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Polystyrene microplastic particles in combination with pesticides and antiviral drugs: Toxicity and genotoxicity in *Ceriodaphnia dubia*☆

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ABSTRACT

Freshwater ecosystems are recognized as non-negligible sources of plastic contamination for the marine environment that is the final acceptor of 53 thousand tons of plastic per year.

In this context, microplastic particles are well known to directly pose a great threat to freshwater organisms, they also indirectly affect the aquatic ecosystem by adsorbing and acting as a vector for the transport of other pollutants ("Trojan horse effect"). Polystyrene is one of the most widely produced plastics on a global scale, and it is among the most abundant microplastic particles found in freshwaters. Nevertheless, to date few studies have focused on the eco-genotoxic effects on freshwater organisms caused by polystyrene microplastic particles (PS-MPs) in combination with other pollutants such as pharmaceuticals and pesticides.

The aim of this study is to investigate chronic and sub-chronic effects of the microplastic polystyrene beads (PS-MP, 1.0 μm) both as individual xenobiotic and in combination (binary/ternary mixtures) with the aciclo-guanosine antiviral drug acyclovir (AC), and the neonicotinoid broad-spectrum insecticide imidacloprid (IMD) in one of the most sensitive non-target organisms of the freshwater food chain: the cladoceran crustacean *Ceriodaphnia dubia.* Considering that the individually selected xenobiotics have different modes of action and/or different biological sites, the *Bliss independence* was used as reference model for this research. Basically, when *C. dubia* neonates were exposed for 24 h to the mixtures during Comet assay, mostly an antagonistic genotoxic effect was observed. When neonates were exposed to the mixtures for 7 days, mostly an additive chronic toxic effect occurred at concentrations very close or even overlapping to the environmental ones ranging from units to tens of ng/L for PS-MPs, from tenths/hundredths to units of μg/L for AC and from units to hundreds of μg/L for IMD, revealing great environmental concern.

1. Introduction

The plastics production increased from 1.5 million metric tons (1950) to 359 million metric tons (2018) and, of this total, 61.8 million metric tons were produced in Europe (Leal Filho et al., 2021). In the environment, plastics are mainly subjected to abiotic degradation (UV, mechanical abrasion, hydrolysis) with consequent fragmentation and formation above all of microplastic particles (MPs, diameter <5 mm) that waste water treatment plants (WWTPs) are not totally capable of trapping trough conventional filtration systems (Browne et al., 2011). In detail, 68–81% of microplastic particles present in the sea (Song et al., 2017) are represented by "secondary" microplastic particles deriving from the fragmentation of large plastic materials such as plastic bags and bottles (Lassen et al., 2012), while 15–31% (Browne, 2015) is represented by "primary" microplastic particles intentionally added, for example, in cosmetic products such as scrubs, shampoos, toothpastes (de Sá et al., 2018).

Therefore, the marine environment is the final acceptor of 53 thousand tons of plastic per year: 4% comes from rivers, 18% from marine activities such as fishing, aquaculture and navigation and 78% from

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coastal activities (Stefanini et al., 2021). Despite the growing number of studies on the effects of MPs in the marine environment, to date few studies have focused on their presence in freshwater ecosystems, although they are also recognized as a non-negligible source of plastic contamination for the marine environment (De Felice et al., 2019). Koelmans et al., 2019 pointed out that MPs are found in fresh and drinking water at concentrations spanning ten orders of magnitude ($1 \cdot 10^{-5}$-$1 \cdot 10^{5}$ p/L) and polystyrene, according to Di and Wang (2018), is among the most abundant microplastic particles found in fresh waters.

Moreover, it has been claimed that MPs may absorb and act as a vector for the transport ("Trojan horse effect") of other pollutants like heavy metals, highly hydrophobic contaminants (e.g. persistent organic pollutants, polychlorinated biphenyls, polycyclic aromatic hydrocarbons), pharmaceutical active compounds, antibiotics, UV filtering substances, flame retardants, organic pesticides promoting their bioaccumulation and biomagnification through the food chain (Menendez- Petriza and Jaumot, 2020; Santos et al., 2021). Many factors influence the interactions between MPs and pollutants: MPs' properties (e.g. density, size, shape, polymer type, surface area available, crystallinity), chemical properties of pollutants (e.g. hydrophobicity, molecular weight and size, pk_a, K_{ow}) and environmental factors (e.g. salinity, pH, ionic strength, temperature) (Hüffer and Hofmann, 2016; Muñoz-Vélez et al., 2018; Menendez- Petriza and Jaumot, 2020; Kumar et al., 2022). The mechanism of interaction between MPs and pollutants not only depends on the characteristics of the particles mentioned above but also on the chemical structure of the pollutants, in particular the functional groups and the chemical bonds that it can establish with other molecules (Wang et al., 2020).

To date, conflicting data on the interaction of MPs and other pollutants as well as on their effects on freshwater organisms is available, but still the simultaneous exposure of aquatic organisms to these toxicants is currently one of the most hazardous threats to the aquatic environment (de Sá et al., 2018; Xiang et al., 2022).

In detail, MPs can allow the adsorption of pharmaceutical products (Liu et al., 2020) and pesticides (Fu et al., 2021) so that it is challenging the study of possible chronic and genotoxic effects of MPs in combinations with the former pollutants in freshwater organisms of the food chain.

In this research, attention was paid to polystyrene, one of the most widely produced plastics on a global scale as well as one of the most commonly found plastics in the environment (Magni et al., 2018). The size of the microplastic particles of polystyrene mainly present in the aquatic environment and studied so far varies from 1 μm to 500 μm (Hidalgo-Ruz et al., 2012; Eitzen et al., 2020; Yoganandham Suman et al., 2020). The selected polystyrene is a monodispersed aqueous solution containing $3.24 \cdot 10^{10}$ particles/mL with a diameter of 1.0 μm, borderline between nano- and microplastics and of high environmental interest because zooplankton species are not able to distinguish it from phytoplankton during the normal feedings and swimming activities (Nugnes et al., 2022). The pollutants chosen for studying their adsorption to polystyrene through binary and ternary combinations were the antiviral acyclovir and the pesticide imidacloprid. Acyclovir is an acicloguanosine, an acyclic analogue of guanosine. It inhibits viral DNA replication following initial phosphorylation mediated by viral thymidine kinase and subsequently by cellular kinases so that the acyclovir triphosphate formed is incorporated by the viral polymerase into the nascent DNA molecule allowing its synthesis to terminate. Only 15–20% of the administered dose of acyclovir is metabolized or bioaccumulated in the human body, while the remaining 80–85% is released into the aquatic environment because it is only partially retained by WWTPs. Its concentration in surface waters ranges from tens to thousands of ng/L (Gupta et al., 2021). Bradley et al. (2014) measured the highest concentration of acyclovir equal to 1.59 μg/L in the Fourmile Creek tributary of the Des Moines River in the United States, while Prasse et al. (2010) demonstrated that its concentrations in Ruhr and in Hessian rivers were 0.020 μg/L and 0.190 μg/L, respectively. In Pearl River Delta in China, Peng and co-authors (2014) reported the concentration of acyclovir equal to 0.113 μg/L.

Imidacloprid, a neonicotinoid broad-spectrum insecticide, is a halogenated heterocyclic compound considered a potential substitute for the organophosphorus pesticide diazinon, suspended in many countries (Jemec et al., 2007), and thus becoming the world's best-selling insecticide in the early 2000s (Tomizawa and Casida, 2005). Chen et al., 2010 demonstrated that imidacloprid can contaminate ground and surface waters through leaching, run-off, and atmospheric drift. Detectable concentrations of imidacloprid in surface waters are from 0.001 to 320 μg/L (Morrisey et al., 2015). Some of the highest reported imidacloprid concentrations were detected up to 320 μg/L in Dutch agricultural surface waters (Van Dijk et al., 2013). In Australia, imidacloprid was detected at concentrations reaching 4.6 μg/L after rainfall events from rivers draining agricultural fields (Sanchez-Bayo and Hyne, 2014). In California imidacloprid was detected in surface water samples (from agricultural regions) at concentrations up to 3.29 μg/L (Starner and Goh, 2012). De Liguoro et al., 2014 detected imidacloprid in Italian samples derived from ground and surface waters from different areas of Veneto region at concentrations ranging from 0.003 to 0.008 μg/L.

