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Toxic impact of polystyrene microplastic particles in freshwater organisms

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**HIGHLIGHTS**

- Freshwater organisms were exposed in vivo to polystyrene beads (1.0 μm).
- Inhibition of reproduction and DNA damage were observed in *C. dubia* at units of μg/L.
- Chronic toxicity and genotoxicity occurred in *C. dubia* at units of μg/L.
- The risk quotient was higher than the threshold value of 1.
- The risk quotient was calculated obtaining a value of 7.2.

**ABSTRACT**

The ongoing COVID-19 pandemic is leading to an increase of the global production of plastics since the use of personal protective equipment (PPEs, i.e. gloves, gowns, masks, packaging items), has become mandatory to prevent the spread of the virus. Plastic breaks down into micro/nano particles due to physical or chemical or biological actions into environment. Due to small dimensions, ubiquitous and persistent nature, the plastic particles represent a significant threat to ecosystems and can enter into food chains. Among the plastic polymers used for PPEs, polystyrene is less studied regarding its eco-geno-toxicity. This study aims to investigate acute, chronic and subchronic effects of the microplastic polystyrene beads (PS-MP, size 1.0 μm) on three freshwater species, the alga *Raphidocelis subcapitata*, the rotifer *Brachionus calyciflorus*, the crustacean *Ceriodaphnia dubia* and the benthic ostracod *Heterocypris incongruens*. Furthermore, the potential genotoxicity and the ROS production due to the PS-MP were also determined in *C. dubia*.

Results revealed that the acute effects occurred at concentrations of PS-MP in the order of dozens of mg/L in *B. calyciflorus* and *C. dubia* and hundreds of mg/L in *H. incongruens*.

Regarding long-term toxicity, increasing chronic effects with EC50s in the order of units (C. dubia), hundreds (B. calyciflorus) and thousands (R. subcapitata) of μg/L were observed. Both for acute and chronic/sub chronic toxicity, daphnids were more sensitive to polystyrene than ostracods. Moreover, when *C. dubia* neonates were exposed to the PS-MP, alterations in genetic material as well as the production of ROS occurred, starting from concentrations in the order of units of μg/L, probably due to inflammatory responses. At last, the risk quotient (RQ) as a measure of risk posed by PS-MPs in freshwater environment, was calculated obtaining a value of 7.2, higher than the threshold value of 1.

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1. Introduction

The COVID-19 pandemic has resulted in a sudden increase in demand for plastics as single-use items for both non-medical applications and medical instruments. Therefore, the increase in use and disposal has led to a strong environmental challenge due to the known long half-life of plastic, its very low degradation and poor waste management with an impact on the environment and ecosystems. This sudden increase in the production and consumption of single-use plastics and personal protective equipment (PPE) such as goggles, gloves, protective suits, safety shoes, face shields, gowns, respirators, medical/surgical masks (World Health Organization, 2020) has brought a huge burden to the already difficult management of plastic waste.

Along the lines of the WHO for countermeasures to protect against the COVID-19 pandemic, the increase in demand for food and groceries delivered at home due to the change in habits also contributed to an increase in the consumption of plastic packaging (40–49%) and people’s disposable preferences to reduce the risk of contamination and infection (Abuwatfa et al., 2021; de Sousa, 2021). Overall, the International Solid Waste Association has estimated 250–300% more consumption of single-use plastics than that used during the pre-pandemic (de Sousa, 2021).

WHO estimates that 89 million medical masks were needed each month in 2020 to cope with COVID-19 (World Health Organization, 2020). For example, China increased its daily mask production to 14.8 million by February 2020 (Selvaranjan et al., 2021) and as of April 2020 Japan had secured over 600 million mask orders per month (METI, 2020). Basically, according to Peng et al. (2021), more than eight million tons of plastic waste associated with the pandemic was generated globally, with more than 25,000 tons entering the global ocean.

The two-year period of global health emergency from COVID-19 has influenced the numerous strategies and guidelines of the environmental program developed in recent decades aimed at reducing the sources of plastic pollution (Directive (EU) 2019/904), at increasing recycling and waste management to achieve a overall reduction in the use of plastic. Most personal protective equipment and plastic-based packaging items are manufactured using multiple types of polymers such as polypropylene (PP), polyvinyl chloride (PVC), polycarbonate (PC), poly styrene (PS) and polyethylene terephthalate (PET) (Parashar and Hait, 2021). For example, disposable surgical masks and packaging materials can be made with different polymeric materials, such as polypropylene and polystyrene (Aragaw, 2020; Abuwatfa et al., 2021).

Unfortunately, although preventive measures for the COVID-19 pandemic such as the use of face masks have been imposed on the population, clear instructions and disposal mechanisms have not been provided.

