq 0.001$], but not significant time x treatment interaction [$H = 8.348$, $P > 0.05$]. Further analysis with Kruskal-Wallis and Dunn's post hoc test indicated that, compared to the control, the body size in the PS-MP-treated group significantly decreased from the 5th observation day to the end of the study. Furthermore, the body size was significantly less in the PS-MP + progestogen-treated group on the last observation day. In the case of Experiment 2 (Fig. 3B), two-way repeated-measures ANOVA revealed significant effects of time (observation days) [$H = 262.577$, $P \leq 0.001$] but not of treatment

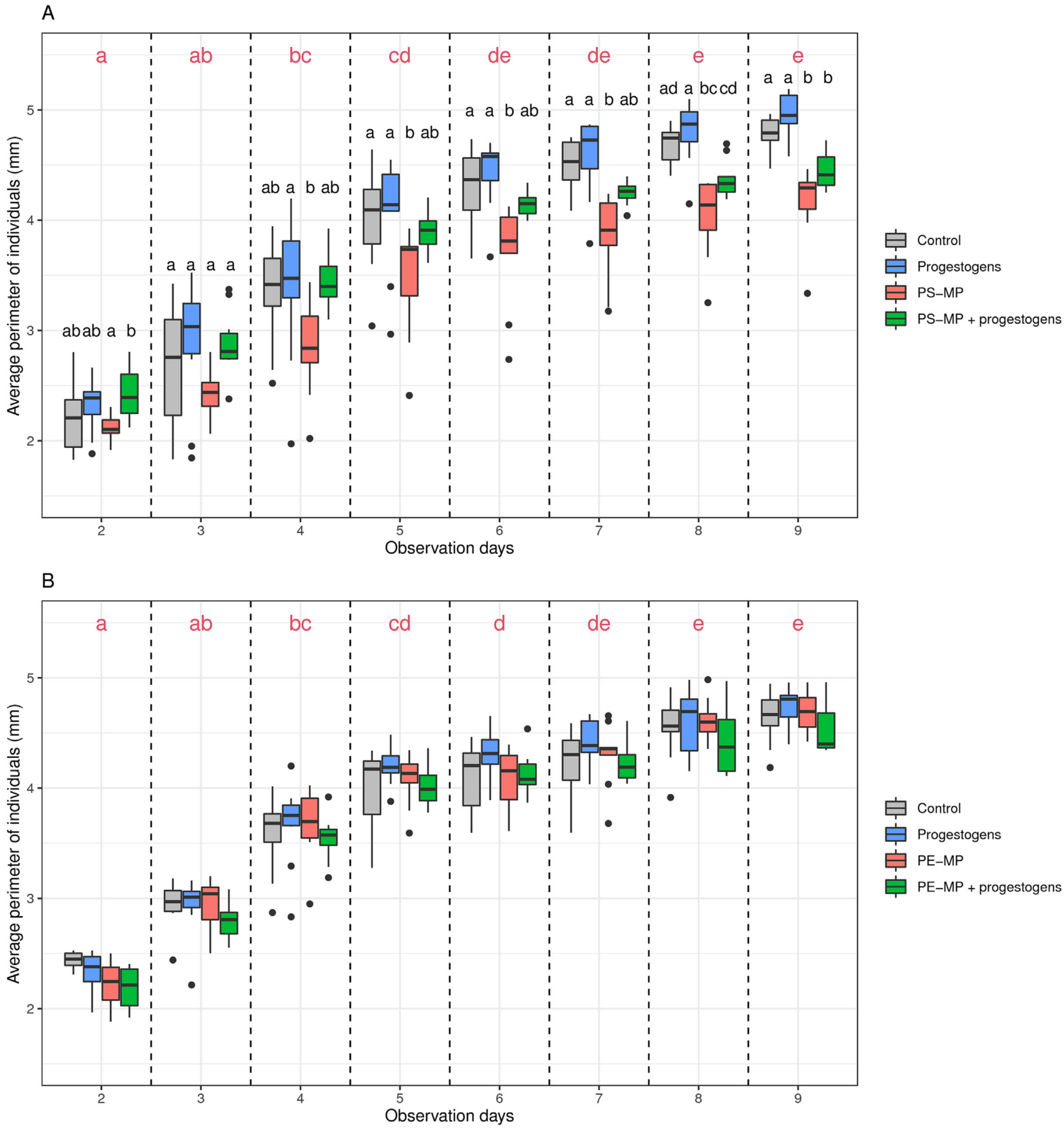

Fig. 3. Average body size (perimeter) of individuals in different experimental groups of Experiment 1 (A) and Experiment 2 (B). Body size was measured every second day during the 21-day exposures. Red letters indicate significant differences between observation days ($P \leq 0.05$). Black letters indicate significant differences between experimental groups on a given observation day ($P \leq 0.05$). In the case of Experiment 2, no significant differences were found.

[$H = 2.329$, $P > 0.05$], nor time x treatment interaction [$H = 1.766$, $P > 0.05$]. Further analysis with Kruskal-Wallis and Dunn's post hoc test indicated that there was no significant difference between the control and the treated groups during the whole observation period.

Regarding reproduction, in the case of Experiment 1 (Table 1), the number of days until production of the first eggs ($\chi^2 = 21.90$, $P < 0.001$) was significantly shorter in the progestogen-treated group (7.88 ± 0.26; $P \leq 0.05$) than the control (10.50 ± 0.68). The number of eggs in the first production [$F(3,34) = 15.22$, $P < 0.001$] significantly decreased in the PS-MP-treated group (2.77 ± 0.43; $P \leq 0.05$) but significantly increased in the progestogen-treated group (8.33 ± 0.74; $P \leq 0.05$) compared to the control (5.70 ± 0.71). The maximum egg number per individual [$F(3,34) = 78.31$, $P < 0.001$] was significantly less in the PS-MP-treated group (3.55 ± 0.55; $P \leq 0.05$) and PS-MP + progestogen-treated group (5.60 ± 0.30; $P \leq 0.05$) than the control group (10.00 ± 0.42), however, it increased significantly in the progestogen-treated specimens (12.55 ± 0.52; $P \leq 0.05$). In the case of Experiment 2 (Table 2), only the animals of the progestogen-treated showed