Although the concentrations of the selected xenobiotics in freshwater systems are rather low, chronic exposures of organisms as well as alterations in their genetic material may be of environmental concern, especially when they occur in mixtures.

The impact of combined toxicants can be expressed as additivity, synergism, potentiation, or antagonism.

Generally, molecules with same modes and biological sites of action can act in mixture in line with the CA model (*Loewe additivity or iso-additivity*), which assumes that all components of the mixture behave as if they are simple dilutions of one another (Loewe and Muischnek, 1926).

This model calculation is carried out according to the following equation:

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} \tag{1}$$

where c_i is the concentration for chemical "*i*" in the mixture leading to the effect "x", ECx_i is the concentration of the component *i* inducing the same effect (x) when applied singly.

On the other hand, molecules with different modes and/or biological sites of action cannot be considered as dilutions of each other and may rather act in mixture according to the IA model (*Bliss independence or heteroadditivity*) (Katsnelson et al., 2011). IA is expressed by the following equation:

$$E(C_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(C_i)) \tag{2}$$

where $E(C_{mix})$ is the effect of the mixture at a concentration $C_{mix} = (c_1, \ldots, c_n)$;

$E(c_i)$ are the effects that the single components would determine if applied individually at their concentration in the mixture. A precondition for this concept to be applied is that the responses E() are on a probability scale or can be expressed as an effect relative to a natural maximum E'_{max} ($E() = E'()/E'_{max}$), where E′ are the original responses.

Chemical combinations may follow or deviate from predictions of these reference models (Ermler et al., 2014; Mater et al., 2014).

Considering that the individually selected xenobiotics have different modes of action and/or different biological sites (Bliss, 1939), the *Bliss independence* (independent action) was used as reference model for this research.

The present study aims to investigate the chronic toxicity and DNA damage performing the 7-day inhibition test and the Comet assay, respectively. Xenobiotics individually and in binary/ternary mixtures were tested on the cladoceran crustacean *Ceriodaphnia dubia*, one of the most sensitive non-target primary consumer of the trophic chain selected for its high reproductive capability, genetic uniformity and short lifespan.

2. Materials and methods

2.1. Test compounds

Polystyrene monodispersed microplastic particles (PS-MP, Product number: 72 938) with analytical standard size of 1.0 μm, particle specific gravity of 1.05 g/cm^3, and a solid content of 2%WT were supplied by Sigma Aldrich (Milano, Italy) as aqueous suspension (21 000 mg/L; $3.24 \cdot 10^{10}$ particles/mL). Solutions were freshly prepared by mixing the appropriate volumes of the aqueous suspension and standard synthetic media.

Acyclovir (AC; CAS: 59 277-89-3) and imidacloprid (IMD; CAS: 138 261-41-3) were purchased from Sigma Aldrich (Milano, Italy). Test solutions were freshly prepared by dissolving powders in Milli-Q water to obtain stock solutions, and test solutions were prepared diluting stock solutions with standard synthetic media. In addition, the stock solutions were prepared at concentrations (500 and 10 mg/L for AC and IMD respectively) far below the solubility limit (1600 and 610 mg/L for AC (22–25 °C) and IMD (20 °C) respectively) to ensure that the powders were completely dissolved in water. The detection of nominal and actual concentrations was performed by Total Organic Carbon Analyzer (TOC-L CSN, Shimadzu, Kyoto, Japan) considering light and/or dark exposure conditions.

2.2. Determination of the optimal dosing regimen of combined exposures

Before starting experiments of mixtures, *C. dubia* clone was exposed to each chemical from five to ten concentrations. For reproductive toxicity, dilutions (obtained after range findings tests) started from 90, 6400 and 10 μg/L for PS-MP, IMD and AC respectively, with a dilution factor equal to 3.2, 2 and 3.2, correspondingly. Tests were carried out in three to four independent experiments (details are reported in Supplementary material). For genotoxicity, the dilutions followed a geometric progression of 10 starting from 8500, 7000 and 200 000 μg/L (concentrations not inducing a mortality greater than 20%) for PS-MP, IMD and AC, respectively. Genotoxicity tests were performed in two independent experiments. Thus, for each independent assay, 2 slides were prepared in duplicate per each concentration (4 slides/concentration), measuring the tail intensity in 50 randomly selected cells/slide.

Subsequently, dose-response curves for reproductive toxicity and genotoxicity data of single chemicals were computed. Log logistic functions were found to be the best fit:

- for reduction in *C. dubia* offspring response at concentration x:

$$R(x) = A(x/\mu)^{\beta} \Big/ \left(1 + (x/\mu)^{\beta}\right) \text{ and } E(x) = R(x) \Big/ A \tag{3}$$

- for % DNA in tail:

$$R(x) = B + A(x/\mu)^{\beta} \Big/ \left(1 + (x/\mu)^{\beta}\right) \text{ and } E(x) = (R(x) - B) \Big/ A \tag{4}$$

where E(x) is for use in the IA reference model and x is the concentration of the compound.

A and B are the upper and lower asymptote, μ is the EC50, and ß is the slope parameter.

Three optimally discriminating endpoints were selected for both long-term and genotoxicity assays for which experiments were designed to result in expected equal effects of combined exposures under the IA assumption at the three specified effect levels: 35, 50, 65% chosen for the reduction of *C. dubia* offspring and 10, 15, 22% DNA in tail. For the chronic toxicity assay, these effect sizes were chosen based on the pooled standard deviation of effects and are ±SD from the median effect size (50% offspring reduction). For the genotoxicity assay, effect sizes corresponding to ½ EC50, EC50 and 2•EC50 were chosen resulting in target values of 10%, 15%, and 22% DNA in tail.

For Bliss independence (independent action, heteroaditivity), the procedure to determine the mixture concentrations expected to result in the target effect sizes is similar to the isobologram method applied in the case of Loewe additivity. However, since Bliss independence is specified on effects not doses, it proceeds in two steps. First, the isoeffect curves (Fig. S1) are drawn as the collection of all single exposure effect sizes of two (or more) components that are expected to result in the same specified total effect. Since only reversible concentration – response functions are allowed for application in reference models, for each effect size on the isoeffect curves, there is a unique dose found by inverting the concentration - response function. In contrast to Loewe additivity, in case of Bliss independence, the isobolograms are no longer straight lines in the case of two components or plains in the case of three (see Fig. S2).

From equation (2) it follows for two substances A and B:

$$E(c_B) = (E(c_{mix}) - E(c_A)) / (1 - E(c_A)) \tag{5}$$

Hence, the relationship is non-linear. Given the standardized two-parameter log-logistic function by insertion into the equation above, and solving for concentration c_B the isobolograms can be constructed showing the collection of points in the c_A-c_B plane expected to result in the same effect $E(c_{mix})$ under the reference model (Fig. S2).

$$E(x) = (x/\mu)^{\beta} \Big/ \left(1 + (x/\mu)^{\beta}\right) \tag{6}$$

2.3. Mixture preparation

The concentrations of polystyrene and acyclovir and/or imidacloprid combined in binary/ternary mixtures at the intended effect levels for reproductive toxicity (Tables S1 and S2) and for genotoxicity (Table S3).

The computed concentrations expected to result in the specified effect sizes under three mixture assumptions for binary mixtures (1:1, 1:3, and 3:1) and 1:1:1 for the ternary mixture. In detail PS-MP + AC, PS-MP + IMD and PS-MP + AC + IMD were tested in chronic toxicity assays, while only PS-MP + AC was tested in genotoxicity assays because imidacloprid genotoxic effect percentages, resulting in equal effects of combined exposures, could not be reached due to its acute individual toxicity.