All of these polymers have increased the presence of microplastics (MPs) and nanoplastics (NPs) in the aquatic environment. MPs and NPs should be precisely defined. To date, there is no clear, recognized and standardized definition of them. Microplastics are generally defined as particles occurring at concentrations of ten orders of magnitude (1 10−5 - 1 106 particles/liter) (Koelmans et al., 2019).

Numerous studies have already focused on the distribution and the occurrence of NP in marine and freshwater environments, sediments and biota (Auta et al., 2017; Karlsson et al., 2017; Jiang, 2018; Li et al., 2018; Murphy and Quinn, 2018; Peng et al., 2018; Xu et al., 2020), others on the toxicity of microplastic particles or single polymers on marine organisms (Anderson et al., 2016; Ajith et al., 2020), other studies have highlighted the potential translocation of microplastic particles in tissues especially of marine invertebrates and fish up to the possible effects of these particles on human health through the seafood (Smith et al., 2018; Vital et al., 2021).

Studies on the effect of COVID-19 on waste and plastic pollution are quite few and of concern for the marine environment, while scientific research on NP contamination in freshwater matrices and organisms of various species is much more limited (Bellasi et al., 2020; Cole et al., 2020; De Felice et al., 2019).

However, freshwater ecosystems represent the way by which most MPs reach the marine environment. Microplastics and nanoplastics have shown causing adverse effects such as decreased growth/reproduction rate and oxidative stress in freshwater pelagic and/or benthic organisms (Zhao et al., 2014; Canniff and Hoang, 2018). These toxic effects are mainly due to the microparticles (found in the environment from fragmentation) which are favoured by their size to overcome the biological barriers of organisms.

The microplastic particles of polystyrene have been found to be genotoxic to aquatic organisms probably due to the production of reactive oxygen species (ROS), oxidative stress and interference in DNA repair (Dong et al., 2019; Solomando et al., 2020).

The present study aimed to broaden the knowledge on the acute, chronic and subchronic effects of microplastic polystyrene beads (PS-MP, size 1.0 μm) on three species of the freshwater chain, the green alga Raphidocelis subcapitata, the rotifer Brachionus calyciflorus, the cladoceran crustacean Ceriodaphnia dubia and on one organism of the sediments, the benthic ostracod Heterocyclops incongruens. Furthermore, as C. dubia is a key organism in the freshwater food chain, PS-MP genotoxicity and ROS production were also determined in this crustacean. Furthermore, the daphnids were analysed to determine whether PS-MP was in their gut or accumulated in different organs using light microscopy. Finally, the risk quotient (RQ) was calculated as a measure of the risk posed by PS-MPs in freshwater environments. In order to establish the possible differences between the nominal and actual concentrations, the concentration of PS-MP in the test solution was also investigated.

2. Materials and methods

2.1. Test compound

Monodispersed micro particles of polystyrene (PS-MP) with a solid content of 2% weight, analytical standard size equal to 1.0 μm and particle specific gravity equal to 1.05 g/cm3, were purchased from Sigma Aldrich (Milano, Italy) as aqueous suspension (21,000 mg/L) containing approximate 3.24·1010 particles/mL (PS, Product number: 72,938).

Test solutions were freshly prepared by mixing the appropriate volumes of the aqueous suspension of polystyrene particles and standard synthetic media. Differences between nominal and actual concentrations of PS-MP in test solutions were detected by Total Organic Carbon Analyzer, TOC-L CSN (Shimadzu, Kyoto, Japan) at the beginning of each test and after 24 h. The chemical analyses were performed on the basis of the different materials used as test wells (polyethylene, polystyrene, glass).

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2.2. Toxicity testing

2.2.1. Acute toxicity tests

The rotifer B. calyciflorus, the crustacean C. dubia and the benthic ostracod H. incongruens were used in acute assays.

B. calyciflorus assay (ASTM E 1440–91, 2004) was performed using organisms hatched from cysts (MicroBioTest Inc, Nazareth, Belgium) under continuous lighting (3000–4000 lux) for 16–18 h in moderately hard synthetic freshwater (80–100 mg/L CaCO$_3$, pH 7.5 ± 0.3) at 25 ± 1 °C. A 0.3 mL test solution (from 33.4 to 144 mg/L, geometric progression of 1.2, chosen after appropriate range finding tests) was used in each test well (36-well plates) with five rotifers/well, in six replicates.

The plates were incubated in darkness at 25 ± 1 °C in synthetic medium (hardness 250 mg/L expressed as CaCO$_3$) with a 16:8 h light: dark cycle (600 lux) and fed with a combination of 5 g/L each of Saccharomyces cerevisiae, alfa-alfa, and flake food, as well as the unicellular green alga Raphidiocelis subcapitata (10$^8$ cells/mL). Tests were performed in 24-well plates with 10 organisms and 1.0 mL of test solution (from 15.6 to 500 mg/L, geometric progression of 2, chosen after appropriate range finding tests) in each well, in triplicates.

