Impact of microplastic beads and fibers on waterflea (*Ceriodaphnia dubia*) survival, growth and reproduction: Implications of single and mixture exposures

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Abstract

There is limited knowledge regarding the adverse effects of wastewater-derived microplastics, particularly fibers, on aquatic biota. In this study, we examined the acute (48 h) and chronic (8 d) effects of microplastic polyester fibers and polyethylene (PE) beads on freshwater zooplankton *Ceriodaphnia dubia*. We also assessed the acute response of *C. dubia* to a binary mixture of microplastic beads and fibers for the first time. Acute exposure to fibers and PE beads both showed a dose-dependent effect on survival. An equitoxic binary mixture of beads and fibers resulted in a toxic unit of 1.85 indicating less than additive effects. Chronic exposure to lower concentrations did not significantly affect survival of *C. dubia*, but a dose-dependent effect on growth and reproduction was observed. Fibers showed greater adverse effects than PE beads. While ingestion of fibers was not observed, scanning electron microscopy showed carapace and antenna deformities after exposure to fibers, with no deformities observed after exposure to PE beads. While much of the current research has focused on microplastic beads, our study shows that microplastic fibers pose a greater risk to *C. dubia*, with reduced reproductive output observed at concentrations within an order of magnitude of reported environmental levels.

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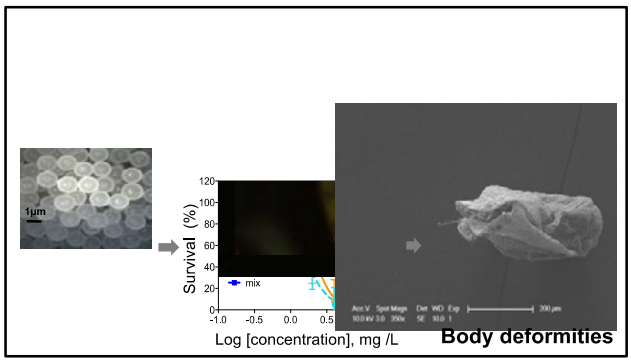

Keywords: *C. dubia*, microplastics, mixture effects, polyester fibers, polyethylene beads

1. INTRODUCTION

Microplastics are widespread emerging contaminants that have been found globally in the marine and freshwater environments.[1] Microplastics can enter the aquatic environment as both primary and secondary microplastics from aquatic and land-based sources.[2,3] Recently, wastewater treatment plant (WWTP) effluent was reported as a significant land-based source of microplastics to both the marine and freshwater environments.[4-6] Wastewater-derived microplastics originate from synthetic clothing and cleansing products, and primarily include polyester fibers and polyethylene (PE) beads and fragments.[4-6] These wastewater-based microplastics may be taken up as food by a variety of aquatic organisms.[7,8] For example, PE microplastics have been detected in the stomach of filter feeders (*Lepas sp*.).[9] Similarly, Taylor et al.[10] found microplastic fibers, including acrylic, polyester and polypropylene, in deep-sea organisms. Uptake of microplastics by aquatic organisms can lead to long-term accumulation of microplastic in their digestive tract, with one study reporting that PE microplastics make up as much as 58% of the stomach content of filter feeders (*Lepas sp*.).[9] This decreases the intake of actual food, which may adversely affect growth and reproduction rates.[11] In the long term, it can also lead to increasing mortality, due to blocking of the digestive tract or decreased nutrient uptake.[12]

Recent studies have demonstrated the trophic transfer of microplastics in aquatic food webs.[13,14] Consequently, it is important to understand the potential effects of microplastics on lower trophic levels organisms, such as zooplankton, as this may have implications for higher level organisms through biomagnification.[13] Ingestion of microplastics, such as fibers and fragments, has been reported in zooplankton in the Northeast Pacific Ocean, revealing the need for toxicity studies on such organisms.[15,16] Recently *Daphnia magna* has been used as a planktonic freshwater model organism for microplastic toxicity tests and this can provide insights into the potential effects of microplastics on lower trophic level organisms.[11,17] Further, the detection of microplastics, particularly fibers and beads, in freshwater ecosystems such as rivers, lakes and estuaries demonstrates the requirement for toxicity studies using freshwater organisms.[18-20]

