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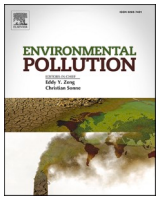

Microplastics originated from Plasmix-based materials caused biochemical and behavioral adverse effects on *Daphnia magna*☆

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ABSTRACT

The implementation of advanced recycling techniques represents a key strategy for mitigating the mismanagement and the environmental impact of plastic waste. A limited array of plastic polymers can be efficiently recycled, while a notable portion of plastic waste remains unrecyclable. In Italy, this residual, heterogeneous fraction is referred to as Plasmix. Because of its complexity and non-homogeneous composition, Plasmix is primarily directed towards low-value applications. However, recent developments in laboratory-scale mechanical recycling have enabled the creation of new plastic materials from Plasmix. Prior to their application, these materials must undergo rigorous eco-safety evaluation. The present study aims to assess the potential toxicity of microplastics (MPs) from Plasmix-based materials on the freshwater crustacean *Daphnia magna*. Specifically, this study investigated sub-individual and individual effects induced by a 21-day exposure to different concentrations of MPs generated from the grinding of naïve and Additivated Plasmix-based materials (hereafter referred to as Px-MPs and APx-MPs, respectively). Sub-individual endpoints focused on changes in oxidative status, including the modulation of antioxidant and detoxifying enzyme activities, as well as oxidative damage, such as lipid peroxidation. Individual level endpoints included alterations in survival and reproduction. Microscopy analyses confirmed the ingestion of both Px-MPs and APx-MPs by *D. magna* individuals. An oxidative stress condition raised in organisms exposed to Px-MPs, whereas no effect was observed in individuals exposed to APx-MPs. Although survival was not affected, a significant impairment in reproductive output was detected at the end of exposure to all the concentrations of both MP types. These findings suggest that even low concentrations of Px-MPs and APx-MPs could negatively affect the health status of *D. magna*, underscoring the need for further research to complete the risk assessment of Plasmix-based materials prior to their use in consumer products.

1. Introduction

Plastic is a predominant material that significantly affects modern society and daily life (Van Rensburg et al., 2020). Plastics are used across various sectors, including food packaging, construction, healthcare, agriculture, automotive, electronics, household items, and recreational activities, and their global production escalated from 225 million tons in 2004 to 440.3 million tons in 2022 (Plastics Europe, 2023). This extensive use often leads to mismanagement and improper disposal of plastic products at their end of life, contributing to environmental contamination of terrestrial, aquatic, anthropogenic, and natural ecosystems, including remote areas (Li et al., 2020; Xu et al., 2020; Rai et al., 2021; Porta, 2021).

To mitigate plastic waste leakage and environmental impact, various strategies have been adopted, such as advancements in recycling technologies and the shift towards biodegradable plastics (Gazzotti et al., 2022). In Europe, remarkable efforts have focused on enhancing the management, sorting, and recycling of plastic waste. The European Directive on packaging and packaging waste (CE/62/94) mandates that by 2025, the 55% of plastic packaging waste must be recycled, with the ultimate goal of ensuring that all plastic packaging in the EU market is recyclable by 2030 (UN Environment, 2018). Currently, the European

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recycling rate for plastic packaging stands at 22.5%, achieving 32.5% of total plastic waste production (European Commission, 2021), with some EU member states, nearing the 2025 target in 2019 (Picuno et al., 2021). Despite this promising trend, increasing recycling rates remains challenging due to the heterogeneous nature of plastic packaging waste and difficulties in effective separation (Colantonio et al., 2020).

In Italy, over 1 million tons of plastic waste are generated annually, with only about half being recycled (Novati and Leonardi, 2022). Currently, only packaging made of polyethylene (PE), polypropylene (PP), and polyethylene terephthalate (PET) are economically viable for sorting and recycling (COREPLA, 2015). The remainder, known as Plasmix, includes materials that are unrecyclable or difficult to recycle because of their heterogeneity and multi-layer composition (COREPLA, 2023). Plasmix comprises a complex mixture of materials (i.e., approximately 57% plastics, 10% paper and cardboard, 3% wood and textiles, and 27% inert materials and other substances; Rossi et al., 2010), whose composition varies over time and depends on sorting plant efficiency (Colantonio et al., 2020). The plastic fraction is predominantly composed of polyolefins (PE and PP; 60–70% of the total volume), followed by PET (4–5%) and PS (2–4%), with minimal contributions from other polymers (COREPLA, 2015). Because of its heterogeneous and variable composition, the separation of polymer fractions in Plasmix is economically unfeasible, leading to its primary disposal through incineration (57%), use as a coal substitute in cement kilns (27%), or landfilling (16%) (COREPLA, 2015).

Considering the increasing volume and complexity of plastic packaging and the inefficiencies of current sorting and recycling processes, the management and valorisation of Plasmix has become a priority. Recently, Plasmix has been used for producing wood adhesives, packaging, construction components such as roof and garden tiles, outdoor furniture, 3D printing filaments, asphalt additives, and hydrogen (Gazzotti et al., 2022; Fiore and Tamborrini, 2024). Moreover, Plasmix has been explored for mechanical recycling to create more homogeneous materials (i.e., Plasmix-based materials) for secondary use (Zilia et al., 2024). However, prior to the application of these materials in product manufacturing, an assessment of their potential environmental risks and toxicity is essential and mandatory. In fact, once in the environment, they can degrade or fragment into microplastics (MPs), which are defined as plastic items ranging from 1 to < 1000 μm (Hartmann et al., 2019). Thus, ecotoxicological assessment is pivotal for understanding the potential environmental risk posed by new plastic materials. The application of ecotoxicological assays can allow characterizing the materials and identifying potential targets and mechanisms of toxic action in model organisms at various biological and ecological levels, ultimately ensuring eco-safety and sustainable outcomes (Corsi and Grassi, 2019). Considering the fate of plastics in the environment, a crucial step concerns the investigation of the potential toxicity of MPs originating from the degradation and fragmentation of items or objects made of Plasmix-based materials. Indeed, a growing number of ecotoxicological studies have investigated the adverse effects caused by the exposure to MPs of varying sizes, shapes, and polymer compositions, as well as associated additives, on both aquatic (e.g., da Costa et al., 2023; Sun et al., 2019; Liu et al., 2022) and terrestrial organisms (e.g., Dissanayake et al., 2022). Overall, all the studies have confirmed that after the ingestion, MPs can induce diverse adverse effects to different organisms, including the onset of oxidative stress (e.g., Ainali et al., 2022; Parolini et al., 2020a,b) and inflammation (Duan et al., 2022), changes in morphometric traits and growth rate (De Felice et al., 2018; Green, 2016), as well as alterations in swimming behaviour and reproduction (de Oliveira et al., 2021; De Felice et al., 2019). As Plasmix-based materials are still limitedly used to create items or objects, the environmental presence of MPs deriving from the degradation or fragmentation of these materials has been neglected. However, considering the amount of Plasmix generated every year and the increase in its use in diverse application, the input of MPs from Plasmix-based materials can increase, and consequently, terrestrial and aquatic organisms can experience exposure conditions. A preliminary study performed on the earthworm *Eisenia foetida* has shown that MPs from Plasmix-based did not cause significant sub-individual or individual adverse effects (De Felice et al., 2024). These findings suggest that, at low exposure levels, MPs from Plasmix-based materials may pose minimal risk to terrestrial organisms. However, the exposure of aquatic organisms to MPs from Plasmix-based materials could result in opposite outcomes, which deserve to be investigated in order to complete the ecotoxicological assessment of these materials. This study yearned at exploring the adverse effects induced by the administration of MPs obtained from two Plasmix-based materials towards a common biological model for aquatic ecotoxicology, such as the freshwater crustacean *Daphnia magna*. This is a keystone species in freshwater ecosystems and, because of its peculiar eco-physiological features, is the most commonly used freshwater indicator species for ecotoxicological studies (Shaw et al., 2008). Microplastics were obtained from the grinding of naïve and additivated Plasmix-based materials (hereafter Px-MPs and APx-MPs, respectively). Increasing concentrations of Px-MPs and APx-MPs were administered to *D. magna* individuals for 21 days and effects at sub-individual and individual levels were investigated. First, the ingestion of MPs and the potential migration of chemicals in aqueous medium were investigated through microscopy analysis and gas chromatography coupled with mass spectrometry (GC-MS), respectively. Then, a battery of oxidative stress biomarkers was applied to assess the onset of an oxidative stress condition through the analysis of alterations in the activity of antioxidant (SOD, CAT, GPx) and detoxifying (GST) enzymes and levels of lipid peroxidation. Changes in survival and reproductive success were considered as endpoints at individual level. Although no toxic effects raised after the exposure to MPs originated from Plasmix-based materials in earthworms (De Felice et al., 2024), considering previous findings obtained of *D. magna* or similar species exposed to diverse MPs, we expect that Px-MPs and APx-MPs can cause impairments at both sub-individual and individual level in our model species.