H. incongruens test (ISO 14371, 2012) was performed on ostracod neonates hatched from cysts (MicroBioTest Inc, Nazareth, Belgium) under continuous illumination at 3000–4000 lux, for 52 h in US EPA (2002) moderately hard synthetic Freshwater (80–100 mg/L CaCO$_3$, pH 7.5 ± 0.3) at 25 ± 1 °C, and pre-fed 4 h prior to the test with the blue-green Spirulina algae (Arthrospira platensis powder from MicroBioTest Inc, Nazareth, Belgium). Tests were performed in 6-well plates with 10 organisms and 2.0 mL of test solution (from 3.9 to 1000 mg/L, geometric progression of 2, chosen after appropriate range finding tests) as well as 1728.88 ± 48.30 mg of reference sediment (river sand, particle size between 0 and 2 mm, MicroBioTest Inc, Nazareth, Belgium) into each well, in triplicate. The algal suspension (2.0 mL) was added as food (1.5 × 10$^7$ cells R. subcapitata/mL) into each well.

Negative controls (only medium) were performed for each acute test. The plates were incubated in darkness at 25 ± 1 °C for 24 h for rotifers and daphnids or 6 days for ostracods. The end-point considered was mortality, and the number of the rotifers/crustaceans obtained in test solutions was compared to those obtained for the respective negative controls in order to determine the effective percentages and to evaluate the Median Lethal Concentration (LC50), resulting in the 50% effect. For C. dubia acute assay the effective percentages for each dilution were calculated using ToxRat Professional software, Version 2.10.05 (Alsdorf, Germany).

2.2.2. Chronic and sub-chronic toxicity tests

The green alga R. subcapitata, B. calyciflorus and C. dubia were used for chronic toxicity assays, H. incongruens was used for sub-chronic toxicity testing.

R. subcapitata growth inhibition test (in line with OECD 201, 2011, with slight modifications reported by Paixao et al., 2008) was performed in 96-well plates in six replicates. The test solutions (7 dilutions, from 195 to 12.5 × 10$^{-3}$ µg/L, geometric progression 2, chosen after appropriate range finding tests) were incubated with 10$^4$ cells/mL of algal suspension under continuous illumination (6000 lux) at 25 ± 1 °C on a microplate shaker (450 rpm). The plates were read at 450 nm using the automated microplate reader (Synergy H1, Biotek, Winooski, USA) immediately before the assay and after 24 h, 48 h and 72 h.

B. calyciflorus offspring reduction test (ISO, 20666, 2008) was performed on less than 2 h old organisms coming from cysts hatched as described above. Tests were performed in 48-well plates with one rotifer/well and 0.9 mL/well of test solutions (10 dilutions in moderately hard dilution water, from 0.9 to 32·10$^{-3}$ µg/L, geometric progression of 3.2, chosen after appropriate range finding tests). All tests were performed in six replicates. The organisms were fed with 0.1 mL of fresh suspension of 10$^3$ cells/mL of the unicellular alga R. subcapitata. Plates were incubated in darkness at 25 ± 1 °C for 48 h.

C. dubia offspring reduction test (ISO, 20665, 2008) was performed over 7 d on 24 h old organisms. Individual neonates from at least the third generation were exposed to 25 mL of test solutions (8 dilutions starting from 0.03 to 90 µg/L, geometric progression of 3.2, chosen after appropriate range finding tests) in beakers in decuplicate. Test media were changed five times/week under semi-static conditions and crustaceans were fed as explained above. From the fourth d-exposure, the neonates produced by each parent organism were counted and removed, and beakers were incubated at 25 ± 1 °C, with a 16:8 h light: dark cycle (600 lux).

H. incongruens growth inhibition test (ISO 14371, 2012) was performed in triplicate on ostracod neonates hatched from cysts, exactly as described for acute assay. Therefore, 6-well plates were used setting each well with 10 organisms, 2.0 mL of test solution, 2 spoons (1000 µL) of reference sediment and 2.0 mL of algal suspension (1.5 × 10$^7$ cells R. subcapitata/mL). The plates were incubated in darkness at 25 ± 1 °C for 6 d. The length of ostracods was measured on living organisms immobilized by Lugol fixative solution (MicroBioTest Inc, Nazareth, Belgium) using a micrometre immediately before the test and after 6 d.

The growth inhibition was determined only when a mortality of less than 30% was found in the acute assay.