2.4. Reproductive toxicity testing

C. dubia reproductive toxicity test was conducted in line with the ISO 20665 (2008). The assay was performed over 7 days at 25 °C, starting from neonates (organisms less than 24 h). The procedure, in line with Nugnes et al. (2022) is reported in detail in Supplementary materials.

Individual xenobiotics were tested as well as their mixtures for which the concentrations used were expected to reveal the inhibition of reproduction at the effect levels of 35, 50 and 65%.

2.5. Genotoxicity testing

Single cells isolated from whole *C. dubia* neonates were used to perform the comet assay. 20 neonates were exposed to individual xenobiotics and to their mixtures for which the concentrations expected to reveal the effect levels of 10, 15 and 22% DNA in tail were tested. The procedure was performed according to Nugnes et al. (2022) and all details are reported in Supplementary materials.

2.6. Combined effects data analysis

ToxRat Professional software was used to obtain the offspring reduction percentages, which were arcsine transformed (to remove the correlation between mean and standard deviation for data that is given as percentages). Combined results were statistically compared (using analysis of variance, ANOVA) to those obtained by single exposures at the same calculated effect sizes. Bartlett tests were used to test homogeneity of variances, while Kolmogorov–Smirnov tests with Lilliefors' corrected p-values were used to test normality of residuals. All analyses were conducted using Statistica10.0 (StatSoft Inc., 2011; Tulsa, OK, USA). p-values<0.05 were considered significant for hypothesis testing.

3. Results

No appreciable difference (0.2–3%) between nominal and actual concentrations was highlighted using TOC-L CSN. When the actual concentrations are less than 20% of the nominal ones they can be considered very close (Li, 2012). Therefore, the effective concentrations are reported as nominal ones.

3.1. Chronic toxicity assessment

Results of individual PS-MP, IMD and AC are expressed as EC50s and reported in Table 1, while their concentration/effect curves are depicted in Fig. 1.

The most chronically active xenobiotics were AC and PS-MP with EC50 values in the order of hundredths and units of μg/L, respectively, and with concentration/effect curves depicted in the left side of Fig. 1, with effective concentrations of the whole trend going from thousandth to dozens

μg/L and from hundredths to dozens μg/L, for AC and PS-MP, respectively. Differently, IMD was the least effective xenobiotic, affecting *C. dubia* offspring at concentrations going from dozens to thousands of μg/L.

PS-MP, IMD and AC were in binary and ternary combinations, their concentrations were selected to result in the targeted effect sizes equal to 35%, 50% and 65% of the inhibition of *C. dubia* reproduction. When PS-MP and AC were mixed in 3:1 and 1:3, mean values for offspring reduction were 29.2, 49.2, and 64.4% for 3:1 and 19.3, 36.5, and 54.8% for 1:3 (Fig. 2), demonstrating that the increased amount of PS-MP caused greater inhibition of crustacean reproduction. In particular, in the 3:1 ratio and in the predicted percentages of 50% and 65%, the obtained effect sizes did not diverge from those expected, confirming heteroadditive interactions between PS-MP and AC. Bliss antagonism was observed only for the first effect size in the case of 3:1, and from the first and third effect sizes in the case of 1:3 ratio (Table 2).

Regarding the PS-MP + IMD binary mixture (Fig. 3), the obtained offspring reduction percentages were 35.1, 49.5, and 64.7% for the ratio 3:1, and 34.0, 49.2, and 64.7% for ratio 1:3, obtaining heteroadditive interactions for all the cases (Table 2).

When the ternary combination (PS-MP + IMD + AC, 1:1:1 ratio) was tested, it induced 32.8, 48.4, and 64.0% inhibition of the *C. dubia* reproduction (Fig. 4) with an heteroadditive interaction (Table 2).

Fundamentally, the chronic toxicity experiments revealed that in mixtures with the lowest concentrations of polystyrene a Bliss antagonistic effect occurred in the presence of acyclovir, while experiments

Table 1
Offspring reduction expressed as EC50 values in μg/L (95% confidence intervals in brackets) for polystyrene microplastic particles (PS-MP), imidacloprid (IMD) and acyclovir (AC).

PS-MP	IMD	AC
Offspring reduction (EC50, μg/L)		
1.68 (1.17–2.42)	1358 (1177–1566)	0.04 (0.03–0.06)

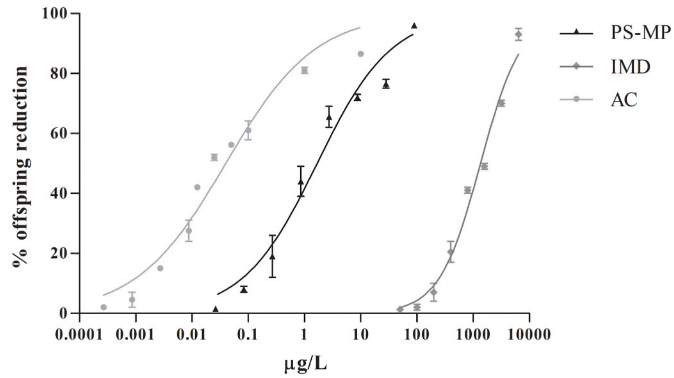

Fig. 1. Concentration/effect curves of polystyrene microplastic particles (PS-MP), imidacloprid (IMD) and acyclovir (AC) on *C. dubia*. Whiskers indicate standard deviations (SD), n = 3.

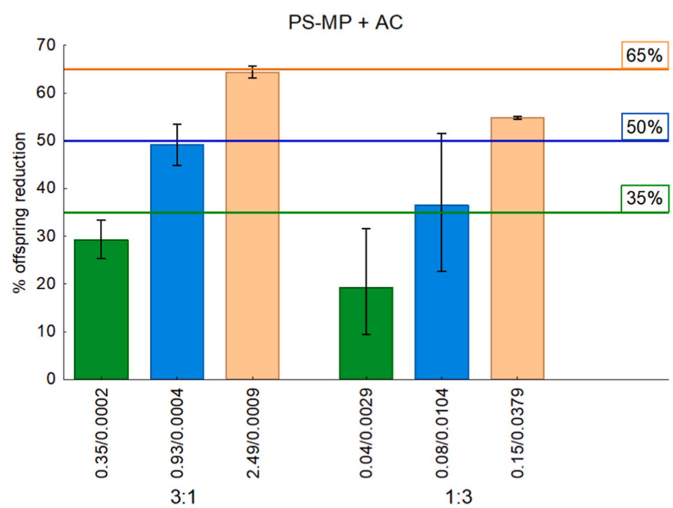

Fig. 2. Combined effect of polystyrene microplastic and acyclovir (PS-MP + AC) obtained by the reproduction of *C. dubia*. Bars represents means and whiskers 95% confidence intervals based on two independent tests. Horizontal lines represent the predicted effects (green 35%; light blue 50%; orange 65%) from Bliss independence. The concentrations of PS-MP/AC tested in mixtures are expressed in μg/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including imidacloprid met the expectations of Bliss independence (Table 2).

3.2. Genotoxicity evaluation

Genotoxicity results of individual exposures are reported in Fig. 5. In detail, PS-MP was the xenobiotic capable of causing the highest damage to DNA on single *C. dubia* cells with % DNA in tail from 2.1 ± 0.20 (0.85 μg/L) to 28.60 ± 1.27 (8500 μg/L). In addition, exposing single cells to AC, % DNA in tail values were from 0.39 ± 0.05 (20 μg/L) to 14.30 ± 0.31(200 000 μg/L). IMD was the only xenobiotic causing a weak DNA damage, reaching the maximum % DNA in tail equal to 2.85 ± 0.09 at the highest tested concentration (7000 μg/L) and, showing too low effects at higher concentrations and was not included in the combination experiments.