In a recent study Rehse et al.[17] examined the short-term impact of two different size ranges of PE microplastics (1-4 μm and 100-106 μm) on *D. magna* and reported that only 1-4 μm microplastics were ingested, which is the size range that is preferably ingested by filter feeders. Rehse et al.[17] also reported no significant physical effects on *D. magna* after a 48 h short-term exposure to 1-4 μm microplastics at concentrations ranging from 12.5 mg/L (2.5×10^{10} microplastic particles/L) to 400 mg/L (8×10^{11} microplastic particles/L). However, after a prolonged exposure of 96 h, 75% immobilization was reported at the 200 mg/L concentration.[17] While ingestion of larger PE microplastics (100 μm) was not observed in Rehse et al.[17], a recent study by Jemec et al.[8] surprisingly reported uptake of large synthetic fibers (62-1400 μm) by *D. magna*, resulting in high mortality after a short-term exposure. Further, Ogonowski et al.[11] examined exposure to 1-5 μm PE microplastics at concentrations ranging from 10^5 to 10^8 particles/L on *D. magna* over 21 d and reported 50% mortality at the highest concentration. This study also found approximately 30% lower food intake after exposure to PE microplastics at 2.2×10^5 particles/L.

It should be mentioned that the high microplastic concentrations used in the reported studies are unlikely to be environmentally realistic. To date, there is no reported data on the concentrations of microplastics in the 1-20 μm size range due to technical limitations to isolate and characterize small microplastics in environmental samples.[21] However, it is generally assumed that the environmental concentrations of smaller microplastic particles are much higher than those currently reported for microplastics in the range of 20 to 300 μm in marine and freshwater ecosystems.[11,22,23]

In this study we examined the toxicity of two common wastewater-derived microplastics, namely PE beads and polyester fibers, following acute and chronic exposure in a freshwater zooplankton (*Ceriodaphnia dubia*) with a focus on mortality, growth and reproduction. We aimed to test lower microplastic concentrations than have previously been tested in *D. magna*, with the lowest fiber concentrations tested during chronic exposure experiments in the range of environmentally relevant concentrations previously reported for surface waters in the Southern North Sea (6.5×10^2 particles/L)[24] and in wastewater effluent (6.1×10^2 particles/L).[25] Higher

concentrations were used for the acute experiments, but it should be noted that the concentration of fibers and PE beads used in the current study were around 100 times lower than previously used in acute and chronic tests with *D. magna*.[8,11]

To date, studies have investigated the effects of individual microplastics on aquatic organisms; however, in the aquatic environment organisms are exposed to combinations of microplastics that may lead to additive, synergistic or antagonistic effects. While polyethylene and polyester have different densities, density modification[26] and other environmental factors such as mixing due to surface circulation and wind[27] can lead to the simultaneous occurrence of different types of microplastics in the water column. Therefore, we also investigated the mixture toxicity response by exposing *C. dubia* to a combination of PE beads and polyester fibers as both of these microplastics are found together in the aquatic environment.[28,29]

2. MATERIALS AND METHODS

2.1. Microplastics preparation for bioassays

Microplastic fibers were prepared by cutting the fleece surface of orange fluorescent clothing (100% polyester, density 1.38 g/cc) and chopping the fibers into small pieces. The chopped fibers were then soaked in ethanol (70%) overnight to remove possible contamination, washed with deionized water and dried at room temperature. Pristine spherical white 1-4 μm PE microplastic beads were supplied by Cospheric, USA (density of 0.987 g/cc). The pristine PE beads and cleaned fibers were used to limit potential contamination from plasticizers. Spherical polyethylene microplastics have been widely reported in cosmetic products with the size reported to be as small as 8 μm.[30] Stock solutions of microplastics at specific concentrations for bioassays were prepared by adding dry microplastics to moderately hard water (MHW), which was also used for bioassays. Since PE beads and polyester fibers have different densities than MHW and have a tendency to aggregate, a small amount (0.1% v/v) of Tween-20 surfactant (Sigma-Aldrich, USA) was used to disperse the microplastics.[11] To achieve a well-dispersed suspension the mixture was vigorously

mixed using a vortex (BioCot, Stuart) for 2 min after the addition of Tween-20 and treated in an ultrasonic bath for 30 min (Figure S1 in the Supporting Information (SI)). The suspension was then re-vortexed immediately before use in the bioassays.