Negative controls (only medium) were performed for each chronic/ sub-chronic test. The number of the rotifer/daphnid offspring, or the algal growth, or the ostracod length outputs obtained in test solutions were compared to the values obtained for the respective negative controls in order to determine the effective percentages and to evaluate the chronic Effective Concentrations (ECx). In addition, to calculate the percentage of growth inhibition of H. incongruens, the growth decrease observed in test solution after 6 d-exposure was compared with the growth increase calculated in the negative control after 6-d exposure respect to the measurement taken at the beginning of the test. For C. dubia chronic assay the effective percentages for each dilution were calculated using ToxRat Professional software, Version 2.10.05 (Alsdorf, Germany).

2.3. Genotoxicity testing

The crustacean C. dubia was the organism selected for the genotox- icity testing performed by the Single Cell Gel Electrophoresis (Comet assay) following Parrella et al. (2015), Kundi et al. (2016), Lavorgna et al. (2016) and Russo et al. (2018).

The comet assay was performed on cells coming from whole organ- isms. Neonates were less than 24 h old and coming from the second to the fifth brood, obtained by parthenogenesis.

About 20 neonates were placed in glass beakers and exposed to polystyrene for 24 h, in darkness at 25 °C. Tests were performed in duplicate at concentrations starting from at least 1/10 the acute EC50 value and arranged in a geometric series with a factor 10. After the treatment, neonates were placed in 1 mL of phosphate-buffered saline (PBS) containing 20 mM ethylene diamine tetra-acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and then subjected to serial pipetting according to Park and Choi (2007) and centrifuged at 5000 rpm. The single cells, obtained by chemical and mechanical exoskeleton disintegration, were re-suspended in 0.7% low melting point (LMP) agarose and spread onto microscope slides pre-coated with 1% normal melting point (NMP) agarose and subjected to the alkaline comet assay (Tice et al., 2000). Subsequently, slides were dipped for 1 h in 10 mM Tris, 100 mM EDTA, 2.5 M NaCl, 10% DMSO, 1% Triton X-100 (pH 10) in order to lyse the cell and nuclear membranes. After that, DNA unwinding was carried out for 20 min at 4 °C in alkaline conditions (300 mM NaOH, 1 mM EDTA, pH ≥ 13). The electrophoresis was performed at 4 °C at 400 mA (25 V, 1
V/cm) for 20 min. The slides were neutralized (Tris–HCl 0.4 M), dehydrated in 70% ethanol, stained with 50 µL ethidium bromide (10 mg/L) and then analysed using a fluorescence microscope (400 × magnification, Eclipse 50i, Nikon, Kanagawa, Tokyo).

### 2.4. Production of ROS

The 2′,7′ dichlorodihydrofluorescein diacetate (H$_2$DCFDA) was used to detect ROS activity in C. dubia neonates less than 24 h and coming from the second to the fifth brood, obtained by parthenogenesis. Tests were performed in duplicate in 24-well plates with 10 organisms and 1.0 mL of the polystyrene solution at concentrations starting from at least 1/10 of the acute LC$_{50}$ value and arranged in a geometric series with a factor of 10. The negative control was prepared using only medium. Plates were incubated for 24 h, in the dark at 25 °C. After exposure, two ROS detection methods were applied. The difference between the two methods is that the first method detects the ROS released into the medium by organisms, the second detects the total ROS amount when the organisms are disintegrated. Briefly:

- First method: 10 whole organisms/well were transferred in 1.0 mL of H$_2$DCFDA (10 µM) in 24-well plates for 4 h at 25 °C in the dark. The contents of the well, with the exception of organisms, were analysed in sixfold (three replicates of 300 µL/well) in 96-well plates. Fluorescence was monitored by a fluorescence microplate reader (Synergy H1, Bioteck, Winooski, USA), (Xie et al., 2006);
- Second method: 10 whole organisms were homogenized in 0.32 mM sucrose, 20 mM HEPES, 1 mM MgCl$_2$ and 0.5 mM Phenylmethylsulfonyl fluoride buffer and centrifuged at 4 °C for 10,000 g. The supernatant was collected and 96-well plates were prepared with 50 µL PBS 1x, 50 µL H$_2$DCFDA 40 µM, 100 µL supernatant, in sixfold (three replicates from each polystyrene incubation well). Plates were incubated for 4 h at 20 °C in the dark (Kim et al., 2011; Lin et al., 2019).

The fluorescence was monitored with a microplate reader setting the excitation wavelength at 485 nm and the emission wavelength at 520 nm. The increase in fluorescence intensity yielded the ROS quantity.

### 2.5. Microscopic analysis

The gut content of C. dubia after 24 h of exposure to different concentrations of PS microparticles (0.0085, 0.85, 85 and 850 mg/L) was visually examined using the LEICA M205C light microscope. Organisms were fixed in 4% paraformaldehyde, held at 4 °C until the microscopic analysis.