In Fig. 6, the concentration/effect relationship of the binary mixture (PS-MP + AC) is shown. The tested mixtures reached DNA in tail percentages of 8.6, 10.9, and 11.1% in the 1:1 effect ratio condition, suggesting an antagonistic effect, values of 8.9, 16.0, and 20.9% for the 3:1 ratio, deviating from Bliss independence only at the lowest effect target of 10% proposing antagonism at this level, and 10.4, 9.8, and 10.8%

Table 2
Results of analysis of variance of C. dubia offspring reduction. Target as offset. P-values from comparison of effect against zero.

Combination	Ratio	Target	**Deviation from target mean (95% CI)**	p-value	Interaction
PS-MP + AC	3:1	35%	−5.8 (−9.7; −1.7)	0.036	Bliss antagonism
		50%	−0.8 (−5.1; 3.5)	0.258	heteroadditive
		65%	−0.6 (−1.9; 0.7)	0.106	heteroadditive
	1:3	35%	−15.7 (−25.5; −3.4)	0.040	Bliss antagonism
		50%	−13.5 (−27.3; 1.6)	0.056	heteroadditive
		65%	−10.2 (−10.5; −9.9)	0.001	Bliss antagonism
PS-MP + IMD	3:1	35%	0.1 (−19.8; 23.3)	0.954	heteroadditive
		50%	−0.5 (−8.1; 7.2)	0.572	heteroadditive
		65%	−0.3 (−13.9; 12.2)	0.822	heteroadditive
	1:3	35%	−1.0 (−3.7; 1.8)	0.138	heteroadditive
		50%	−0.8 (−6.7; 5.1)	0.345	heteroadditive
		65%	−0.3 (−2.8; 2.2)	0.362	heteroadditive
PS-MP + IMD + AC	1:1:1	35%	−2.2 (−13.6; 10.3)	0.263	heteroadditive
		50%	−1.6 (−9.7; 6.5)	0.237	heteroadditive
		65%	−1.0 (−4.4; 2.3)	0.157	heteroadditive

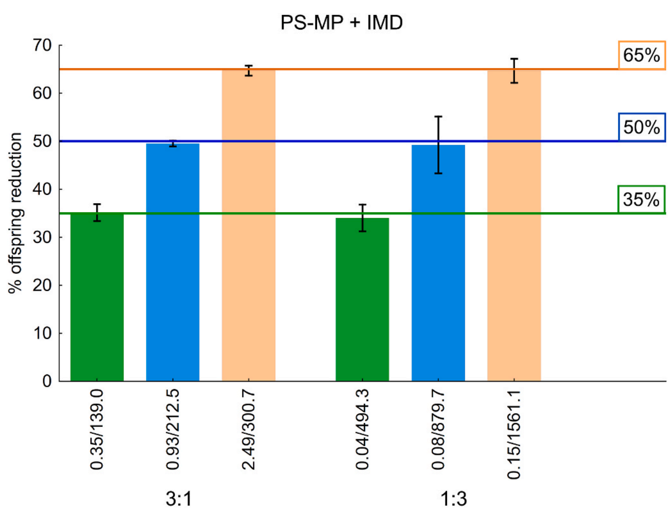

Fig. 3. Combined effect of polystyrene microplastic and imidacloprid (PS-MP + IMD) obtained by the reproduction of *C. dubia*. Bars represents means and whiskers 95% confidence intervals based on two independent tests. Horizontal lines represent the predicted effects (green 35%; light blue 50%; orange 65%) from Bliss independence. The values expressed as x/y are the concentrations of PS-MP/IMD in μg/L tested in the mixture. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DNA in tail at the 3:1 ratio, suggesting antagonism except for the lowest effect target of 10% (Table 3).

4. Discussion

Microplastic particles represent a global threat for the aquatic environment. The effects of the of polystyrene monodisperse micro particles on the health of freshwater organisms have been investigated in many aquatic species of the trophic chain, observing that the cladoceran *C. dubia* was the most sensitive organism to PS-MP (Nugnes et al., 2022). In detail, Nugnes and collaborators (2022), exposing this crustacean to PS-MP and using light microscopy, observed that microplastic particles were in *C. dubia* gut (already at concentrations of units of μg/L) likely due to indiscriminate ingestion of these particles during the swimming and feeding activities. Hence, daphnids are able to uptake and ingest small suspended particles (1–70 μm in size) from the water with the inability to distinguish between size and quality (Ebert, 2005; Wright et al., 2013; Lei et al., 2018).

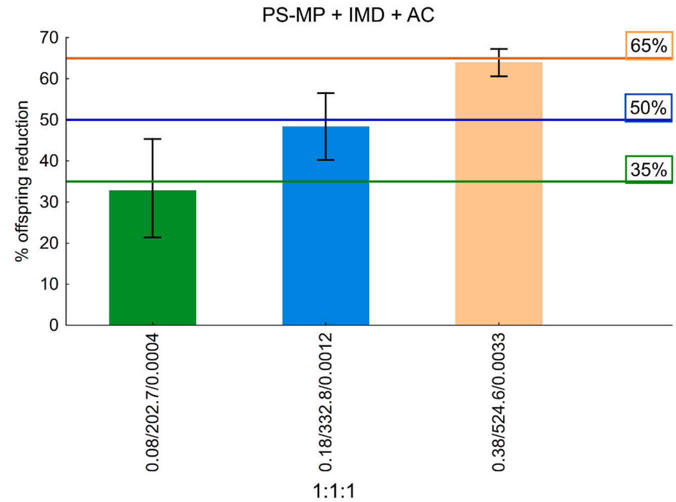

Fig. 4. Combined effect of polystyrene microplastic, imidacloprid and acyclovir (PS-MP + IMD + AC) obtained by the reproduction of *C. dubia*. Bars represents means and whiskers 95% confidence intervals based on two independent tests. Horizontal lines represent the predicted effects (green 35%; light blue 50%; orange 65%) from Bliss independence. The concentrations of PS-MP/IMD/AC tested in mixtures are reported in μg/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

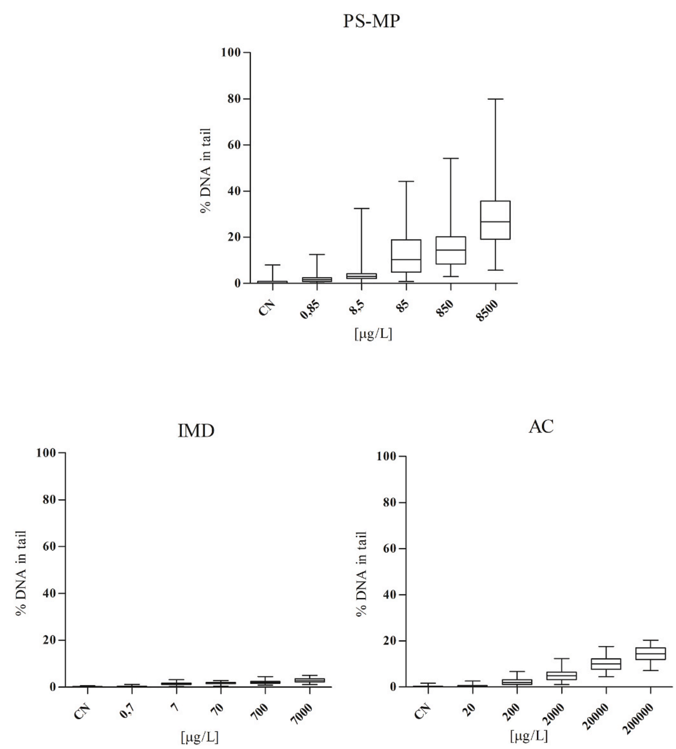

Fig. 5. Effects of polystyrene microplastic particles (PS-MP), imidacloprid (IMD) and acyclovir (AC) on induction of DNA strand breaks in *C. dubia* single cell gel electrophoresis (comet assay). Results are expressed as % DNA in tail. Concentrations of xenobiotics are reported in μg/L.