2. Materials and methods

2.1. Preparation of microplastics from Plasmix-based materials

Microplastics derived from Plasmix-based materials were produced following the methodology described by De Felice et al. (2024). Plastic waste from a sorting facility was supplied by the Consorzio Nazionale per la Raccolta, il Riciclaggio e il Recupero degli Imballaggi in Plastica (COREPLA, 2023). Prior to processing, the plastic waste was manually re-sorted to eliminate non-plastic components. The remaining plastic mixture, referred to as Plasmix, underwent several washes using a solution of dishwashing detergent in tap water, followed by a thorough rinse with tap water. After drying, the plastic mixture was subjected to several cycles of freezing (using liquid nitrogen) and grinding, according to the protocol described by Parolini et al. (2020a, b).

The grinded Plasmix mixture was processed with a twin-screw lab-scale extruder (Thermo Scientific Process 11 double screw extruder with screw diameter of 11 mm and L/D = 40). The extrusion occurred at a temperature ranging between 200 °C (at the feeding of the extruder) and 240 °C (in the central zone of the extruder), with twin screws rotating at 210 rpm. The extruded, referred to as naïve Plasmix-based material, was then cooled at room temperature and then grinded into small granules using a laboratory grinder. Before the extrusion, a part of the dried, chopped Plasmix (about 200 g) was dry mixed with 4% wt. of Vibatan peroxide. The obtained blend was then extruded at a temperature ranging between 220 °C (at the feeding of the extruder) and 260 °C (in the central zone of the extruder), with twin screws rotating at 100 rpm. The extruded (i.e., Additivated Plasmix-based material) was cooled at room temperature and then grinded in granules. The granules of both naïve and Additivated Plasmix-based materials were processed through injection molding to obtain dogbone specimens (ISO 527-5A).

Specifically, 150 g of each granule type was molded using a Babyplast injection molding machine, operating at temperature ranging between 180 °C and 190 °C, and pressure ranging between 35 and 37 bar. The dogbone specimens were subsequently used to generate MPs from Plasmix-based (Px-MPs) and Additivated Plasmix-based materials (APx-MPs). To create MPs, the dogbone specimens were subjected to a series of freezing (in liquid nitrogen) and grinding cycles, resulting in micronized particles (Parolini et al., 2020a,b). The resulting items were then sieved using a 1 mm mesh sieve to isolate particles within the typical size range of MPs (1 μm–1 mm; Hartmann et al., 2019). The chemical spectra of Px-MPs and APx-MPs were obtained using Fourier Transform Infrared Spectroscopy (FT-IR; PerkinElmer Spectrum 100). Additionally, images of Px-MPs and APx-MPs were captured using a scanning electron microscope (LEO1430, Zeiss, Oberkochen, Germany)