### 2.6. Data analysis

For each eco-toxicity assay, effect percentages coming from three independent experiments were pooled using Prism5 software (Graphpad Inc, CA, USA) to estimate the concentrations giving x% effect (LE(C)x) by non-linear regression (log agonist vs. normalized response-variable slope). The LC$_{50}$ value, corresponding to 50% of mortality, was the test parameter for each acute test. For long term toxicity, EC$_{50}$, EC$_{20}$, and EC$_{10}$ were the concentrations that gave 50%, 20% and 10% inhibition of reproduction/growth respectively in rotifers, daphnids and algae. Moreover, the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) were estimated by ANOVA and the Dunnett’s multiple comparison test. Results were also examined in a multiple comparison procedure with Tukey’s HSD test (honestly significantly difference) to verify differences among test concentrations (p < 0.0001).

### 3. Results

The TOC-L CSN analysis revealed non-appreciable differences between nominal and actual concentrations of the PS-MP at the beginning of each test. After 24 h, PS-MP concentrations diverged from the nominal concentrations by a maximum of 3%. When the actual concentrations are less than 20% of the nominal ones they can be considered very close, as reported by Li (2012). Thus, in the present study, the effective concentrations were reported as nominal concentrations.

#### 3.1. Toxicity testing

##### 3.1.1. Acute toxicity tests

Acute toxicity testing was performed on pelagic and benthic invertebrates. Results, coming from three independent experiments, are expressed in mg/L, and are reported in Table 1. Pelagic invertebrates were the most sensitive organisms to PS-MP with LC$_{50}$ values in the order of dozens of mg/L, with the highest lethal effect observed for the rotifer B. calyciflorus (LC$_{50} = 75.35$ mg/L) after 24 h-exposure. A lower lethal effect was observed for the benthic crustacean (6 d-exposure) with LC$_{50}$ values in the order of hundreds of mg/L. Despite the similar phylogenetic affiliation, the pelagic crustacean (daphnid) was more sensible to polystyrene than the benthic crustacean (os TR).  

| Pelagic organism | Benthic organism |
|------------------|------------------|
| B. calyciflorus  | C. dubia         |
| 24 h             | 24 h             |
| 7.35 (70.71-80.30) | 84.85 (70.75-101.8) | 136.30 (92.55-200.80) |

#### 3.1.2. Chronic and sub-chronic toxicity tests

Chronic toxicity tests were performed on freshwater organisms belonging to different levels of the trophic chain: the producer green alga R. subcapitata and two primary consumers such as the rotifer B. calyciflorus and the crustacean C. dubia exposed to PS-MP at completely different exposure times (respectively equal to 72 h, 48 h and 7 d).

| Table 2 | EC$_{50}$, EC$_{20}$, EC$_{10}$ values expressed in µg/L obtained in chronic toxicity testing, with 95% confidence intervals. |
|---------|---------------------------------------------------------------------------------------------------------------------|
| Pelagic organism | R. subcapitata | B. calyciflorus | C. dubia |
| 72 h  | 4.02 (3.52-4.58) | 16.86 (9.37-28.77) | 1.75 (1.16-2.64) |
| 48 h  | 1.41 (1.03-1.72) | 16.86 (9.37-28.77) | 0.19 (0.09-0.36) |
| 7 d   | 1.72 (0.76-3.88) | 0.53 (0.03-0.14) | 0.05 (0.03-0.14) |
7 d) in line with the respective standard guidelines and referring to one third of the life span of the organisms.

The results of EC50, EC20 and EC10 from three independent experiments are shown in Table 2 and are expressed in μg/L. The percentage of inhibition of growth was calculated comparing the growth decrease observed in test solutions after 6 d-exposure with the growth increase observed in the negative control after 6 d-exposure respect the measurement taken before the test. H. incongruens at different concentrations expressed in μg/L results are presented as mean ± SD (n = 3). Significance from negative control (**p < 0.001 and ***p < 0.0001) was determined using ANOVA and Dunnett’s multiple comparison tests.

### Table 3

| Benthic organism | μg/L | Survival (%) | Length (μm) | Inhibition of growth (%) |
|------------------|------|--------------|-------------|--------------------------|
| NC               | 100.00 ± 0.00 | 357.50 ± 3.00 | 6.60 ± 40   |
| 3.91 × 10^3      | 95.00 ± 3.00  | 334.70 ± 10.00 (**) | 15.40 ± 20 |
| 7.81 × 10^3      | 85.00 ± 7.00  | 325.60 ± 16.00 (***) | 21.40 ± 60 |
| 15.62 × 10^3     | 70.00 ± 6.00  | 305.60 ± 30.00 (***) | 27.40 ± 50 |

3.1.3. Genotoxicity testing

The genotoxicity evaluation was performed by the comet assay on the freshwater crustacean C. dubia wholly exposed in vivo to polystyrene for 24 h. As reported in Fig. 2, significant (**p < 0.0001) DNA strand breaks were evident starting from the concentration of 8.5 μg/L (LOAEC value, 4.2% DNA in tail) with a significant increase in effect with increasing concentration (**p < 0.0001), reaching 27.25% DNA in the tail at the highest concentrations tested.