2.2. Microplastics counting procedure

While microplastic toxicity studies typically use concentrations in mg/L units, microplastics detected in the aquatic environment are generally reported in number of particles/L. Therefore, it is necessary to convert between mg/L and number of particles/L to put the bioassay results into an environmental context. To determine the number of 1-4 μm PE beads in the stock solution we used a hemocytometer based on the same approach used for cell counting.[11] Counting was done with three replicates and the total number of microplastics per litre of stock solution was then calculated. The number of microplastics in each concentration (x) used for bioassays was then calculated using Equation 1, where $TMPs_{stock}$ is the total number of microplastics in the stock solution, C_x is the concentration (x) of microplastics in the bioassay and C_{stock} is the concentration of microplastics in the stock solution. More details about the concentrations of the stock solutions and the microplastics calculations are provided in the Section S1 of the SI.

$$\text{MPs (particles/L)} = \frac{(TMPs_{stock}(\text{particles/L}) \times C_x\ (\text{mg/L}))}{C_{stock}\ (\text{mg/L})} \quad (1)$$

Since fibers had a larger size range than the PE beads, the hemocytometer was not appropriate. Fiber counting was done using a subsample approach. Five subsamples of the 100 μL were taken from stock solution and microplastics were counted using a camera-connected Stereo Microscope (Olympus, SZX9, Japan). The 100 μL subsample was chosen as it could provide the best visual counting area under the microscope. To reduce error, the number of fibers in each subsample was recorded using the point-counting tool available in the CellSens Standard image analysis software. The counting was repeated twice for each of the five aliquots and the average

number of fibers was then calculated. The number of fibers at each concentration was calculated according to Equation 1.

2.3. Fiber characterization and size distribution determination

Fourier transform infrared spectroscopy (FTIR) with attenuated total reflection (ATR) mode on a Nicolet iS50 spectrometer, equipped with both an in-built diamond single bounce sampling accessory and a Continuum infrared microscope (Thermo Fisher Scientific, Madison WI, USA) was used to confirm the polymer type of the fibers. A sample of fiber was taken from the clothing used for bioassays, and pressed on to diamond crystal of the ATR accessory and their spectrum was obtained at 4 cm^{-1} resolution and 64 scans. Fluorescent fibers were also visually examined using a Nikon Eclipse 80i fluorescent microscope at 465-495 nm.

To determine the size distribution of the fibers used for the bioassays, 10 subsamples of 100 µL of stock solution were taken and the size of fibers in each subsample was obtained by measuring the length of fibers using image analysis software (Figure S2). This procedure was done in triplicate for each aliquot to determine the average size range of microplastic fibers in the stock solution.

2.4. Test organism (*C. dubia*)

The stock of *C. dubia* neonates was obtained from the laboratory stock at the Commonwealth Scientific and Industrial Research Organization (CSIRO), Adelaide, SA. Culturing was performed in 800 mL beakers using diluted mineral water and was maintained at 25°C in a photoperiod of 16 h light and 8 h darkness according to the US Environmental Protection Agency (USEPA) guidelines.[31] MHW supplemented with 2 µg/L selenium (Na_2SeO_4) was used as the test media for bioassays. The MHW was prepared in the laboratory using analytical grade reagents based on USEPA standard protocol.[31] All toxicity tests were performed using third brood neonates less than 24 h old.