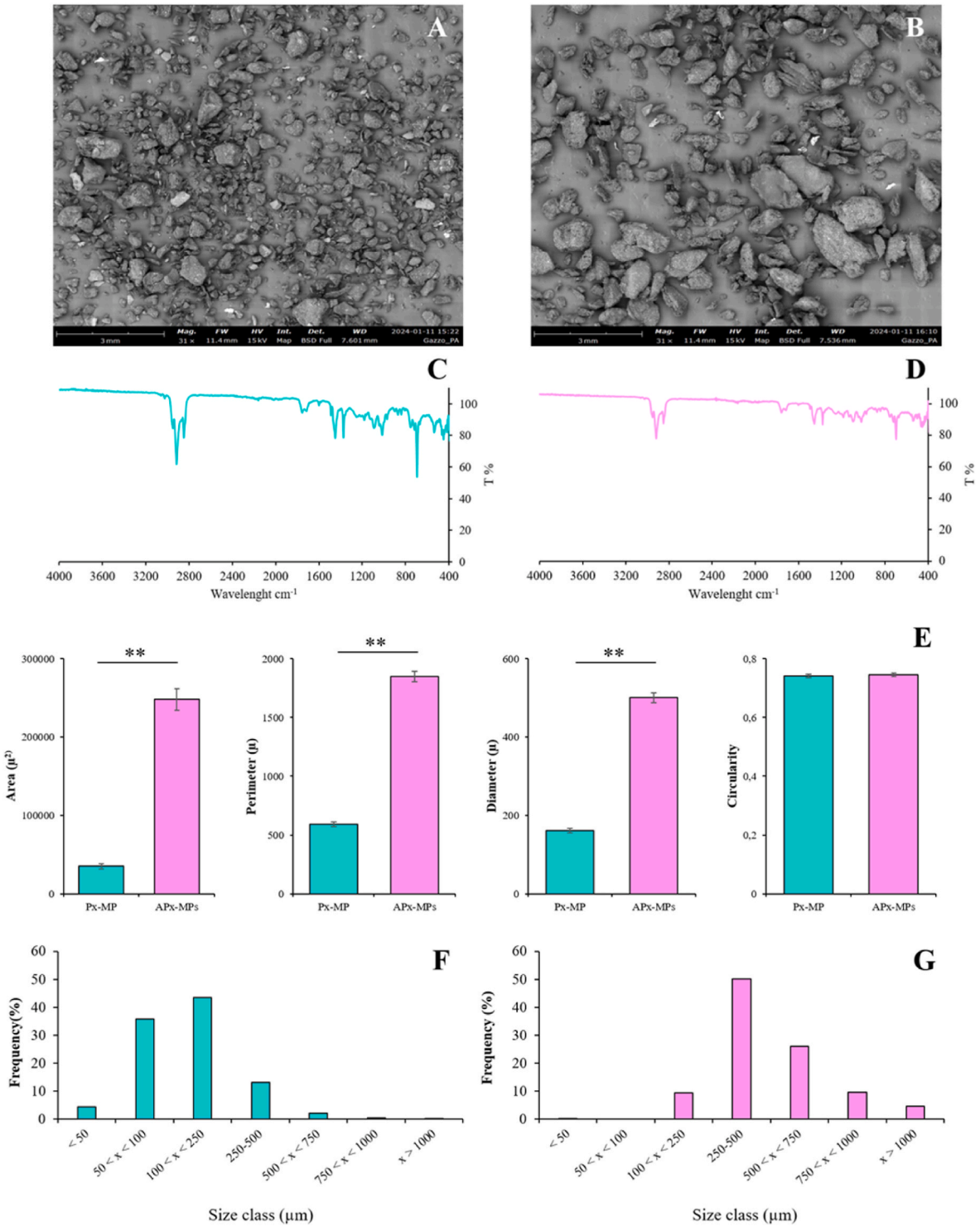

Fig. 1. Scanning Electric Microscope (SEM) image of Plasmix-based microplastics (Px-MPs, A) and Additivated Plasmix-based microplastics (APx-MPs, B), spectra of Px-MPs (C) and APx-MPs (D) polymer composition obtained through Fourier Transformed Infrared Spectroscopy (FTIR), morphometric features of both the MPs typologies (E) and distribution in size classes of Px-MPs (F) and APx-MPs (G). Asterisks represent significant differences of different parameters between the MPs types (unpaired Student's *t*-test; $P < 0.05$).

for morphometric analysis. These images were analyzed with ImageJ software to calculate area, diameter, perimeter, and circularity (calculated as $4\pi \times \text{area/perimeter}^2$) for approximately 500 Px-MPs and APx-MPs. Given the irregular shape of Px-MPs and APx-MPs, their size was calculated as the diameter of a spherical particle with an equivalent area (De Felice et al., 2024). The shape, polymer composition, morphometric characteristics, and size distribution of Px-MPs and APx-MPs are presented in Fig. 1.

2.2. Gas-chromatographic-mass spectrometric (GC-MS) analysis

GC-MS analyses were performed employing a Finnigan Trace GC Ultra equipped with a Phenomenex ZB-WAX MS capillary column (30 m, 0.25 mm i.d., 0.25 μm thickness) and coupled with a single quadrupole Trace DSQ mass spectrometer. The injection was performed at 250 °C, in splitless mode and helium was employed as carrier gas at the flow of 1.0 mL/min. The MS transfer line was set at 300 °C. The MS signal was acquired in EI^+ mode (ionization energy of 70.0 eV) with the ion source temperature of 290 °C. The acquisition was performed in full scan mode in the 35–500 m/z range. About 0.50 mg of Px-MPs or APx-MPs was put in contact with 10.00 mL of ultrapure water for 48 h. After centrifugation, 6.00 mL of supernatant was collected and put in contact with 2.00 mL of dichloromethane (CH_2Cl_2). A liquid-liquid extraction was performed by shaking for 3 min by a vortex mixer. The CH_2Cl_2 phases were separated and filtered (0.2 μm, PTFE) and 3 μL were injected in the GC-MS apparatus. Each chromatogram was integrated and the area of each peak was registered and scaled for the sample weight (Aw). Each material was tested in triplicate. The obtained values are the average value and represent semi-quantitative and comparable results among the experiments.

2.3. Experimental plan

Individuals of *Daphnia magna* used in the experiments performed in the present work came from the husbandry located in the laboratory of the University of Milan. Adults (30 individuals/L) were maintained in glass beaker filled with San Benedetto® commercial water, placed into a thermostatic chamber at 20.0 ± 0.5 °C and 16:8 h light:dark photoperiod, and fed *ad libitum* three times a week with the yeast *Saccharomyces cerevisiae* and the unicellular green alga *Pseudokirchneriella subcapitata* according to De Felice et al. (2022). For further experiments, newborns (i.e., daphnids) from the third generation were used. As no information on the environmental concentrations of Plasmix-based MPs is currently available, a preliminary acute toxicity test (Test No. 202: *Daphnia* sp. Acute Immobilization Test) was performed following the OECD Guidelines for the Testing of Chemicals (OECD, 2004). Daphnids (individuals <24 h old) were exposed to a wide range (i.e., 0 μg/mL, 0.05 μg/mL, 0.1 μg/mL, 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 5 μg/mL, 7 μg/mL, 10 μg/mL and 15 μg/mL, 30 μg/mL and 50 μg/mL) of arbitrarily selected Px-MPs and APx-MPs concentrations for 48 h. For each experimental group, five replicates with five daphnids each were prepared. The immobilization of individuals was recorded after 48 h of exposure and data were used to calculate the half-maximal immobilization concentration (IC_{50}). As the acute toxicity test did not show complete mortality in none of the experimental groups, the IC_{50} value could not be calculated (see Results section). Thus, the concentrations used to perform chronic toxicity were selected to match those used in previous studies aimed at investigating the toxicity of MPs made of single polymers composing the Plasmix on *D. magna* (De Felice et al., 2018, 2019; 2022; Parolini et al., 2023) and other aquatic organisms (Messinetti et al., 2018; Parolini et al., 2020a, b). The selection of the same concentrations tested in previous studies allowed us to compare the toxicity of PX-MPs and/or APx-MPs with that of MPs made of single polymers composing the Plasmix on *D. magna*, under the same experimental conditions. Daphnids were exposed to three concentrations (0.125, 1.25 and 12.5 μg/mL) of Px-MPs and APx-MPs for 21 days following the procedure described by De Felice et al. (2022). Each experimental group was performed in triplicate including 20 daphnids (<24 h old) in 100 mL beaker (60 daphnids for each treatment). At the end of the exposures, individuals were randomly collected from each beaker (i.e., experimental replicate) and photographed under a stereomicroscope (Leica EZ4 W) to confirm the ingestion of MPs. At the end of photo capture, all the individuals (n = 20) from each replicate were pooled in an Eppendorf tube, frozen and stored at −80 °C until the application of oxidative stress biomarkers (i.e., activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and lipid peroxidation). Biomarker analyses were performed according to the protocols reported by De Felice et al. (2022). A detailed description of biomarker methods is reported in Supporting Information.