Comparing the LOAEC value (8.5 μg/L) obtained in the genotoxicity tests with the LOEC value (0.3 μg/L) obtained in the chronic toxicity tests, it is evident that the reproductive damage develops in C. dubia at concentrations significantly lower than those observed causing DNA damage.

3.1.4. Production of ROS

ROS production was observed wholly exposing C. dubia neonates in vivo to the PS-MP for 24 h and subsequently transferring whole organisms in H2DCFDA (first procedure) or homogenising neonates and collecting only supernatant (after centrifugation) and transferring it in H2DCFDA (second procedure). Differences between the two listed methods were observed and results were reported in Fig. 3.

Despite both methods were able to detect the statistically increase in ROS production at increased concentrations (p < 0.05), higher fluorescence percentages were reached using the supernatant method. Nevertheless, by significance from negative control (Dunnett’s multiple comparison tests), both approaches revealed NOAEC and LOAEC values tested, C. dubia was the organism with the lowest NOEC and LOEC values showing the highest significant no effect concentration and the lowest significant effect concentration in the order of tenths and hundreds of μg/L, respectively (Table 4).

### Table 4

| Pelagic organism | NOEC (μg/L) | LOEC (μg/L) | Benthic organism | NOEC (μg/L) | LOEC (μg/L) |
|------------------|-------------|-------------|------------------|-------------|-------------|
| R. subcapitata    | 195.31      | 29.00       | R. subcapitata    | 3.90 × 10^3 | 3.90 × 10^3 |
| B. calyciflorus   | 390.62      | 95.00       | C. dubia         | 3.90 × 10^3 | 3.90 × 10^3 |
| C. dubia         | 72 h        | 48 h        | H. incongruens   | 3.90 × 10^3 | 3.90 × 10^3 |

![Fig. 1. Concentration/effect curves of the PS-MP in R. subcapitata, B. calyciflorus and C. dubia.](image1)

![Fig. 2. Effect of the microplastic polystyrene on induction of DNA strand breaks in C. dubia. Results are expressed as % DNA in tail and are from two independent experiments (400 nuclei). Data are presented as quartile box plot. Significant difference from control was determined with Dunnett’s test **p < 0.0001. Different letters mean significant differences for p < 0.05 among concentrations expressed in μg/L (Tukey’s HSD multiple comparison test).](image2)
Fig. 3. ROS production in *C. dubia* exposed to the PS-MP for 24 h. Fluorescence percentage was reported after normalization on negative controls. Results are presented as mean ± SD (n = 3). The statistical significant difference between the two procedures is reported as *p* < 0.05, **p** < 0.0001 (Two-way RM ANOVA/ Bonferroni posttests). Different letters mean significant differences for *p* < 0.05 among concentrations expressed in μg/L (Tukey’s HSD multiple comparison test).

Fig. 3. ROS production in *C. dubia* exposed to the PS-MP for 24 h. Fluorescence percentage was reported after normalization on negative controls. Results are presented as mean ± SD (n = 3). The statistical significant difference between the two procedures is reported as *p* < 0.05, **p** < 0.0001 (Two-way RM ANOVA/ Bonferroni posttests). Different letters mean significant differences for *p* < 0.05 among concentrations expressed in μg/L (Tukey’s HSD multiple comparison test).

## 4. Discussion

In this study we observed the acute, chronic, sub-chronic and genotoxic effects caused by PS-MP (1 μm) in organisms belonging to different levels of the freshwater trophic chain. Considering that the distribution of PS in freshwaters is highly heterogeneous (Klein et al., 2018; Triebskorn et al., 2019) and the differences among particles occur in terms of type, size and shape, it is rather difficult to compare the findings of this study to the PS Measured Environmental Concentrations (MECs) reported in the literature using various units of measurement (Blancho et al., 2021). Generally, polystyrene microparticles detected in water are reported as particle number/L (Di and Wang, 2018; Pivokonsky et al., 2018) while toxicity results are typically but not exclusively reported as concentrations (μg/L) (Ziajahromi et al., 2017). Hence, although the outcomes of this research were reported in μg/L, for ease of understanding, they were also converted to p/L, following equation (1) (Ziajahromi et al., 2017):  

$$\text{MPs (particles/L)} = \left(\text{TMPs } \text{stock (particles/L)} \times \text{ Cx (mg/L))} / \text{Cstock (mg/L)}\right)$$  

Where TMPs stock is the total number of microplastics in the stock solution, Cx is the concentration (x) of microplastics in the bioassay and Cstock is the concentration of microplastics in the stock solution.