177 **2.5. Bioassays**

178 **2.5.1. Single and mixture acute bioassays**

179 Three separate 48 h bioassays were designed to examine the short-term effects of
180 microplastics in *C. dubia*. The experimental design included single exposure to PE beads and
181 polyester fibers separately as well as exposure to a mixture containing both PE beads and polyester
182 fibers. For single acute exposure, *C. dubia* were exposed to a concentration range of 0.5 to 16 mg/L
183 of PE beads and 0.125 to 4 mg/L of polyester fibers, which corresponds to 1.7×10^4 to 5.4×10^5
184 particles/L for PE beads and 1.1×10^3 to 3.4×10^4 particles/L for polyester fibers. The studied
185 concentrations in both mg/L and number of particles/L can be found in the SI (Table S1).

186 The concentration range was selected based on preliminary range finding experiments
187 (Section S2; Table S2 in the SI). A 48 h acute immobilization test using copper(II) sulfate
188 pentahydrate ($CuSO_4.5H_2O$) as a known reference toxicant with a concentration range of 5 to 20
189 µg/L was carried out according to the USEPA guidelines to ensure that the *C. dubia* neonates were
190 appropriately sensitive.[32] Assay negative controls including a water control (MHW only) and a
191 solvent control (Tween-20, 0.1% v/v).

192 All bioassays were conducted in 50 mL glass beakers containing 25 mL test media and 5
193 cultured neonates were randomly transferred to each test vessel. No food was added during the
194 acute experiments and all treatments were done with four replicates. All treatment groups were
195 incubated at 25°C under constant conditions. After the 48 h exposure, water quality parameters such
196 as dissolved oxygen (DO), pH and electrical conductivity (EC) were measured (Hach, HQ40d, US).
197 The survival of neonates in each treatment group was recorded after 48 h using a stereo microscope
198 (Lecia Wild M3Z, US). Neonates that failed to move after 15 s of physical stimulation (gentle
199 prodding with a plastic pipette) were considered dead.[32] At the end of the test, alive and dead
200 individuals were collected for gut analysis and microscopy. The LC_{50} values and 95% confidence
201 interval (CI) for both the PE beads and polyester fibers were calculated. To reduce potential
202 microplastic contamination, all experiments were conducted in glass beakers, which were washed

with ultrapure water (18.2 MΩ•cm) prior to each test and were covered with cling wrap during handling and incubation. Additionally, new and unopened glass scintillation vials were used for microplastic stock solution preparation to avoid potential contamination.

A 48 h mixture exposure with both PE beads and polyester fibers was also designed to test the potential toxicity of microplastics in equitoxic mixtures. The concentrations used for the mixture toxicity tests were selected based on individual LC_{50} values for PE beads and polyester fibers, with identical fractions of their individual LC_{50} values for each microplastic.[33,34] Four concentrations below the LC_{50} value (1/16 LC_{50}, 1/8 LC_{50}, 1/4 LC_{50} and 1/2 LC_{50}), one at the LC_{50} value and one concentration above the LC_{50} value ($2\times LC_{50}$) were used (Table S3). The mixture exposure was conducted using the same procedure as described for the single acute tests.

2.5.2. Chronic bioassays

Two parallel 8 d exposure bioassays were conducted for PE beads and polyester fibers, according to USEPA protocol.[32] For both types of microplastics, *C. dubia* were exposed to six concentrations. The exposure concentrations used for chronic bioassays were 62.5 to 2000 μg/L for PE beads and 31.25 to 1000 μg/L for polyester fibers, which corresponds to 2.1×10^3 to 6.7×10^4 particles/L for PE beads and 2.7×10^2 to 8.6×10^3 particles/L polyester fibers. The details of used concentrations can be found in the SI (Table S4). It should be noted that the lowest tested fiber concentrations, including 31.25 μg/L (2.7×10^2 particles/L) and 62.5 μg/L (5.4×10^2 particles/L), were within the range of reported environmental concentrations; however, the higher concentrations were likely to be above environmental realistic levels.

At each experimental concentration, 1 neonate (<24 h old) was transferred to a 50 mL glass beaker containing 25 mL test media, with the media changed every 48 h. All treatment groups including negative controls (both MHW and Tween-20) were carried out with ten replicates. Before starting the test and every 48 h after media renewal, all test solutions were spiked with green algae (*Selenastrum capricornutum*) at a concentration of 8×10^5 cells/mL and orange algae (*Dunaliella*

salina) at a concentration 1 mL/L. Survival and the number of new offspring were recorded on a daily basis during the exposure period. At the end of the test, all adults and neonates were collected and fixed in glutaraldehyde (2.5%) and kept at 4°C for further inspection. The 8 d reproduction EC_{50} values and 95% confidence interval (CI) for both the PE beads and polyester fibers were calculated.