A companion experiment was then performed to assess the potential effects induced by the exposure to Px-MPs and APx-MPs on the reproductive success of *D. magna* individuals. A chronic toxicity test was performed according to the OECD guidelines (i.e., Test No. 211: *Daphnia magna* Reproduction Test) (OECD, 2004). Fifteen replicates including one individual each (<24 h old) were prepared. Individuals were exposed for 21 days to 0.125 and 1.25 μg/mL of Px-MPs and APx-MPs under the same experimental conditions described above. Because of a constraint related to the amount of MPs from both the Plasmix-based materials, we were forced to test only the two lowest concentrations for assessing potential reproductive impairments. The exposure was performed under semi-static renewal condition, with a daily renewal of the exposure medium including food *at libitum* and the proper amount of Px-MPs or APx-MPs. Every day, until the end of the exposure, the number of alive or immobile (i.e., dead) offspring was recorded. The mean number of offspring and of reproductive events were considered as endpoints.

2.4. Statistical analysis

The effects of the treatment (0.125, 1.25 and 12.5 μg/mL), the type of MPs (Px-MPs and APx-MPs), and their interaction, on oxidative stress biomarkers were investigated through a two-way analysis of variance (ANOVA). The effect on the reproductive success, in terms of the mean number of offspring and reproductive cycles, was analyzed through generalized linear models, assuming a Poisson distribution of data. The normal distribution and the homoscedasticity of data were preliminarily checked through the application of Shapiro–Wilk and Levene's tests, respectively. Significant differences among groups (*P < 0.05 and **P < 0.01) were assessed by running Tukey's *post-hoc* test. All the statistical analyses were run in R 4.03 (R Core Team, 2020) using the *lmer* package.

3. Results

3.1. Morphometric features of Px-MPs and APx-MPs

Px-MPs and APx-MPs differed in size and morphometry (Fig. 1). The area (unpaired Student *t*-test; t = 14.563; P < 0.001), the perimeter (t = 20.137; P < 0.001) and the diameter (t = 11.092; P < 0.001) of APx-MPs were significantly larger than Px-MPs. In contrast, the shape of MPs types was similar and approximated a spherical shape, with the circularity that was close to 0.74 (t = 0.150; P = 0.880).

3.2. Release of contaminants evaluation

Px-MPs and APx-MPs were subjected to an aqueous extraction procedure to simulate the exposure of *D. magna* individuals in the experiments and to estimate the release of contaminants from MPs. To obtain reliable data, the amount of MPs placed in water were much higher than in the exposure experiments. Then, the extracted samples were analyzed by GC-MS. The resulting chromatograms are reported in Fig. 2.

By comparing the mass spectra collected for each peak with the mass spectra contained in the Wiley and NIST libraries, the peak identification

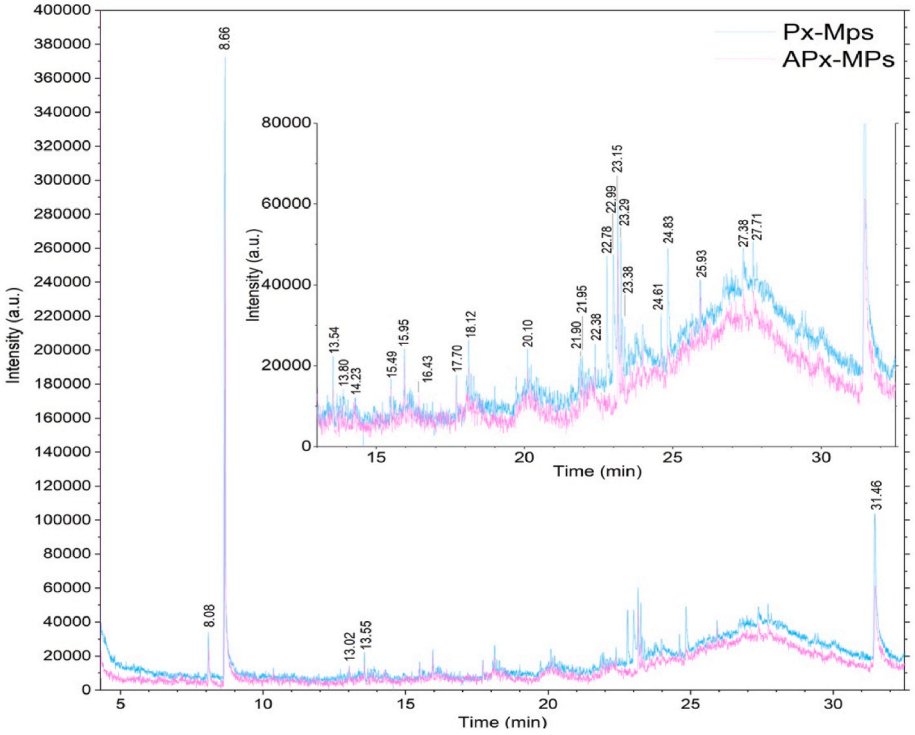

Fig. 2. Chromatograms of chemical compounds released in the aqueous medium from Plasmix-based microplastics (Px-MPs, blue line) and Additivated Plasmix-based microplastics (APx-MPs, purple line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was performed. A peak height >10,000 has been set as a threshold for proceeding with the identification. Table 1 reports the name of the identified molecules and the CAS number of the 20 peaks identified. As expected, they are at very low concentration due to the low affinity with the aqueous solvent used for the extraction. The most intense compound is 1,4-Dioxane-2,5-dione, 3,6-dimethyl- (also called Dilactide, CAS n° 95-96-5) which alone represents 44% and 47% of the total peak area of Px-MP and APx-MP, respectively. Thiscompound is included in the restriction list of the SVHCs (Substances of Very High Concern) typically contained as additive in plastics (http://echa.europa.eu). The other identified compounds are molecules that typically evolve from plastic-made samples (Graham et al., 2000; Dutra et al., 2011) belonging mainly to the class of the aliphatic hydrocarbon.

The total area values obtained by adding the areas of all identified peaks is 1,647,275 for Px-MPs and 907,976 for APx-MPs indicating a significant lower migration of contaminants in the additivated sample.

3.3. Ingestion of Px-MPs and APx-MPs

Microscopy analyses confirmed that *Daphnia magna* individuals were able to ingest Px-MPs and APx-MPs in both the experimental groups. Indeed, MPs were noted in of the digestive tract of treated individuals, while no items were detected in individuals from the control group (Fig. 3).

3.4. Acute effects of Px-MPs and APx-MPs

Acute toxicity tests showed that none of the tested concentrations of Px-MPs or APx-MPs administered in the range 0.05–50 μg/mL induced the mortality of all the exposed individuals. The survival of daphnids after 48 h of exposure to Px-MPs and APx-MPs resulted as always higher than 76% and 72% compared to the beginning of the exposure, respectively (Fig. 4). Thus, the EC_{50} could not be calculated for neither Px-MPs nor APx-MPs.