Table 1
Changes in the reproductive performance of *D. magna* during Experiment 1. The reproduction was monitored every day. Note: values represent mean ± SEM; significant results (bold), in comparison with the control, are indicated by asterisks (ANOVA or Kruskal-Vallis, **P* ≤ 0.05).

Treatments	First egg production (days)	Egg number in the first production	Maximum egg number per individual
Control	10.50 ± 0.68	5.70 ± 0.71	10.00 ± 0.42
Microplastics (3 μm PS-MPs)	12.66 ± 0.92	**2.77 ± 0.43***	**3.55 ± 0.55***
Progestogens	**7.88 ± 0.26***	**8.33 ± 0.74***	**12.55 ± 0.52***
Microplastics + progestogens	8.60 ± 0.30	4.00 ± 0.44	5.60 ± 0.30*

significant changes in the reproductive parameters investigated. The number of days until production of the first eggs (χ^2 = 9.46, *P* < 0.05) was significantly shorter (7.88 ± 0.35; P ≤ 0.05) than the control (9.50 ± 0.40). Both number of eggs in the first production [F(3,33) = 4.67, *P* < 0.01] (8.22 ± 0.66; *P* ≤ 0.05) and the maximum egg number per individual [F(3,33) = 5.86, P < 0.01] (9.77 ± 1.05; P ≤ 0.05) significantly increased compared to the control (5.40 ± 0.61 and 5.80 ± 0.71, respectively).

3.4. Single and combined effects of MPs and progestogens on enzymatic activity of D. magna

The effects of the exposures on the enzymatic activity are shown in Fig. 4. In the case of Experiment 1 (Fig. 4A–C), there was no significant difference between the control and treated groups in both GST (Fig. 4A) and SOD (Fig. 4C) activity. In contrast, the CAT activity (Fig. 4B) significantly increased in the PS-MP-treated group (0.53 ± 0.01; *P* ≤ 0.05) compared to the control (0.34 ± 0.01). In the case of Experiment 2 (Fig. 4D–F), compared to the control (0.17 ± 0.01), the progestogen treatment significantly increased the GST activity (0.20 ± 0.00; P ≤ 0.05) (Fig. 4D). Animals in the PE-MP-treated group (0.70 ± 0.02; P ≤ 0.05) and PE-MP + progestogen-treated group (0.69 ± 0.03; P ≤ 0.05) showed significantly higher CAT activity (Fig. 4E) compared to the control animals (0.49 ± 0.03). The SOD activity (Fig. 4F) significantly decreased in the PE-MP + progestogen-treated group (3.00 ± 0.64; P ≤ 0.05) compared to the control (1.87 ± 0.34).