2.6. Visual analysis of *C. dubia*:

The gut content of *C. dubia* after acute and chronic exposures to microplastics was visually examined using a camera-connected stereo microscope (Olympus, SZX9, Japan) and image analysis software (cellSens Standard). All *C. dubia* samples were washed three times with ethanol (99%, Sigma Aldrich) before microscopy to remove glutaraldehyde. The gut of *C. dubia* exposed to different concentrations of PE beads and polyester fibers was visually inspected and compared to the negative control sample (MHW). To visually determine the fullness of the gut for PE exposed *C. dubia*, the gut was divided to five parts with specific percentile (Figure S3) and the percentage of gut fullness was determined accordingly.[35] To further inspect fluorescent fibers, *C. dubia* exposed to fibers were also inspected under a fluorescent microscope (Nikon Eclipse 80i) at 465-495 nm.

Growth was assessed by measuring the body size of all adults and neonates (up to 25 individuals) from each chronic treatment group using the stereo microscope in the same way as described for fiber size measurement.

2.7. Morphological analysis of *C. dubia*

Since visual inspections only provide information about the uptake of microplastics by *C. dubia*, scanning electron microscope (SEM) imaging was conducted on adult *C. dubia* to assess morphological alterations, such as deformities, after chronic exposure to better understand the adverse effects of microplastics. The samples were washed with 2% osmium tetroxide and gently dehydrated in an increasing series of ethanol (30, 40, 50, 60, 70, 80, 90, 100%). In the next step, the

samples were dried to the critical point in a critical point dryer (Leica EM CPD300). Prior to using SEM the samples were coated with a thin layer of platinum (approximately 10 nm) using a Cressington 208HR sputter coater. SEM images were obtained using a Philips XL30 FEG SEM, using secondary electron (SE) mode, a 10kV beam and spot size 3 at a 10mm working distance. Images were collected at various magnifications including at 200×, 350× and 800× for each sample.

2.8. Data analysis

Data were analyzed using GraphPad Prism (version 7) statistical software. Log-logistic concentration-effect curves were used to determine the LC_{50} and EC_{50} values and the 95% confidence intervals using non-linear regression. To determine the significance of effects in the chronic bioassays, data were analyzed by one-way analysis of variance (ANOVA) and statistical difference was set at $\alpha = 0.05$.

2.9. Mixture modeling:

The mixture effects of PE beads and polyester fibers were evaluated based on the toxic unit (TU) model, which is defined as the total of the effect contributions of each component in the mixture. The TU for mixture of microplastics was calculated using the following equation.[33]

$$TU = \frac{LC_{50}\ \text{PE (mix)}}{LC_{50}\ \text{PE (alone)}} + \frac{LC_{50}\ \text{fiber (mix)}}{LC_{50}\ \text{fiber (alone)}} \quad (2)$$

Using this model, TU less than one indicates more than additive effects (e.g. synergism), while a TU greater than one indicates less than additive effects (e.g. antagonism).

Further, the two common mixture toxicity models, concentration addition (CA) and independent action (IA), were applied to predict the effect of the binary mixture. CA assumes that the mixture components are acting according to the same mode of action, while IA assumes that the components have different modes of action.[36,37] Due to the different morphology of the microplastics, a common mode of action was not expected. The LC_{50} value based on the CA prediction ($LC_{50,mix}$) was calculated using Equation 3, where p_i is the fraction of each microplastic

component in the mixture and LC_{50i} is the LC_{50} value of each mixture component i. The effect based on independent action was calculated using Equation 4, where E_i represents the effect of each mixture component i.

$$LC_{50,mix} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{LC_{50i}}} \quad (3)$$

$$E_{IA} = 1 - \prod_{i=1}^{n} (1 - E_i) \quad (4)$$

It should be noted that all mixture toxicity calculations were conducted in units of particles/L, rather than mg/L. This is because we expect that the effect is related to the number of microplastics present, rather than their mass.