3.5. Effects of Px-MPs and APx-MPs on oxidative stress-related endpoints

No mortality was noted over the 21 days of exposure to all the experimental conditions, including control group. Significant modulation of antioxidant and detoxifying enzymes occurred (Fig. 5). A significant effect of the concentration ($F_{3,16} = 7.403$; $P = 0.002$), type of MP ($F_{1,16} = 11.775$; $P = 0.003$), and their interaction ($F_{3,16} = 8.628$; $P = 0.001$) on SOD activity was observed. The SOD activity measured in individuals exposed to Px-MPs was higher compared to individuals treated with APx-MPs ($P = 0.034$), independently of the exposure concentrations. Overall, the activity measured in individuals exposed to 1.25 μg/mL was higher compared to that of conspecifics from the control ($P = 0.034$), 0.125 μg/mL ($P = 0.037$) and 12.5 μg/mL ($P = 0.001$). A significant increase of SOD activity was noted in individuals exposed to 0.125 μg/mL ($P = 0.008$) and 1.25 μg/mL ($P = 0.004$) of Px-MPs compared to the conspecifics from the control group, while no effects of the interaction between the exposure concentration and the MP type was noted for the APx-MPs treatment ($P > 0.05$ in all the cases). Similarly, to SOD, a significant effect of the concentration ($F_{3,16} = 22.803$; $P < 0.001$), type of MP ($F_{1,16} = 22.633$; $P < 0.001$), and their interaction ($F_{3,16} = 17.301$; $P < 0.001$) on CAT activity was noted. The activity measured in individuals exposed to Px-MPs was higher compared to individuals treated with APx-MPs ($P < 0.001$), independently of the exposure concentrations. Overall, the activity measured in individuals exposed to 1.25 μg/mL was higher compared to that of conspecifics from the control ($P < 0.001$), 0.125 μg/mL ($P = 0.002$) and 12.5 μg/mL ($P < 0.001$). A significant increase of CAT activity was noted in individuals exposed to 0.125 μg/mL ($P = 0.009$) and 1.25 μg/mL of Px-MPs compared to the conspecifics from the control group ($P < 0.001$) while no effects of concentration × MP type interaction was noted at the end of the exposure to APx-MPs ($P > 0.05$ in all the cases). Neither the exposure concentration ($F_{3,16} = 2.567$; $P = 0.091$), nor the MP type ($F_{1,16} = 3.418$; $P = 0.083$), nor their interaction ($F_{3,16} = 2.175$; $P = 0.131$). The activity of GST was modulated according to the

Table 1
retention time, name and CAS number of the 20 peaks (i.e., molecules) identified after the release from Plasmix-based microplastics (Px-MPs) and Additivated Plasmix-based microplastics (APx-MPs).

Peak #	Retention time (min.)	Name	CAS #	Px-MPs (ApW)	APx-MPs (Apw)
1	8.1	Oxetane, 2,3,4-trimethyl-	53778-61-3	48,412	51,167
2	8.7	1,4-Dioxane-2,5-dione, 3,6-dimethyl-	95-96-5	786,926	485,498
3	13.5	Decane, 2,9-dimethyl-	1002-17-1	24,931	18,098
4	13.8	Tetracosane, 12-decyl-12-nonyl-	55320-13-3		15,987
5	15.5	Octacosane, 2-methyl-	1560-98-1	19,702	
6	15.9	Docosane, 11-decyl-	55401-55-3	31,287	20,352
7	17.7	Nonadecane, 2-methyl-	1560-86-7	20,302	17,612
8	18.1	Undecane, 4,7-dimethyl-	55401-55-3	28,432	18,275
9	20.1	1-Iodo-2-methylundecane	73105-67-6	15,616	
10	21.9	Triacontane, 1,30-dibromo-	121473-35-6		33,566
11	22.0	Hexacosane, 13-dodecyl-	55517-73-2	18,265	
12	22.4	Benzene, (3-nitropropyl)-	22818-69-5	26,579	
13	22.7	2-Tridecene, (Z)-	41446-59-7	46,420	
14	22.9	1,5-Hexadiyne	628-16-0	117,235	
15	23.1	Oxetane, 2-methyl-4-propyl-	7045-79-6	104,688	72,429
16	23.2	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-	131758-71-9	74,713	36,253
18	25.9	Hexaethylene glycol monododecyl ether	3055-96-7		15,460
19	27.7	Pentaethylene glycol monododecyl ether	3055-95-6	15,954	15,783
20	31.4	Benzene, 1,3-bis(1,1-dimethylethyl)-	1014-60-4	267,813	107,496

concentration ($F_{3,16} = 8.837$; $P = 0.001$), the MP type ($F_{1,16} = 15.433$; $P = 0.001$) and the interaction between the two main predictors ($F_{3,16} = 8.688$; $P = 0.001$). Overall, the activity measured in individuals exposed to Px-MPs was higher compared to individuals treated with APx-MPs ($P = 0.001$) and it was higher in individuals exposed to 1.25 μg/mL compared to that of conspecifics from the control ($P = 0.001$), 0.125 μg/mL ($P = 0.044$) and 12.5 μg/mL ($P = 0.003$). A significant increase of CAT activity was noted in individuals exposed to 0.125 μg/mL ($P = 0.034$) and 1.25 μg/mL ($P < 0.001$) of Px-MPs compared to the conspecifics from the control group, while no effects of concentration × MP type interaction was noted at the end of the exposure to APx-MPs ($P > 0.05$ in all the cases).

No effect of the exposure concentration ($F_{3,16} = 2.723$; $P = 0.078$) and the MP type ($F_{1,16} = 1.432$; $P = 0.248$) on lipid peroxidation was observed. However, a significant effect of the interaction between the predictors occurred ($F_{3,16} = 5.532$; $P = 0.008$), with levels measured in individuals exposed to 12.5 μg/mL of Px-MPs significantly higher compared to those from conspecifics of the control group ($P = 0.043$; data not shown).