4. Discussion

Most of efforts on the research of MPs have been placed on marine environment, hence still much less data are available on their abundance in freshwaters (Lambert and Wagner, 2018; Li et al., 2018). In the present study, for the first time, we showed the presence of 7 polymer types of MPs in the 50 μm – 100 μm size range in Lake Balaton, the largest shallow lake in Europe. Based on the literature data, polypropylene (PP)- and PE-MPs are the most frequent types of polymer in freshwaters (Koelmans et al., 2019; Scherer et al., 2020), our results also reflect this. PS-MPs are also considered to be one of the most frequent MPs in the freshwaters (Koelmans et al., 2019), but interestingly we did not detect this polymer type in the size range investigated. However, we would like to mention that PS-MPs are also present in Lake Balaton with a size of >100 μm (our own still unpublished results). However, we included only the MP data of the 50 μm – 100 μm size range in the present paper because this is the relevant one to the filtering capacity (up to 70 μm) of *D. magna* (Ebert, 2005). The detailed distribution of MP particles between 50 μm and 1 mm collected from the lake and its catchment area will be published in another paper. Regarding the detectability and quantification of the presence of MPs in Lake Balaton, we determined the LOD and LOQ values: 5 of the 8 samples reached the LOD value (2, 3, 5, 6, 8 water samples) and 3 of them also reached the LOQ value (2, 5, 6 water samples). To be able to compare the detected particle number in Lake Balaton with that of other European lakes, we made a raw, polymer-independent calculation for the average particle number of Lake Balaton, which was 5.5 particles m^{-3} (size range: 50 μm – 100 μm). This value is included in the average European range (0 and 7.3 particles m^{-3}; detected size range: 45 μm - 780 μm; (Tanentzap et al., 2021)). The composition of MP polymer types showed that PP-MPs and PE-MPs were the most in Lake Balaton in the size range of 50–100 μm investigated (Supplementary Fig. 5).

Many previous studies demonstrated the long-term single effects of MPs, as well as their long-term combined effects with different chemicals, on *D. magna* at different biological levels (An et al., 2021; Chenxi et al., 2022; De Felice et al., 2019; Jeong et al., 2022; Liu et al., 2022a; Liu et al., 2022b; Liu et al., 2022c; Ma et al., 2016; Martins et al., 2022; Parolini et al., 2022; Schwarzer et al., 2022; Trotter et al., 2021; Yin et al., 2020; Zhang et al., 2020). In the present study, we investigated the potential effects of MPs alone and in combination with three progestogen compounds on this model species. To the best of our knowledge, this is the first study to examine the combined effects of MPs and any type of vertebrate sex steroids on *D. magna*. Earlier studies presented that vertebrate sex steroid hormones, including PRG, are also adsorbed onto MPs (Lara et al., 2021; Leng et al., 2022; Siri et al., 2021); hence we hypothesized that they could have joint toxicity to aquatic species. We used two types of MPs (PS-MPs and PE-MPs) in our experiments. The choice of PS-MPs without any further modification was due to consideration that this polymer type (without modification) is the predominant one used in ecotoxicological studies, hence we also investigated their effects this way to make our results comparable with the previous findings. The choice of PE-MPs with home-made modification was due to consideration that this polymer type is present in Lake Balaton in the size range investigated and that fragmentation occurs in the environment. Hence, we wanted to make the exposure environmentally more relevant. Importantly, the effects of MPs are known to be not only shape and size but also polymer dependent (Imhof et al., 2017; Schwarzer et al., 2022; Zimmermann et al., 2020).