3. RESULTS AND DISCUSSION

3.1. Properties of fiber microplastics and size distribution

ATR-FTIR analysis confirmed that the textile fibers used for toxicity tests were polyethylene terephthalate (PET), a common polymer in the polyester family. The FTIR spectra are shown in Figure S4. Examining the size range of fibers used for bioassays showed a range from 25.7±10 to 1,150±160 μm with an average length of 280±50 μm. The majority of fibers were within the 100-400 μm size range (Figure S2).

3.2. Single acute effects

The acute and chronic LC_{50} values of reference toxicant (copper sulfate) in this study were in the normal range of 12.2 (95% CI: 10-14.8) and 13.1 (95% CI:11.9-14.4) μg/L, respectively. Mortality of negative controls (both MHW and Tween-20) was ≤5% and no significant difference was observed between the negative controls with and without Tween-20 (t-test, p=0.37). Further,

negative controls were checked visually under the microscope and no microplastic contamination was found in the control treatments. The recorded water quality parameters such as pH, EC and DO for acute bioassays with polyester fibers and PE beads after 48 h were comparable and within the recommended range based on USEPA protocols (Table S5).

The 48 h LC_{50} values for PE beads and polyester fibers were 2.2 mg/L (95% CI: 1.9-2.6) and 1.5 mg/L (95% CI: 1.3-1.7), respectively, which corresponds to 7.4×10^4 PE beads/L and 1.3×10^4 fibers/L (Table 1). The mortality of *C. dubia* after acute exposure to PE beads and polyester fibers increased with increasing concentration in a dose-dependent manner (Figure 1A). This differs from the findings of Jemec et al.[8] who did not observe a dose-dependent response in *D. magna* mortality during acute exposure to microplastic PET fibers with length range of 62-1400 μm, which is similar to the fibre size range in the current study. This could be attributed to the larger size of *D. magna* compared to *C. dubia*, as well as the different exposure conditions such as variable exposure of *D. magna* to microplastics fibers during bioassays due to sedimentation of microplastics,[8] with no sedimentation of PE beads or fibers observed in the current study.

Complete (100%) mortality was observed at concentrations of 4 mg/L (i.e. 3.4×10^4 particles/L) for polyester fibers and 8 mg/L (i.e. 2.7×10^5 particles/L) for PE beads during acute bioassays. Moreover, *C. dubia* exposed to fibers often showed abnormal swimming behavior, especially at the higher concentrations, and were found entangled in the fibers, resulting in inability to swim and complete immobilization. This observation may explain the higher toxicity of polyester fibers to *C. dubia* compared to PE bead microplastics. Previous research on a freshwater organism (*H. azteca*) also showed greater toxicity of microplastic fibers compared to PE microbeads during acute exposure, which was attributed to the longer residence time and slower egestion of fibers.[38]

3.3. Acute mixture effects

The TU of the equitoxic mixture of polyester fibers and PE microplastics was calculated at 1.85, indicating less than additive effects. In other words, the effect of the binary mixture was less

than expected based on the effects of the individual microplastics (Figure 1A). To further explore the mixture effects the models of CA and IA were applied, which assume that mixture components are either acting according to similar or dissimilar modes of action, respectively. These models are typically applied to chemical mixtures and to our knowledge have not been applied to mixtures of microplastics. While the modes of action by which these microplastics induce an effect in *C. dubia* is unclear given apical effects were studied, it appears that the microplastics are impacting on *C. dubia* through different exposure pathways. For example, *C. dubia* ingested PE beads, while fibers appeared to restrict the mobility of *C. dubia* through entanglement. Consequently, IA is expected to be a more representative model. The experimental LC_{50} value of the mixture was 8.7×10^4 particles/L, with the IA LC_{50} prediction within a factor of 1.3 of the experimental mixture (LC_{50} 1.2×10^5 particles/L). In contrast, the CA prediction was approximately a factor of 2 lower than the experimental mixture LC_{50} (LC_{50} 4.2×10^4 particles/L). This fits with observations from the literature for chemical mixtures that CA is the more conservative model,[39] though IA appears to be more representative in the current study.