As reported by Rainieri et al. (2018), a serious and underestimated

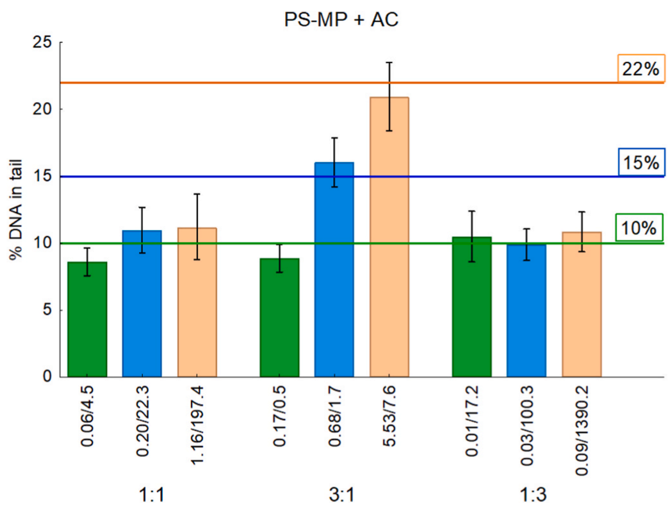

Fig. 6. Combined effect of polystyrene microplastic and acyclovir (PS-MP + AC) obtained by the comet assay. Bars represent means and whiskers 95% confidence intervals of % DNA in tail based on two independent experiments with two separate slides each with 50 cells evaluated. Horizontal lines represent the predicted effects (green 10%; light blue 15%; orange 22%) from Bliss independence. The concentrations of PS-MP/AC tested in mixtures are expressed in mg/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Results of analysis of variance of genotoxicity assay (% DNA in tail). Target as offset. P-values from comparison of effect against zero.

Combination	Ratio	Target	Deviation from target mean (95% CI)	p-value	Interaction
PS-MP + AC	1:1	10%	−1.4 (−2.4; −0.4)	0.024	Bliss antagonism
		15%	−4.1 (−5.7; −2.3)	0.006	Bliss antagonism
		22%	−10.9 (−13.2; −8.3)	0.001	Bliss antagonism
	3:1	10%	−1.1 (−2.2; −0.1)	0.042	Bliss antagonism
		15%	1.0 (−0.8; 2.9)	0.174	heteroadditivity
		22%	−1.1 (−3.6; 1.5)	0.265	heteroadditivity
	1:3	10%	0.4 (−1.4; 2.4)	0.519	heteroadditivity
		15%	−5.2 (−6.3; −4.0)	0.001	Bliss antagonism
		22%	−11.2 (−12.7; −9.7)	$<$0.001	Bliss antagonism

issue is the absorption of chemical pollutants to plastic particles with the risk of ingesting pollutants from plastics increasing the adverse effects due to the plastic or to the pollutants individually considered.

In this work, the combined eco-geno/toxicological effects of the 1 μm-polystyrene beads, the insecticide imidacloprid, and the antiviral drug acyclovir were explored in *C. dubia*. Before starting combined exposure experiments of PS-MP in mixtures with IMD and/or AC, we exposed *C. dubia* to single xenobiotics. When these xenobiotics were individually tested in *C. dubia* 24 h-neonates, AC and PS-MP were the most chronically active substances after 7 d-exposure, with the median concentration of offspring reduction in the order of hundredth and units of μg/L, respectively. PS-MP was the most geno-toxicologically active xenobiotic after 24 h-exposure, obtaining units/tens % DNA in tail at concentrations ranging from tenths to thousands of μg/L. According to Nugnes et al. (2022), PS-MP was able to reduce the *C. dubia* offspring and to cause DNA damage at concentrations in the order units of μg/L and in line with the findings of this study. Basically, the same authors explain that the offspring reduction and DNA lesions could occur due to an increase in cellular Reactive Oxygen Species (ROS) levels associated to the exposure to plastic particles. To the best of our knowledge, very little is known about chronic toxicity and genotoxicity of Almeida et al., 2019 found *C. dubia* chronic EC50 in the order of units of mg/L, findings higher than those obtained in this study in the order of hundredths of μg/L, while Tomicic et al., 2002 studied the genotoxic effect of Acyclovir on metabolically competent Chinese hamster ovary (CHO) cells that express the thymidine kinase gene of the herpes simplex virus type 1 (CHO-HSVtk), through the Comet assay demonstrating that, after 24 h of exposure and at a concentration of 22 521 μg/L, acyclovir was a highly genotoxic agent inducing single-strand breaks of DNA.

In the present research, IMD was the least toxic xenobiotic, without affecting *C. dubia* reproduction rate and DNA integrity, except to thousands of μg/L as in line with Agatz et al., 2014 who observed a reduced reproduction by up to 57% in *Daphnia magna* at concentrations equal or higher than 4000 μg/L. On the other hand, Ansoar-Rodríguez et al. (2015) exposed *Oreochromis niloticus* erythrocytes to IMD observing DNA damage at hundreds of μg/L and explaining that the toxic effects could be due to the increase in the lipid peroxidation and to the formation of ROS, which are able to affect genetic material.

In light of the above, the xenobiotics here studied may affect *C. dubia* along different modes of action (some of which are still unknown) and at different concentrations, and therefore the study of toxic effects caused by the interactions between PS-MP and other xenobiotics was performed by using independent action (Bliss independence) as reference model.

In detail, when PS-MP and AC were mixed (1:3 and 3:1 ratios), the *C. dubia* offspring reduction sizes obtained did not diverge from those expected highlighting heteroadditive interactions, while Bliss antagonism was observed only for the lowest effect size in the case of 3:1, and from the first and third effect sizes in the case of 1:3 ratio. When PS-MP and IMD were mixed the obtained offspring reduction percentages did not diverge statistically from those expected, obtaining heteroadditive interactions for all the studied effect sizes. Similarly, when PS-MP, IMD and AC were combined in ternary mixture there was a heteroadditive inhibition of the *C. dubia* reproduction. Therefore, in chronic toxicity experiments of mixtures with low concentrations of polystyrene caused a Bliss antagonistic effect in the presence of acyclovir, while experiments including imidacloprid showed heteroadditivity.

Binary combinations of PS-MP and AC were tested on *C. dubia* single cells for studying interactions on DNA damage and a Bliss antagonistic effect was detected whenever the concentration of AC was comparatively high. Hence, Bliss antagonism was observed in the 1:1 mixture, the lowest effect size in the 3:1 and the higher effect sizes in the 1:3 mixture experiments while in the conditions of PS-MP dominance heteroadditivity could not be rejected.

In the recent literature, limited and conflicting researches regarding the combined effects of micro/nanoplastics and other organic pollutants are reported. According to Huang et al. (2021), the combined toxicities of microplastic particles and organic pollutants on the aquatic organisms may lead to chemical-specific and species-specific interactions depending on complex factors like microplastic and pollutant properties and tested species. Mei et al. (2020) stated that additive/antagonist/synergic toxic effects between micro/nanoplastic particles and other pollutants could be explained assuming that plastic particles can behave as carriers for organic compounds changing the tissues of organisms that have ingested them.

Regarding the combined exposure to PS nanoparticles and drugs, Brandts et al. (2018) reported that this combination was able to induce significant downregulation in the expression of some genes for biotransformation, DNA damage, and cell-tissue repair in the gills of Mediterranean mussels. In 2018 Brandts and colleagues studied the effects of PS nanoparticles (110 nm, 0.05 mg/L) in combination with carbamazepine on hemocytes of the mussel *M. galloprovincialis* revealing that although the co-exposure caused genotoxicity and oxidative damage, the percentage of DNA in tail after exposure to the mixture was lower than that resulting from the exposure to the drug, suggesting an antagonistic effect. In Zhu et al., 2019, combining different types of MPs (polystyrene, polyethylene, polyvinyl chloride; 74 μm) and antibiotic drug (triclosan), observed an antagonistic effect on growth inhibition and oxidative stress in algae *S. costatum*; in the same year Zhang et al., 2019 studied the effects caused by polystyrene and roxithromycin (an antibiotic drug), demonstrating significant acute and chronic effects in *D. magna* with polystyrene microplastic of size 1 μm. More recently, Zhou et al. (2020) exposed the edible clam *Tegillarca granosa* to PS microplastic particles (500 nm) and veterinary antibiotics (oxytetracycline and florfenicol) indicating that microplastic particles aggravated the bioaccumulation of these antibiotics suppressing the clam glutathione-S-transferase activity as well as other detoxification processes. In addition, Shi et al. (2020) found synergistic effects of polystyrene and sertraline (antidepressant drug) with apoptosis of haemocytes and a reduction in function of the immune system; in 2021 Santos and collaborators reported that the interaction of microplastics and drugs could potentiate toxic effects on aquatic organisms.