Literature data on the single effects of MPs on *D. magna* mortality show variability (summarized in Supplementary Table 3). Similarly to some of the previous findings (De Felice et al., 2019; Liu et al., 2022a), MPs caused no significant lethality during the long-term exposures in the present paper. It is worth mentioning that a previous study using nanoplastics (50 nm) described significant lethality in *D. magna* (Ma et al., 2016). As a supplementary investigation, we also performed such an experiment but was not able to reproduce this previously reported result (Supplementary Table 4). Also similarly to literature data (Kashian and Dodson, 2004; Svigruha et al., 2021b; Torres et al., 2015), no significant lethality was observed in the progestogen-treated groups during both Experiment 1 and 2. No joint effect of MPs and progestogens could be observed in this endpoint either.

Similarly to the mortality data, previous findings regarding the single effects of MPs on the body size of *D. magna* also show high variability (summarized in Supplementary Table 3). Overall, similarly to some of the previous studies (Chenxi et al., 2022; Jeong et al., 2022; Liu et al., 2022b; Trotter et al., 2021), our long-term exposure to PS-MPs caused a continuous (i.e. day-to-day) significant decrease in the body size in Experiment 1. Progestogens did not change the body size in either Experiment 1 or 2, this finding coincides with previous results (Kashian and Dodson, 2004; Svigruha et al., 2021b). Although the effect was not continuously significant, the body size of the animals significantly decreased in the PS-

Table 2
Changes in the reproductive performance of *D. magna* during Experiment 2. The reproduction was monitored every day. Note: values represent mean ± SEM; significant results (bold), in comparison with the control, are indicated by asterisks (ANOVA or Kruskal-Vallis, *P ≤ 0.05).

Treatments	First egg production (days)	Egg number in the first production	Maximum egg number per individual
Control	9.50 ± 0.40	5.40 ± 0.61	5.80 ± 0.71
Microplastics (≤ 100 μm PE-MPs)	8.88 ± 0.20	6.44 ± 0.58	7.55 ± 0.41
Progestogens	**7.88 ± 0.35***	**8.22 ± 0.66***	**9.77 ± 1.05***
Microplastics + progestogens	8.66 ± 0.23	4.88 ± 0.53	6.55 ± 0.89