The results of this research show that the rotifer and the cladoceran crustacean were the organisms acutely affected by 24 h - PS-MP exposure with median lethal concentrations in the order of tens of mg/L corresponding to approximately 1.10\(^{-1}\) p/L. To the best of our knowledge, few studies are in the literature regarding the occurrence and toxicity of polystyrene microparticles and in particular on dimensions around 1 μm (Singh et al., 2022). MPs (from 1 to 500 μm) including PS have been detected in freshwater at concentrations from a few units to hundreds of p/L (Zha et al., 2014; Schymanski et al., 2018; Di and Wang, 2018; Pivokonsky et al., 2018) and polystyrene microparticles at concentrations between 5.84 and 13 ng/L (Schirinzi et al., 2019), our acute toxicity results are very far from environmental concentrations and therefore not harmful to the environment.

From a toxicological point of view, the median immobilization of *Daphnia pulex* was found by Liu et al. (2019), after 48 h exposure, at concentrations of polystyrene nanoparticles (75 nm) equal to 76.69 mg/L (LC50), a value within the confidence limits of that obtained in this study. A similar study on the acute toxicity of PS nanoparticles was carried out for 48 h by Lin and collaborators in 2019 in the cladoceran crustacean *D. magna* with an LC50 in the order of mg/L units, underlining an increase in toxicity in relation to a time of longer exposure. Since organisms in the natural environment are usually exposed to lower concentrations for a longer time, aquatic organisms were subjected to these conditions in this study. The aquatic organism most affected by the PS-MP was the crustacean *C. dubia* with EC50 after 7 d-exposure in the order of units of μg/L (10\(^{4}\) p/L) and a LOEC value in the order of tenths of μg/L (10\(^{5}\) p/L) which is at effective concentrations close to those of environmental concern.

Surely, as reported by Gorokhova (2015) and De Felice et al. (2019) of particular concern is the exposure and the subsequent effects due to microparticles in zooplanktonic filter-feeder species, which indiscriminately ingest them during their normal swimming and feeding activity. In fact, as reported by Ebert (2005), daphnids have the ability to uptake and ingest small (1–70 μm in size) suspended particles from the water with the inability to distinguish between size and quality, which implies a lack of selection and likely ingestion of MPs. Once ingested, microplastics can affect these organisms causing the inhibition of nutrient absorption, the reduction of growth and of the reproduction rate (Wright et al., 2013; Lei et al., 2018). In 2018, Canniff and Hoang exposing *D. magna* to polyethylene particles (63–75 μm) during a long-term assay, observed that the digestive tract resulted filled with microplastics. In 2019 De Felice et al. observed that *D. magna* was able to efficiently ingest 1–10 μm PS with subsequent behavioural changes in terms of swimming activity, phototactic behaviour, and reproduction. The same authors explain that the swimming behaviour is closely related to filter-feeding activity and food uptake is one of the main driving forces of growth and reproduction.

Also in our study, we evidenced, through microscopic analysis, that the *C. dubia* gut was filled by the PS-MP after 24 h-exposure at concentrations ranging from 8.5 μg/L, through 85 mg/L (corresponding to 50% mortality) to 850 mg/L (corresponding to 100% mortality). In 2014 Besseling and coauthors observed that when the green chlorococcal alga *Scenedesmus obliquus* was exposed to PS (70 nm) from 0.22 to 103 mg/L for 5 d a decrease in terms of growth and chlorophyll density occurred, and when *D. magna* was fed by the same treated algae, malformation in body shapes, decrease in body size and in reproduction were detected. Therefore, from algae to zooplankton and fish, bioaccumulation of MPs in the aquatic trophic chain occurs unavoidably (Cederwall et al., 2012; Llorca et al., 2014; Lu et al., 2016). Hence, the ingestion of microplastic particles and the subsequent bioaccumulation occurs across taxa within different trophic chains, reaching humans via seafood and drinking waters (Chae et al., 2018; Koelmans et al., 2019; Shams et al., 2021). Furthermore, it has been demonstrated that the exposure of aquatic organisms to PS microparticles can cause not only short- and long-term toxicity, but also additional negative cellular effects including DNA damage and the reduction of various enzymes related to antioxidant and immune responses (Avio et al., 2015). In the present study we demonstrated that when *C. dubia* neonates were exposed to the PS-MP for 24 h, alterations in genetic material and production of ROS occurred starting from concentrations in the order of units of μg/L.