Also regarding the effects of combined exposures of microplastic particles and pesticides, little is known, and conflicting information is present in literature. On one hand, some studies reported that the combined exposure of the microplastic particles and pesticides can increase the toxicity in aquatic organisms. Hence, Luo et al., 2021 demonstrated that PS and IMD affected the growth of zebrafish, the glycolipid metabolism with the oxidative stress-related biochemical parameters. In Felten et al., 2020 studied the effects of polyethylene microplastic particles combined with the pesticide deltamethrin on *Daphnia magna* and found synergic adverse effects on the survival, brood number and fertility. Zocchi and Sommaruga, 2019 observed a significant increase of mortality after exposure to polyethylene and/or polyamide microplastic and glyphosate in the crustacean *D. magna*. To the best of our knowledge the mode of action of this kind of combination is still unknown. Nevertheless, Sun and collaborators (2021) co-testing microplastics and the pesticide dufulin, observed an increase of oxidative stress with an increase of the content of malondialdehyde, superoxide dismutase, and a decrease of glutathione with alterations of different metabolic pathways including phenylalanine, glycine, serine and threonine metabolism as well as tyrosine and tryptophan biosynthesis in earthworms. In this context, microplastics may favour the accumulation of pesticides in earthworms causing an exacerbated oxidative damage (Sun et al., 2021). Surely, as explained by Birben et al. (2012), when organisms are stimulated by external factors, they try to generate free radicals so that the antioxidant defence system is able to remove free radicals protecting cells from oxidative damage. On the other hand, other studies showed that mixture MPs/NPs + pesticides could determine a decrease in the adverse effects on organisms (lethal effects and/or inhibition of physiological functions of organisms). In fact, organic pesticides (glyphosate, chlorpyrifos and bifenthrin) in combination with polystyrene microplastics could determine a decrease in toxic effects on the insect *Chironomus tepperi,* the cyanobacterium *Microcystis aeruginosa* and on the alga *Isochrysis galbana* (Zhang et al., 2018; Garrido et al., 2019; Ziajahromi et al., 2019). Similarly, Nobre et al. (2020) found a lower genotoxic effect in the oyster *Crassostrea brasiliana* after the exposure to polyethylene (150–250 μm) and triclosan compared to the exposure of the only microplastic particles.

However, to date why and whether antagonistic/additive/synergic interactions between micro/nanoplastic particles and organic pollutants occur still remains under debate (Huang et al., 2021). Therefore, it must be said that the impact of combined exposures of micro/nanoplastic particles and drugs and/or pesticides on aquatic organisms of the trophic chain remains a field that is still unexplored and studies are scarce and need to be increased. Although the modes of action of the co-tested xenobiotics in aquatic organisms is still largely unknown and knowledge needs to be deepened, what emerges from this study is of scientific concern. Basically, when *C. dubia* neonates were exposed for 24 to the binary mixtures during comet assay, mostly an antagonistic genotoxic effect was observed, but when neonates were exposed to mixtures for 7 days, an additive chronic toxic effect occurred. Suffice is to say that when PS-MP and IMD where combined in chronic toxicity assays, their mixture was able to induce the 50% of inhibition of reproduction of *C. dubia* at concentrations of individual drugs (tenths/hundredths of μg/L and hundreds of μg/L, respectively for PS-MP and IMD) at least one order of magnitude lower than that required to cause the same effect when the xenobiotics were exposed individually. The same consideration could be pointed out for the ternary mixture, able to induce a 50% inhibition of reproduction of *C. dubia* when PS-MP, AC and IMD where co-tested at concentrations in order of tenths of μg/L, units of ng/L and hundreds of μg/L, respectively for PS-MP, AC and IMD. Comparing these effect concentrations with those of the environment, it is to be remembered that in freshwaters PS-MPs concentrations ranged from units to tens of ng/L (Schirinzi et al., 2019), AC concentrations ranged from tenths/hundredths to units of μg/L (Prasse et al., 2010; Bradley et al., 2014; Peng et al., 2014), while IMD concentrations ranged from units to hundreds of μg/L (Starner and Goh, 2012; Van Dijk et al., 2013; Sanchez-Bayo and Hyne, 2014). Therefore, the effect concentrations of xenobiotics in combinations are very close or even overlapping to environmental concentrations, revealing that the long term exposure of *C. dubia* neonates to mixtures of polystyrene microplastic particles, acyclovir and/or imidacloprid is of great environmental concern.

When it comes to comparisons of studies applying combined exposures, actual concentrations may be of utmost importance, since the presented results indicate a subtle dependency of the type of interaction effect on the concentrations of the compounds and their ratio. Furthermore, it must be stressed that even under antagonistic interaction, the concentration of a compound in the mixture leading to a certain combined effect can be much lower than the concentration needed to produce the same effect when the substance is the sole exposure. For example, in the chronic toxicity experiments, the antagonistic interaction observed at the highest effect target of the 1:3 mixture exerted a 54.8% offspring reduction at concentrations of 0.15 and 0.0379 μg/L for PS-MP and AC, respectively. Such an effect is expected to occur at about 2 μg/L for PS-MP (i.e. at more than the tenfold concentration) and at 0.8 μg/L for AC (i.e. at more than the 20-fold concentration). Under many circumstances even an antagonistic interaction must be interpreted as increased danger of the combined exposure as compared to single exposure conditions. Therefore, conclusions drawn from combined exposure experiments must consider the concentrations carefully.

Combining PS-MP and AC in the genotoxicity assay revealed a subtle dependency of the AC concentration in the mixture. As long as the PS-MP concentration was comparably high, this compound dominated the effect. When AC concentration increased, the effect became comparatively smaller. This could suggest that AC bound to the microplastic leads to shielding of each other's reactive structures reducing the effective concentration. Mixture interaction will, according to the mass action law, in general be concentration dependent where at low concentrations the compounds will be mostly present as single substances while at higher concentrations the combination product will prevail.

In our experiments, the relevant dose range for the assays applied was spanned by the combined exposures almost totally and by studying different concentration relations (1:3, 1:1, and 3:1) we accounted for a wide range of possible environmental conditions. Still the study has some limitations. Due to the limited number of replications, we could only detect deviations from the target of about 2% effect size for chronic toxicity and 1% for genotoxicity assays. Furthermore, acute toxicity of IMD precluded its inclusion in combined experiments with the comet assay.

5. Conclusions

Microplastics can be a potential vector of organic pollutants, posing a threat to the environment and human health. In this scenario, the chronic and genotoxic effects of the microplastic polystyrene, the drug acyclovir and the pesticide imidacloprid, individually and in binary/

ternary mixtures, were evaluated on a key freshwater food chain organism, the crustacean *C. dubia*.

The present work highlighted that the interaction of the PS-MP with acyclovir and imidacloprid as well as the different ratios in which they were tested, leads to different modes of interaction in the mixtures considered.

In addition, this study shows that mixtures of the selected xenobiotics caused the inhibition of reproduction and damage to the DNA of the crustacean *C. dubia* at concentrations similar to those found in the environment, stressing that simultaneous exposure to several pollutants poses a serious threat to the entire ecosystem.

These data may be used to implement regulatory policies to protect and preserve the aquatic ecosystem. Future research should investigate the sublethal effects of the studied mixtures on other organisms of different trophic levels of the aquatic food chain. In addition, since the size of plastic polymers can influence the interaction with organic pollutants and hence the potential environmental toxicity of mixtures, polystyrene particles smaller than 1 μm should be investigated.