3.6. Effect of Px-MPs and APx-MPs on reproductive success

Significant effect of the concentration ($F_{2,75} = 17.948$; $P < 0.001$), but not of the MP type ($F_{1,75} = 0.386$; $P = 0.536$) and the interaction between the two main predictors ($F_{2,75} = 2.392$; $P = 0.098$) on the mean number of offspring was noted. The mean number of offspring generated from individuals exposed to 0.125 μg/mL and 1.25 μg/mL was significantly lower compared to conspecifics from the control group ($P < 0.001$ for both the cases), independently of the MP type (Fig. 6). Similar effects

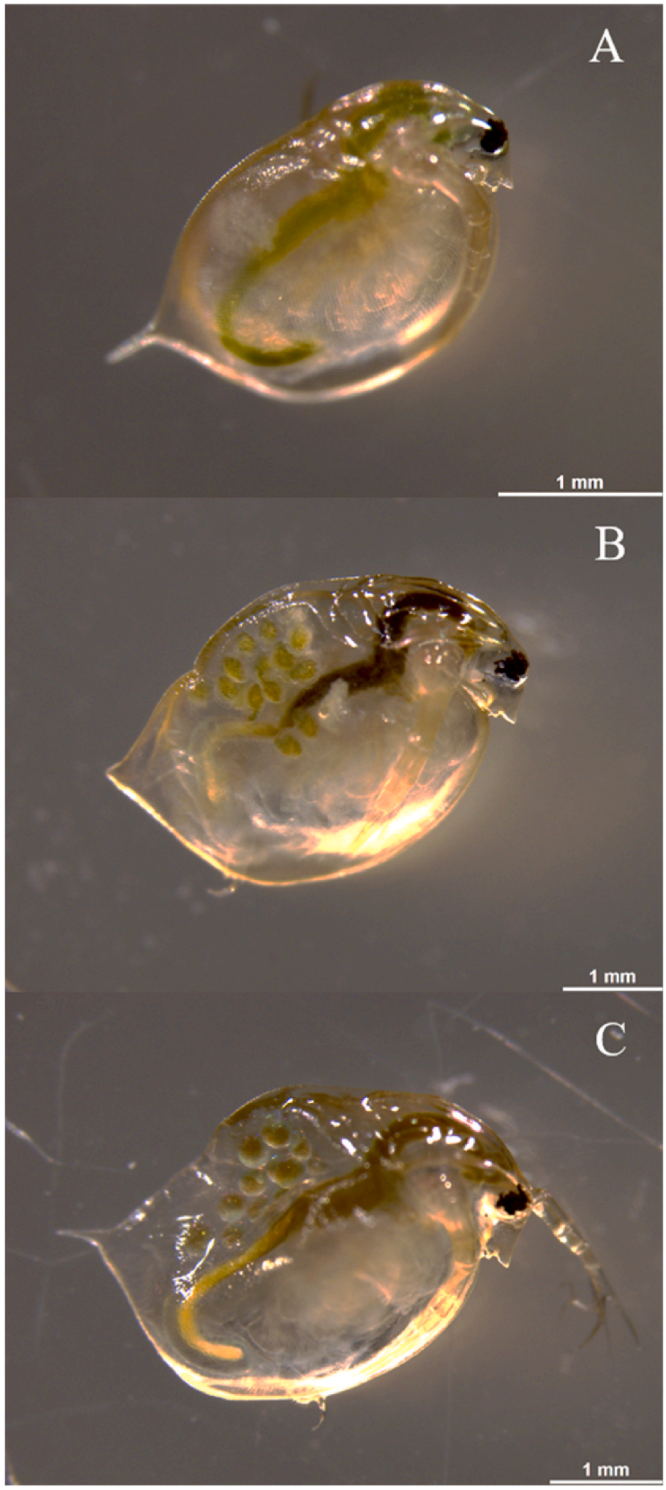

Fig. 3. microplastics (MPs) made of Plasmix-based material (Px-MPs, panel B) or Additivated Plasmix-based material (APx-MPs, panel C) in the digestive tract of 21-days old *D. magna* individuals. PX-MPs and APx-MPs filled the proximal part of the digestive tract of individuals (brownish/dark area in the proximal part of the digestive tract in B and C panel). An individual from the control group is reported in panel A.

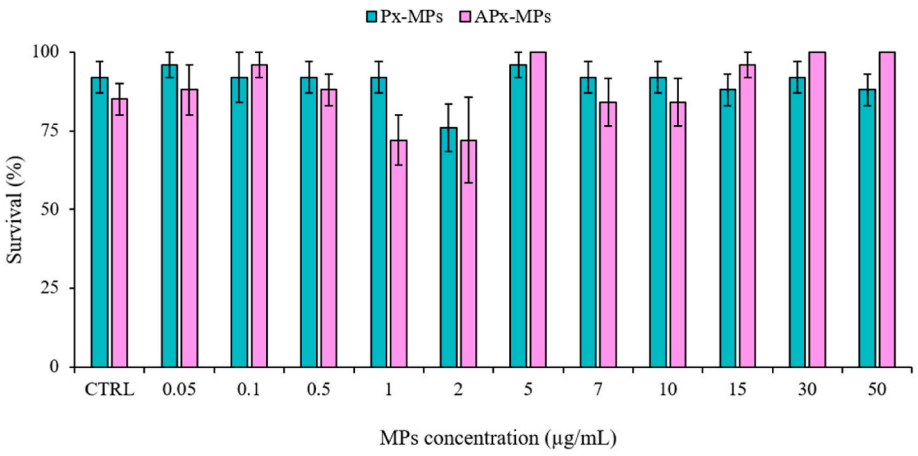

Fig. 4. survival of daphnids at the end of the exposure to increasing concentrations (range 0.05–50 µg/mL) of Px-MPs and APx-MPs.

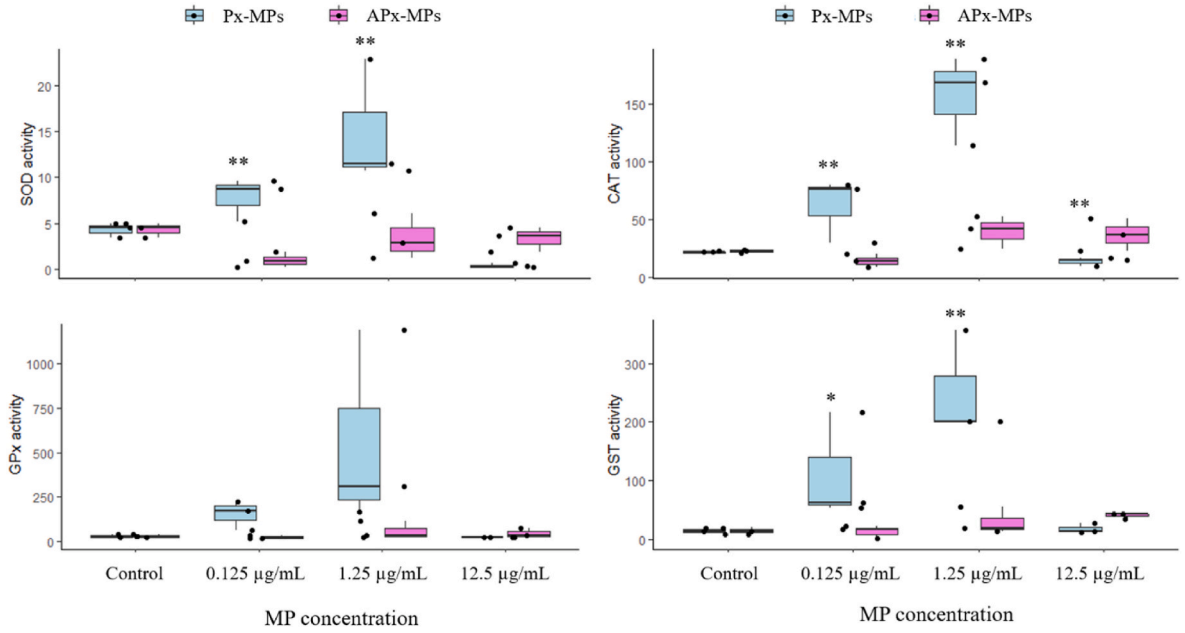

Fig. 5. box-plots of the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) measured in 21-days old *Daphnia magna* individuals after the exposure to microplastics originated from Plasmix-based material (Px-MPs) and Additivated Plasmix-based material (APx-MPs). Asterisks above the box-plots represent significant differences between the treatment and the corresponding control group (*P < 0.05; **P < 0.01).