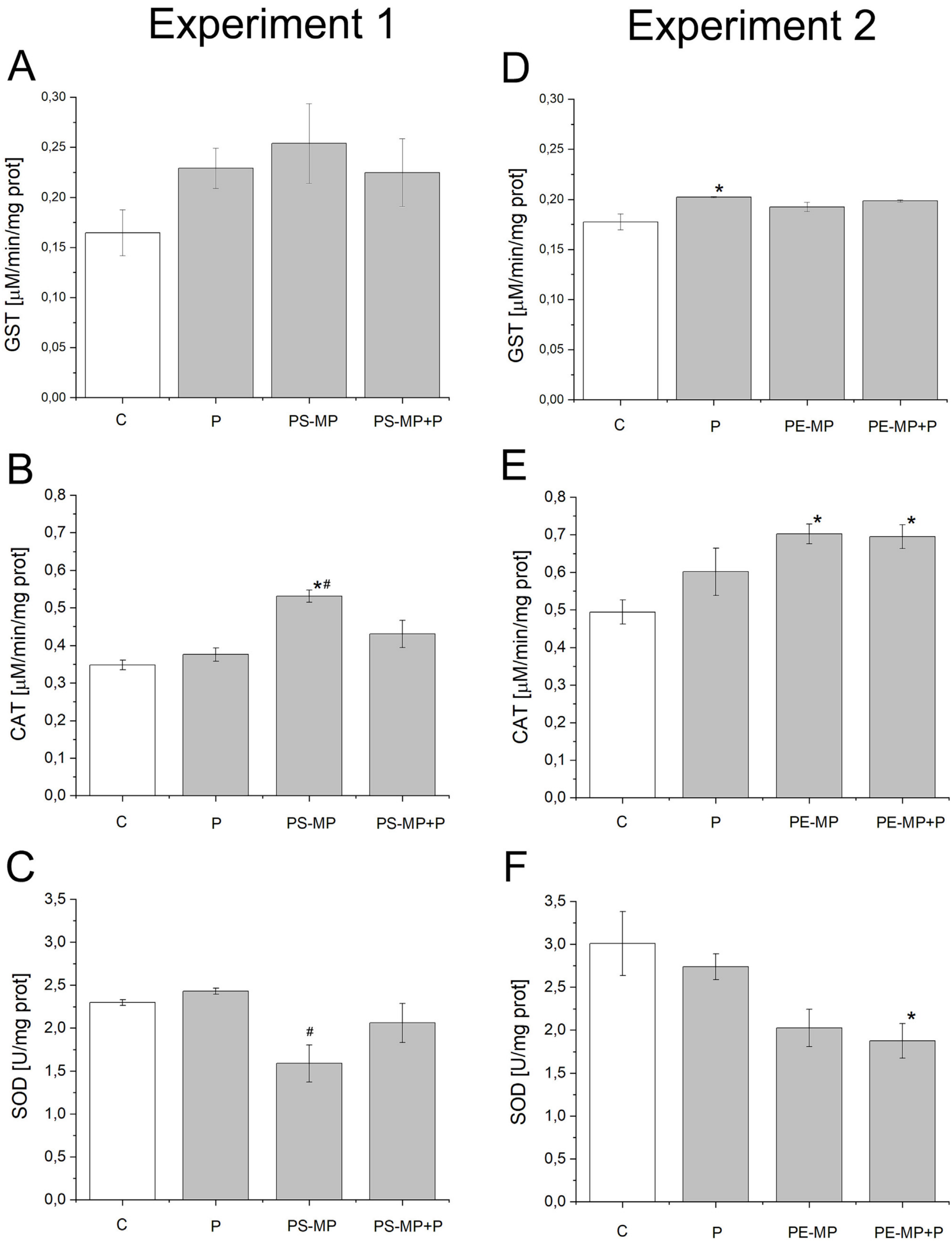

Fig. 4. Changes of GST (A, D), CAT (B, E), and SOD (C, F) activity in *D. magna* specimens during Experiment 1 (A-C) and Experiment 2 (D–F). Each bar represents mean ± SEM. The white column represents the control while the grey columns represent the treated groups. Significance of differences to the control or to the progestogen-treated group is indicated by asterisks and hashtags, respectively (*,#P ≤ 0.05). Abbreviations: C – control; P – progestogens; PS-MP – polystyrene microplastics; PE-MP – polyethylene microplastics.

MP + progestogen-treated group at the end of the exposure; but this cannot be considered as a joint effect.

Literature data on the long-term effects of MPs on reproduction of *D. magna* are presented in Supplementary Table 3; the changes of the parameters investigated were also found not to be consistent. In Experiment 1, similarly to some of the previous studies (Jeong et al., 2022; Liu et al., 2022b; Liu et al., 2022c; Trotter et al., 2021; Yin et al., 2020), PS-MPs significantly decreased both of the number of eggs in the first production and the maximum egg number per individual. In contrast to some previous studies reporting a significant delay in the time of the first egg production (De Felice et al., 2019; Liu et al., 2022b; Liu et al., 2022c), PS-MPs did not change this parameter. Interestingly, PE-MPs caused no significant alterations in Experiment 2 at all. Similarly to our previous study (Svigruha et al., 2021b), progestogens significantly increased all reproductive parameters investigated. However, it is worth mentioning that there was no significant change in the number of eggs in the first production previously. The lack of the consistency may come from the different animal number and the fact that arthropods do not have nuclear progesterone receptors (Baker, 2019; Markov et al., 2009; Markov et al., 2017; Ren et al., 2019; Taubenheim et al., 2021). Although homolog sequences to membrane progesterone receptor gamma and progesterone membrane receptor component 1 are found in crustaceans (Ren et al., 2019), the progesterone-binding ability of this protein has not yet been tested. Hence, we believe that the changes induced by progestogens in *D. magna* are non-specific and can be altered in different experiments. Although the PS-MPs + progestogens treatment also decreased significantly, the maximum egg number per individual, an antagonistic joint effect was observed.