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Author statement

The authors' responsibilities were as follows:ML, MK and MI: Conceptualization, Supervision, Writing - review & editing.MK, RN, CR, EO: Data check; Formal analysis; Writing - draft;RN, CR: Experimental work, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Agatz, A., Ashauer, R., Brown, C.D., 2014. Imidacloprid perturbs feeding of Gammarus pulex at environmentally relevant concentrations. Environ. Toxicol. Chem. 33 (3), 648–653.

Almeida, M., Martins, M.A., Soares, A.M., Cuesta, A., Oliveira, M., 2019. Polystyrene nanoplastics alter the cytotoxicity of human pharmaceuticals on marine fish cell lines. Environ. Toxicol. Chem. 69, 57–65.

Ansoar-Rodríguez, Y., Christofoletti, C.A., Marcato, A.C., Correia, J.E., Bueno, O.C., Malaspina, O., Fontanetti, C.S., 2015. Genotoxic potential of the insecticide imidacloprid in a non-target organism (*Oreochromis niloticus*-Pisces). J. Environ. Prot. Sci. 6 (12), 1360.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organization J 5 (1), 9–19.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26 (3), 585–615.

Bradley, P.M., Barber, L.B., Duris, J.W., Foreman, W.T., Furlong, E.T., Hubbard, L.E., Kolpin, D.W., 2014. Riverbank filtration potential of pharmaceuticals in a wastewater-impacted stream. Environ. Pollut. 193, 173–180.

Brandts, I., Teles, M., Gonçalves, A.P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., Martins, M.A., Soares, A.M.V.M., Tort, L., Oliveira, M., 2018. Effects of nanoplastics on Mytilus galloprovincialis after individual and combined exposurewith carbamazepine. Sci. Total Environ. 643, 775–784.

Browne, M.A., 2015. Sources and pathways of microplastics to habitats. In: Bergmann, M., Gutow, L., Klages, M. (Eds.), Marine Anthropogenic Litter. Springer, Cham, pp. 229–244. https://doi.org/10.1007/978-3-319-16510-3_9.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. Environ. Sci. Technol. 45 (21), 9175–9179.

Chen, X.D., Culbert, E., Hebert, V., Stark, J.D., 2010. Mixture effects of the nonylphenyl polyethoxylate, R-11 and the insecticide, imidacloprid on population growth rate and other parameters of the crustacean. *Ceriodaphnia dubia*. Ecotoxicol. Environ. Saf. 73, 132–137.

De Felice, B., Sabatini, V., Antenucci, S., Gattoni, G., Santo, N., Bacchetta, R., Ortenzi, M. A., Parolini, M., 2019. Polystyrene microplastics ingestion induced behavioral effects to the cladoceran *Daphnia magna*. Chemosphere 231, 423–431.

De Liguoro, M., Dalla Bona, M., Gallina, G., Capolongo, F., Gallocchio, F., Binato, G., Di Leva, V., 2014. A monitoring of chemical pollutants in water used for field irrigation and livestock watering in the Veneto region (Italy), using bioassays as a screening tool. Environ. Sci. Pollut. Res. 21, 3546–3557.

de Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029–1039.

Di, M., Wang, J., 2018. Microplastics in surface waters and sediments of the three gorges reservoir, China. Sci. Total Environ. 616, 1620–1627.

Ebert, D., 2005. Some parasites of Daphnia. Bethesda (MD): national center for biotechnology information (US), chapter 3. In: Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. National Center for Biotechnology Information (US).

Eitzen, L., Ruhl, A.S., Jekel, M., 2020. Particle size and pre-treatment effects on polystyrene microplastic settlement in water: implications for environmental behavior and ecotoxicological tests. Water 12 (12), 3436.

Ermler, S., Scholze, M., Kortenkamp, A., 2014. Genotoxic mixtures and dissimilar action: concepts for prediction and assessment. Arch. Toxicol. 88, 799–814.

Felten, V., Toumi, H., Masfaraud, J.F., Billoir, E., Camara, B.I., Férard, J.F., 2020. Microplastics enhance *Daphnia magna* sensitivity to the pyrethroid insecticide deltamethrin: effects on life history traits. Sci. Total Environ. 714, 136567.

Fu, L., Li, J., Wang, G., Luan, Y., Dai, W., 2021. Adsorption behavior of organic pollutants on microplastics. Ecotoxicol. Environ. Saf. 217, 112207.

Garrido, S., Linares, M., Campillo, J.A., Albentosa, M., 2019. Effect of microplastics on the toxicity of chlorpyrifos to the microalgae Isochrysis galbana, clone t-ISO. Ecotoxicol. Environ. Saf. 173, 103–109.

Gupta, A., Vyas, R.K., Gupta, A.B., 2021. Occurrence of acyclovir in the aquatic environment, its removal and research perspectives: a review. J. Water Proc. Eng. 39, 101855.

Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the marine environment: a review of the methods used for identification and quantification. Environ. Sci. Technol. 46 (6), 3060–3075.

Huang, W., Song, B., Liang, J., Niu, Q., Zeng, G., Shen, M., Deng, J., Luo, Y., Wen, X., Zhang, Y., 2021. Microplastics and associated pollutants in the aquatic environment: a review on their ecotoxicological effects, trophic transfer, and potential impacts to human health. J. Hazard Mater. 405, 124187.

Hüffer, T., Hofmann, T., 2016. Sorption of non-polar organic compounds by micro-sized plastic particles in aqueous solution. Environ. Pollut. 214, 194–201.

ISO 20665, 2008. Water Quality-Determination of Chronic Toxicity to Ceriodaphnia Dubia in 7 Days-Population Growth Inhibition Test. International Organization for Standardization, Geneva, Switzerland.

Jemec, A., Tisler, T., Drobne, D., Sepcic, K., Fournier, D., Trebe, P., 2007. Comparative toxicity of Imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. Chemosphere 68, 1408–1418.

Katsnelson, B.A., Privalova, L.I., Varaksin, A.N., Kazmer, J.I., Kireyeva, E.P., Panov, V.G., 2011. An approach to characterizing the type of combined environmental toxic-ity based on epidemiologically assessed exposure-response relationships. Open J. Epidemiol. 4, 60–69.

Koelmans, A.A., Nor, N.H.M., Hermsen, E., Kooi, M., Mintenig, S.M., De France, J., 2019. Microplastics in freshwaters and drinking water: critical review and assessment of data quality. Water Res. 155, 410–422.

Kumar, P., Inamura, Y., Bao, P.N., Abeynayaka, A., Dasgupta, R., Abeynayaka, H.D.L., 2022. Microplastics in freshwater environment in asia: a systematic scientific review. Water 14, 1737.

Lassen, C., Hansen, S.F., Magnusson, K., Norén, F., Hartmann, N.I.B., Jensen, P.R., Nielsen, T.G., Brinch, A., 2012. Microplastics-Occurrence, effects and sources of. Signif 2, 2.

Leal Filho, W., Salvia, A.L., Bonoli, A., Saari, U.A., Voronova, V., Klõga, M., Kumbhar, S. S., Olszewski, K., Quevedo, D.M.D., Barbir, J., 2021. An assessment of attitudes towards plastics and bioplastics in Europe. Sci. Total Environ. 755, 142732.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Huahong, S., Kathleen, M.R.S., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. Sci. Total Environ. 619, 1–8.

Li, M.H., 2012. Survival, mobility, and membrane-bound enzyme activities of freshwater planarian, dugesia japonica, exposed to synthetic and natural surfactants. Environ. Toxicol. Chem. 31 (4), 843–850.

Liu, P., Lu, K., Li, J., Wu, X., Qian, L., Wang, M., Gao, S., 2020. Effect of aging on adsorption behavior of polystyrene microplastics for pharmaceuticals: adsorption mechanism and role of aging intermediates. J. Hazard Mater. 384, 121193.

Loewe, S., Muischnek, H., 1926. Über kombinationswirkungen. Mitteilung: hilfsmittel der fragestellung. Archiv f. experiment. Pathol. u. Pharmakol. 114, 313–326.