Basically, an increase in intracellular ROS levels is associated with oxidative stress condition where highly reactive species react with...
cellular macromolecules (Livingston et al., 2001) including DNA with formation of base adducts, single stranded breaks and base oxidation (Kehrer and Biswal, 2000). Our results are in line with previous studies where the exposure to 1 μm PS-microparticles or to PS-nanoparticles of shrimps and fish caused significant DNA damage, and production of ROS and pro-inflammatory reactions, respectively hypothesizing that plastic particles may increase the amount of ROS causing the breakage of DNA strands (Berber, 2019; Lee et al., 2019). Lin and collaborators in 2019 stated that PS increased the ROS level of D. magna because of mitochondrial dysfunction as well as the decrease of integrity of mitochondrial membranes. As reported by Kehrer and Biswal (2000) and Zhao et al. (2014), the induction of ROS would lead to lipid peroxidation leading to the destruction of cellular membranes. In the present study we used two different methods for the ROS production evaluation, reaching the highest ROS yield after homogenization of the organisms wholly exposed to PS-MPs thanks to the hydrolysis of DCFDA by intracellular esterase in DCFH that remains trapped within the cells and reacts with H₂O₂, generating the fluorescent 2′,7′-dichlorofluorescin (DCF) (Katerji et al, 2019).

In light of the results obtained in this study, we were curious to assess the risk quotient (RQ) as a measure of the risk posed by the PS-MP in a freshwater environment following the standard deterministic guidelines for the Environmental Risk Assessment (ERA), including the European Medicines Agency ((EMA guidelines 2006)) as well as the EU Technical Guidance Document (TGD) (2003) and, in second instance, according to Everaert and collaborators (2018) and Zhang and collaborators (2020) who specifically estimated the risk assessment of microplastics. Thus, the RQ value was calculated by the ratio between polystyrene micro-particles Measured Environmental Concentration (MEC), the actual detected concentration in the environment and the Predicted No Effect Concentration (PNEC) for which no adverse effects are expected to occur, ensuring environmental protection. The PNEC value is the ratio between a given endpoint (such as NOEC or EC₉₀) and an assessment factor. The size of the assessment factor depends on the available toxicity data, the number of trophic levels tested and the taxonomic groups. The proposed assessment factors are presented in Table S1.

Therefore, the concentration found for polystyrene micro-particles in freshwater equal to 13 ng/L (Schirinzi et al., 2019) was used as MEC. PNEC value was calculated by the ratio between the lowest long-term NOEC value and the appropriate assessment factor (AF). Although European regulatory guidelines require the evaluation of chronic effects on Daphnia magna (21 d), C. dabia can be considered a suitable surrogate for D. magna, providing comparable data within one-third the experimental time (Constantine and Hoggett, 2010). In this study, the lowest long-term NOEC value (0.09 μg/L) (Table 4) was found for C. dabia after 7 days of exposure. In addition, the size of the AF depended on the number of trophic levels considered which, in the present study, were two (alga, rotifers and crustaceans, producers and primary consumers) (Table S1). Therefore, considering all the parameters just mentioned, the value of PS-MP RQ was equal to 7.2 absolutely above the threshold value of 1, with a severe environmental concern for the freshwater ecosystem.

In light of the above, there is no doubt that during the COVID-19 era, the request, the production and the consumption of plastic materials, including polystyrene products have grown exponentially worldwide, along with the relative abundance in the environment of micro and nanoparticles derived from them. Our results have been referred to occurrence data from pre-Covid pandemic literature as, to the best of our knowledge, no further data is available. Therefore, considering the current increase of PS-MPs in freshwater it could be assumed that the estimated QR could be much higher than the calculated one.

5. Conclusions

Polystyrene is one of the most widely used plastic polymers, and the sudden Sars-CoV-2 pandemic has increased its release into the environment due to the widespread use of PPEs and food packaging. This research has provided solid information on the eco-genotoxicological implications of the 1 μm polystyrene, potentially considerable borderline size between micro- and nanoplastic particles, demonstrating that this contaminant poses a serious hazardous risk to freshwater organisms in the trophic chain. Since plastic particles could easily bypass conventional treatment plants, pollute aquatic environment, and enter the food chain, they could potentially act also as carriers of toxins, pathogens, hazardous substances, and pollutants. Future research should be conducted to understand the possible synergistic/antagonistic effects that result from their interactions. Since there are currently no effective solutions for the removal of microplastic particles from the environment, it would be necessary to adopt useful strategies to stimulate sustainable actions that prevent further plastic pollution after the Covid-19 pandemic.

Author statement

The authors’ responsibilities were as follows: MI and ML: designed the study, supervised the work, edited the paper; RN: designed the study, analysed data, and wrote the paper; CR and EO: contributed to study design, and wrote the paper; All authors read and approved the final manuscript. The authors declare that they have no conflicts of interest with the publication of this work.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.134373.

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