Previous results on the long-term effects of MPs on detoxification-related enzyme activity of *D. magna* are presented in Supplementary Table 5. Coinciding with previous studies (De Felice et al., 2019; Liu et al., 2020b), neither MPs caused significant change in the GST activity in Experiment 1 or 2. Previous long-term experiments with MPs demonstrated a high fluctuation of CAT activity over time, but the activity consistently significantly decreased at the end of the 21-day exposures (Chenxi et al., 2022; Liu et al., 2022c). In contrast, we found that CAT activity significantly increased in both Experiment 1 and 2. The existing data on the SOD activity shows high variability (Chenxi et al., 2022; Liu et al., 2022c; Liu et al., 2022a), neither MPs caused significant changes in our experiments. Similarly to our previous study (Svigruha et al., 2021b), progestogens significantly increased the GST activity in Experiment 2. This effect was not observed in Experiment 1. The lack of the consistency may come from the abovementioned reasons. The best of our knowledge, there has been no information about the effects of progestogens alone and in combination with MPs on CAT or SOD activity in *D. magna*. In our experiment, neither CAT nor SOD activity changed due to the progestogen treatments. Although the PE-MPs + progestogens treatment also significantly increased the CAT activity, this cannot be considered as a joint effect. In contrast, an additive or synergistic joint effect was detected in the case of SOD manifested in the decrease of its activity.

Based on our results, *D. magna* specimens seem to be sensitive to different types of MPs, but further investigations are required since there are many controversies and inconsistencies in the literature (collected in Supplementary Table 3 and Supplementary Table 5). Previous studies investigating the combined effects of MPs and different chemicals on aquatic species demonstrated that antagonistic, additive/synergistic, or no effects can occur (Chen et al., 2022a; Hanslik et al., 2020; Hanslik et al., 2022; Lu et al., 2022; Magara et al., 2018; Magara et al., 2019; Na et al., 2021). In the present study, one antagonistic effect (maximum egg number per individual) and one additive or synergistic effect (SOD activity) were observed. Given that one effect was in the case of PS-MPs and at the behavioral level while that other effect was in the case of PE-MPs and at the biochemical level, it is hard to take a comprehensive conclusion from the possible joint effects. Nevertheless, in our opinion, the potential threat of MPs as vectors for many chemicals may be overestimated.

5. Conclusions

In summary, many polymer types of MPs under <100 μm are present in Lake Balaton. Considering the filtering capacity of *D. magna*, the currently detectable smallest MPs (50–100 μm) due to the methodical limitations of the research field (only ≥50 μm particles can be detected during the sampling because of the filtering) are already physiologically relevant (i.e. they are suitable for getting filtered by *Daphnia spp*). Our ecotoxicological experiments confirm that MPs can affect *D. magna* at different biological levels. Based on our findings, *D. magna* specimens seem to be more sensitive to PS-MPs at the behavioral level, while they seem to be more sensitive to PE-MPs at the biochemical level. Our overarching conclusion is that MPs may lead to reduced fitness in the aquatic biota in a natural multi-stressor freshwater environment (such as Lake Balaton), but their combined physiological effects with different chemicals, at least in the case of progestogens, can be overestimated. Therefore, locally, further studies should aim at investigating the spatio-temporal variations (e.g., seasonality) of MPs in Lake Balaton and, globally, their single and combined effects on different invertebrate and vertebrate aquatic species should be better understood.

CRediT authorship contribution statement

RS: Conceptualization, Methodology, Investigation, Writing - original draft, Data curation, Visualization; **BP:** Investigation; Data curation, Visualization; Writing - review & editing; **AF**: Methodology, Investigation, Writing - review & editing; **ÁA**: Investigation, Writing - review & editing; **IF**: Conceptualization, Methodology, Writing - original draft, Data curation, Visualization; **KT**: Writing - review & editing, Data curation, Visualization; **JS**: Methodology, Investigation; Writing - review & editing**; GB:** Methodology, Writing - review & editing; **JH**: Investigation; Writing - review & editing; **PH:** Investigation; Writing - review & editing; **EK**: Supervision; Writing - review & editing; **SS**: Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition; **ZP**: Conceptualization, Methodology, Investigation; Writing - review & editing, Data curation, Visualization, Supervision, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

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