were noted on the number of reproductive events, which was affected by the exposure concentration ($F_{2,75} = 16.375$; $P < 0.001$), but not by the MP type ($F_{1,75} = 0.306$; $P = 0.581$) and concentration × MP type interaction ($F_{2,75} = 2.823$; $P = 0.065$). Individuals exposed to 0.125 µg/mL and 1.25 µg/mL of MPs, independently of their type, showed a lower number of reproductive events compared to control conspecifics ($P < 0.001$ for both the cases).

4. Discussion

Our study showed that the exposure to MPs originated from Plasmix-based materials did not induce acute effects, but can alter the oxidative status and induce changes in the reproductive success towards the freshwater crustacean *Daphnia magna*.

The short-term exposure to Px-MPs and APx-MPs induced only a slight mortality of daphnids, with survival higher than 75% in all the tested concentrations. Similarly, very low mortality occurred in treated individuals over the 21 days of exposure performed to investigate the onset of sub-lethal effects, with mortality remaining lower than 10% in all the experimental groups. These results aligned with previous studies, which showed that the exposure to differently sized MPs made of single polymers, including those prevalent in Plasmix such as PE, PP and PS, did not affect the survival of *D. magna* individuals (e.g., Canniff and Hoang, 2018; De Felice et al., 2019; Parolini et al., 2023). The lack of acute effects following short-term exposure (i.e., 48 h) could be related to the limited ingestion of Px-MPs and APx-MPs of daphnids, as confirmed by our microscopy analyses showing no items in their digestive tract (data not shown). The lack of ingestion by daphnids can be reasonably due to the large size of administered items. In fact, although more than the 80% and the 10% of Px-MPs and APx-MPs were lower than 250 µm, they exceed the typical size range of food particles that can be ingested by *D. magna*, at least at its juvenile stages, which has been estimated ranging between 700 nm and 70 µm (Gophen and Geller, 1984). In contrast, adults were able to ingest MPs from both the materials, mainly of Px-MPs, corroborating previous findings obtained on the same model species after the administration to polyethylene MPs in the 10–106 µm size range (Frydkjær et al., 2017) and up to 250 µm in size (Kokalj et al., 2018). Thus, the slight mortality observed during

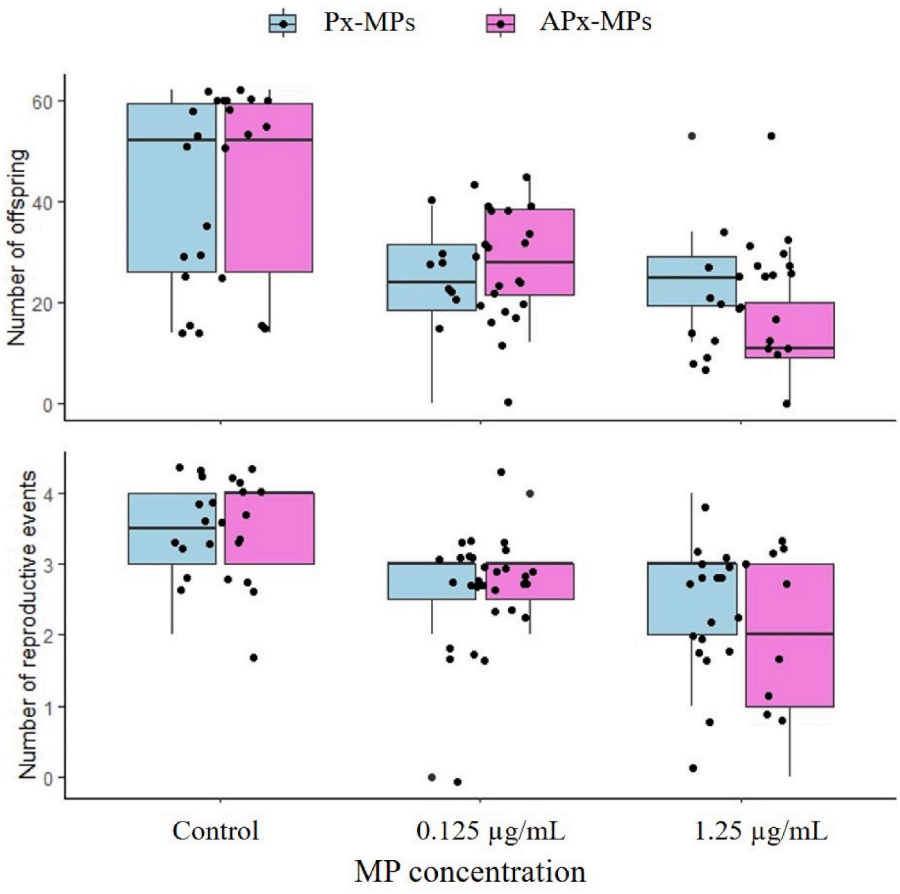

Fig. 6. box-plots of number of offspring and number of reproductive events counted in *Daphnia magna* individuals after 21 days of exposure to microplastics originated from Plasmix-based material (Px-MPs) and Additivated Plasmix-based material (APx-MPs).

short-term and long-term exposure could likely not primarily due to MP ingestion, but rather to interactions between MPs and the body or appendages of daphnids (De Felice et al., 2024) or, alternatively, to the release of chemicals into the water.