Luo, T., Weng, Y., Huang, Z., Zhao, Y., Jin, Y., 2021. Combined hepatotoxicity of imidacloprid and microplastics in adult zebrafish: endpoints at gene transcription. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 246, 109043.

Magni, S., Gagné, F., André, C., Della Torre, C., Auclair, J., Hanana, H., Parenti, C.C., Bonasoro, F., Binelli, A., 2018. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). Sci. Total Environ. 631–632, 778–788.

Mater, N., Geret, F., Castillo, L., Faucet-Marquis, V., Albasi, C., Pfohl-Leszkowicz, A., 2014. In vitro tests aiding ecological risk assessment of ciprofloxacin, tamoxifen and cyclophosphamide in range of concentrations released in hospital wastewater and surface water. Environ. Int. 63, 191–200.

Mei, W., Chen, G., Bao, J., Song, M., Li, Y., Luo, C., 2020. Interactions between microplastics and organic compounds in aquatic environments: a mini review. Sci. Total Environ. 736, 139472.

Menéndez-Pedriza, A., Jaumot, J., 2020. Interaction of environmental pollutants with microplastics: a critical review of sorption factors, bioaccumulation and ecotoxicological effects. Toxics 8 (2), 40.

Morrissey, C.C., Mineau, P., Devries, J.H., Francisco, Sanchez-Bayo, Liess, M., Cavallaro, M.C., Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ. Int. 74, 291–303.

Muñoz-Vélez, M.F., Hidalgo-Salazar, M.A., Mina-Hernández, J.H., 2018. Effect of content and surface modification of fique fibers on the properties of a low-density polyethylene (LDPE)-Al/Fique composite. Polymers 10 (10), 1050.

Nobre, C.R., Moreno, B.B., Alves, A.V., de Lima Rosa, J., da Rosa Franco, H., Abessa, D.M. D.S., Pereira, C.D.S., 2020. Effects of microplastics associated with triclosan on the oyster *Crassostrea brasiliana*: an integrated biomarker approach. Arch. Environ. Contam. Toxicol. 79 (1), 101–110.

Nugnes, R., Lavorgna, M., Orlo, E., Russo, C., Isidori, M., 2022. Toxic impact of polystyrene microplastic particles in freshwater organisms. Chemosphere, 134373.

Peng, X., Wang, C., Zhang, K., Wang, Z., Huang, Q., Yu, Y., Ou, W., 2014. Profile and behavior of antiviral drugs in aquatic environments of the Pearl River Delta, China. Sci. Total Environ. 466, 755–761.

Prasse, C., Schlüsener, M.P., Schulz, R., Ternes, T.A., 2010. Antiviral drugs in wastewater and surface waters: a new pharmaceutical class of environmental relevance? Environ. Sci. Technol. 44 (5), 1728–1735.

Rainieri, S., Conlledo, N., Larsen, B.K., Granby, K., Barranco, A., 2018. Combined effects of microplastics and chemical contaminants on the organ toxicity of zebrafish (Danio rerio). Environ. Res. 162, 135–143.

Sanchez-Bayo, F., Hyne, R.V., 2014. Detection and analysis of neonicotinoids in river waters-development of a passive sampler for three commonly used insecticides. Chemosphere 99, 143–151.

Santos, L.H.M.L.M., Rodriguez-Mozaz, S., Barcelò, D., 2021. Microplastics as vectors of pharmaceuticals in aquatic organisms – an overview of their environmental implications. Case Stud. Chem. Environ. Eng. 3, 100079.

Schirinzi, G.F., Llorca, M., Seró, R., Moyano, E., Barceló, D., Abad, E., Farré, M., 2019. Trace analysis of polystyrene microplastics in natural waters. Chemosphere 236, 124321.

Shi, W., Han, Y., Sun, S., Tang, Y., Zhou, W., Du, X., Liu, G., 2020. Immunotoxicities of microplastics and sertraline, alone and in combination, to a bivalve species: size-dependent interaction and potential toxication mechanism. J. Hazard Mater. 396, 122603.

Song, Y.K., Hong, S.H., Jang, M., Han, G.M., Jung, S.W., Shim, W.J., 2017. Combined effects of UV exposure duration and mechanical abrasion on microplastic fragmentation by polymer type. Environ. Sci. Technol. 51 (8), 4368–4376.

Starner, K., Goh, K.S., 2012. Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010–2011. Bull. Environ. Contam. Toxicol. 88, 316–321.

Stefanini, R., Borghesi, G., Ronzano, A., Vignali, G., 2021. Plastic or glass: a new environmental assessment with a marine litter indicator for the comparison of pasteurized milk bottles. Int J LCA 26, 767–784.

Suman, T.Y., Jia, P.P., Li, W.G., Junaid, M., Xin, G.Y., Wang, Y., Pei, D.S., 2020. Acute and chronic effects of polystyrene microplastics on brine shrimp: first evidence highlighting the molecular mechanism through transcriptome analysis. J. Hazard Mater. 400, 123220.

Sun, W., Meng, Z., Li, R., Zhang, R., Jia, M., Yan, S., Zhu, W., 2021. Joint effects of microplastic and dufulin on bioaccumulation, oxidative stress and metabolic profile of the earthworm (*Eisenia etida*). Chemosphere 263, 128171.

Tomicic, M.T., Bey, E., Wutzler, P., Thust, R., Bernd, K., 2002. Comparative analysis of DNA breakage, chromosomal aberrations and apoptosis induced by the anti-herpes purine nucleoside analogues acyclovir, ganciclovir and penciclovir. Mutat. Res. 505, 1–11.

Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selctive action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268.

Van Dijk, T.C., Van Staalduinen, M.A., Van der Sluijs, J.P., 2013. Macro-invertebrate decline in surface water polluted with imidacloprid. PLoS One 8 (5), e62374.

Wang, S., Xue, N., Li, W., Zhang, D., Pan, X., Luo, Y., 2020. Selectively enrichment of antibiotics and ARGs by microplastics in river, estuary and marine waters. Sci. Total Environ. 708, 134594.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492.

Xiang, Y., Jiang, L., Zhou, Y., Luo, Z., Zhi, D., Yang, J., Lam, S.S., 2022. Microplastics and environmental pollutants: key interaction and toxicology in aquatic and soil environments. J. Hazard Mater. 422, 126843.

Zhang, Q., Qu, Q., Lu, T., Ke, M., Zhu, Y., Zhang, M., Zhang, Z., Du, B., Pan, X., Sun, L., Qian, H., 2018. The combined toxicity effect of nanoplastics and glyphosate on *Microcystis aeruginosa* growth. Environ. Pollut. 243, 1106–1112.

Zhang, P., Yan, Z., Lu, G., Ji, Y., 2019. Single and combined effects of microplastics and roxithromycin on Daphnia magna. Environ. Sci. Pollut. Res. 26 (17), 17010–17020.

Zhou, W., Han, Y., Tang, Y., Shi, W., Du, X., Sun, S., Liu, G., 2020. Microplastics aggravate the bioaccumulation of two waterborne veterinary antibiotics in an edible bivalve species: potential mechanisms and implications for human health. Environ. Sci. Technol. 54 (13), 8115–8122.

Zhu, Z.L., Wang, S.C., Zhao, F.F., Wang, S.G., Liu, F.F., Liu, G.Z., 2019. Joint toxicity of microplastics with triclosan to marine microalgae *Skeletonema costatum*. Environ. Pollut. 246, 509–517.

Ziajahromi, S., Kumar, A., Kumar, P.A., Leusch, F.D.L., 2019. Effects of polyethylene microplastics on the acute toxicity of a synthetic pyrethroid to midge larvae (*Chironomus tepperi*) synthetic and river water. Sci. Total Environ. 671, 971–975.

Zocchi, M., Sommaruga, R., 2019. Microplastics modify the toxicity of glyphosate on *Daphnia magna*. Sci. Total Environ. 697, 134194.