These factors likely contributed to the onset of sub-lethal effects observed in adults at the end of the 21 days of exposure to increasing concentrations of Px-MPs and APx-MPs. Significant imbalances of the oxidative status was observed in individuals exposed to Px-MPs, but not to APx-MPs. A bell-shaped modulation in the activity of antioxidant and detoxifying enzymes was observed. Specifically, the significant increase of SOD activity observed after 21 days of exposure to 0.125 and 1.25 µg/mL of Px-MPs followed by the inhibition observed as the consequence of 12.5 µg/mL exposure suggested an overproduction of superoxide anion. On one hand, the activation of SOD observed at low concentrations confirmed the initiation of the dismutation of superoxide anions into hydrogen peroxide, while on the other hand, the inhibition observed at the higher concentration suggests an excessive production and accumulation of radicals that the organism could not effectively neutralize. The bell-shaped trend of SOD follows the typical response of enzymes after the exposure of the organism to a xenobiotic over the time and/or in response to increasing exposure concentrations (Parolini et al., 2015, 2016; 2024). Thus, the inhibitory effect and/or the negative feedback caused by the production of hydrogen peroxide (Vlahogianni and Valavanidis, 2007) induced by the exposure to the highest Px-MPs concentration could explain the lower SOD activity compared to the lower tested concentrations. This effect may be associated with an excess of pro-oxidant molecules that compromise the functions of the affected individuals, inhibiting enzyme synthesis and/or promoting the inactivation of antioxidant enzymes (Verma and Dubey, 2003). The similar bell-shaped trend observed for CAT and GPx should confirm the overproduction of hydrogen peroxide in *D. magna* individuals exposed to increasing concentrations of Px-MPs. Indeed, CAT and GPx play a complementary role in the detoxification of hydrogen peroxide, with CAT being predominantly activated in response to high levels of this pro-oxidant molecule (Pereira et al., 2013). Whilst the increase in the activity of both the enzymes found in response to lower concentrations suggested that the antioxidant defences of the organisms were able to counteract the overproduction of hydrogen peroxide, the inhibition observed at the end of the exposure to the highest tested concentration confirmed an excess that was not tackled. In addition, a similar bell-shaped trend was also observed for the activity of the detoxification enzyme GST, which plays a pivotal role in the elimination of lipid hydroperoxides (Cheng et al., 2020). Overall, the inhibition of all the enzymes observed after the exposure to the highest tested concentration suggested that treated organisms were not able to counteract the overproduction of pro-oxidant molecules. Consequently, they suffered an oxidative stress condition, which results in the onset of oxidative damage to cellular macromolecules. The significant increase in lipid peroxidation levels observed only at the end of the exposure to highest tested concentration confirmed that the exposure to Px-MPs induced oxidative stress to *D. magna*. Interestingly, only the exposure to Px-MPx promoted the onset of an oxidative stress condition, while APx-MPs did not affect any biochemical response. These findings could be due to different factors related to the size and the composition of MPs. Being larger than Px-MPs, APx-MP could be less ingested by crustaceans and be less reactive into the organism body compared to their non-additivated counterpart. Alternatively, GC-MS analyses showed that Px-MPs released in the water more compounds and in higher amount

compared to APx-MPs. These differences could be due to the different procedure implemented to create the Plasmix-based materials. Indeed, Px-MPs originated from a Plasmix-based material created through a single extrusion process at a temperature ranging between 200 °C and 240 °C. In contrast, APx-MPs derived from a materials that experienced two extrusion processes (the second one was necessary to integrate the additive to the naïve material) at higher temperatures (ranging between 220 °C and 260 °C). Thus, we can suppose that during the production processes of the Additivated Plasmix-based material, the most volatile chemicals evolve and, consequently, the total migration was lower compared to those from naïve Plasmix-based one.

Our results disagreed a previous study in which the same Px-MPs and APx-MPs were administered to the terrestrial earthworm *Eisenia foetida* (De Felice et al., 2024). Indeed, independently of the MP type, despite an efficient ingestion, neither a modulation in the oxidative status nor the increase in lipid peroxidation occurred in treated earthworms compared to controls. This discrepancy could be due to the different exposure methods, including time and concentration of exposure, and/or sensitivity to MPs, with cladocerans that resulted as more sensitive than earthworms.

Although only Px-MPs induced significant biochemical effects, the exposure to both the MP types caused a significant decrease in the reproductive success of *D. magna* individuals. These results disagreed those observed in our previous experiment on earthworms, whereby no alteration of reproductive output (i.e., the number of cocoon) was observed (De Felice et al., 2024). The reproductive impairment occurred at the end of the exposure to both the tested concentrations should suggest that over a long-term exposure, cladocerans can intake an amount of MP able to induce intestinal blockage and food intake obstruction (Yin et al., 2023). Therefore, the energy allocated to the reproduction might decrease because the individual needs to maintain normal growth and to overcome the adverse physiological and biochemical responses occurring when energy reserves are limited (Zhao and Wang, 2011). Moreover, the MP exposure can lead to cell damage and increase in metabolic costs, leading to reproductive impairments in *D. magna* (Saebelfeld et al., 2017; Aksakal, 2020). A large number of studies agreed our findings, showing that the exposure to MPs having different size and made of different polymers impaired the reproduction of *D. magna* (e.g., Lyu et al., 2021; Jaikumar et al., 2018; An et al., 2021; Schür et al., 2021). Although the exposure to Px-MPs and APx-MPs resulted in a slight effect on the survival of *D. magna*, the changes in the features of individual life history, such as the reproductive success, could significantly influence the species' population dynamics and potentially threaten the long-term viability of populations under natural conditions (Pan et al., 2020). These results suggest that potential release of MPs caused by the fragmentation or degradation of Plasmix-based materials in the environment might pose a risk for the population of *D. magna*.

5. Conclusion

Our study showed that MPs originated from naïve (Px-MPs) and additivated Plasmix-based materials (APx-MPs) were efficiently ingested by *D. magna* individuals, returning differential responses at sub-individual level. Indeed, only Px-MPs were able to induce an oxidative stress situation, mainly in individuals exposed to the highest tested concentration. This discrepancy could be due to the release of chemicals from the MP types, which originated from Plasmix-based materials created with different processes. However, independently of the sub-individual effects, the exposure to both the concentrations of either the MP types caused significant impairments of the reproductive success of cladocerans. These results support the hypothesis that the ingestion and the interaction of MPs into the organisms, independently of their size and polymer composition, can affect food intake and energy allocation to different functions. Overall, our findings suggest that MPs from Plasmix-based materials could induce adverse effects at different levels of the biological organization in a freshwater model species. On these bases, long-term exposures (i.e., longer than 21 days) and/or multi-generational experiments, should be necessary to complete the assessment of risk associated with the Plasmix-based materials and related MPs to aquatic organisms. In addition, further experiments should be performed to explore the mechanism of explore the mechanisms of toxicity of MPs from Plasmix-based materials, focusing on investigating how chemical and physical properties can affect biological effects. Lastly, considering that the exposure to APx-MPs induced negligible adverse effects, at least at sub-individual level, compared to Px-MPs, further improvements of chemical recycling of this mixture of plastic waste should be implemented to increase both the chemical-physical features of the materials and their environmental safety.

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CRediT authorship contribution statement

Marco Parolini: Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Beatrice De Felice:** Writing – review & editing, Investigation, Data curation. **Stefano Gazzotti:** Writing – review & editing, Investigation. **Maddalena Roncoli:** Investigation. **Eleonora Conterosito:** Writing – review & editing, Investigation. **Marysol Ferretti:** Investigation. **Marco Aldo Ortenzi:** Writing – review & editing, Conceptualization. **Valentina Gianotti:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marco Parolini reports financial support was provided by University of Milan. Marco Parolini reports a relationship with University of Milan that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125146.

Data availability

Data will be made available on request.

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