The current study is the first to examine the mixture effects of microplastics. Similar to the transition from ecotoxicology to nanotoxicology [40], the application of conventional ecotoxicology methods and mixture toxicity models to microplastics requires further investigation. For example, the physicochemical properties of microplastics, such as their size and morphology, as well as their potential to aggregate and undergo sedimentation, can affect toxicity. Consequently, the application of mixture toxicity models developed for chemicals to microplastics needs further work, including using different microplastic mixture ratios, as only one equitoxic mixture was considered in the current study.

3.4. Chronic effects

Sensitive endpoints of growth, reproduction and time to first brood were examined during chronic exposure of *C. dubia* to PE beads and polyester fibers (Figure 2, Tables 3 and 4). Mortality

of the negative controls (both MHW and Tween-20) was observed to be ≤5% and no microplastic contamination was found in the negative control samples. The mean time to first brood did not significantly change (ANOVA p=0.3) with increasing test concentrations for PE beads and polyester fibers, and was calculated between 4.0-4.5 d for PE beads and 4.0-4.4 d for polyester fibers (Tables S6 and S7). A dose-response relationship was observed during chronic exposure with a significant reduction in number of neonates with increasing microplastic concentration (Figures 1B and 2, Tables 3 and 4). The survival rate of *C. dubia* adults was observed to be ≥90% for all studied concentrations except at the highest concentration for both PE beads and polyester fibers, which both induced 40% mortality (Tables 3 and 4). Despite the excellent survival of adults during chronic exposure, the body size of adults and the number of neonates were negatively affected by exposure to both PE beads and polyester fibers (Figure 2). With polyester fibers, a significant reduction in neonate numbers and adult body size was observed at a concentration of 500 μg/L (i.e. 4.3×10^3 particles/L) and above (Figure 2B), while higher exposure to PE microbeads was needed to produce a similar effect (1000 and 2000 μg/L (i.e. 3.3×10^3 and 6.7×10^4 particles/L) for neonate numbers and adult body size, respectively; Figure 2A).

Although exposure to both PE beads and polyester fibers resulted in decreased body size and reduced the total number of neonates, the effect with polyester fibers was more pronounced. For example, a concentration of 1000 μg/L (i.e. 3.3×10^4 particles/L) of PE microbeads produced a 56% reduction in the total number of neonates compared to the negative control (MHW), whereas the same exposure concentration of polyester fibers significantly (ANOVA, P=0.0001) reduced number of neonates by 84% compared to the negative control (Figure 2). The EC_{50} values for reproduction also indicated greater adverse effect of polyester fibers (EC_{50} 429 μg/L (95% CI: 345-539)) compared to PE beads (EC_{50} 958 μg/L (95% CI: 760-1353)) (Table 2). No significant difference was found in the body length of neonates after both PE bead and polyester fiber exposure (Tables S6 and S7). It should be noted that microplastic fibers within the range of environmentally relevant concentrations ($6.1\times10^2 – 6.5\times10^2$ particles/L[24,25]) did not have a significant effect on the exposed

organisms, with adverse effects on reproduction and adult body size occurring at concentrations around six times higher than previously reported in the environment.

Exposure to PE beads was expected to lead to accumulation of microplastics in the digestive tract, given they are in the size range of *C. dubia*'s typical food source. The inability for self-cleaning and egestion of microplastics may lead to blockage of the digestive tract and inhibition of food uptake.[41] The reduced food consumption rate in the presence of microplastics has previously been reported in other aquatic organisms such as crab and lugworm.[41,42] During chronic exposure, the lower food uptake would negatively impact the level of energy reserves, likely forcing *C. dubia* to preferentially invest more of the limited available energy in survival rather than growth and reproduction, resulting in reduced number of offspring. This was previously observed for *D. magna* after exposure to silver nanoparticles.[43] In the current study, chronic (8 d) exposure to both PE beads and polyester fibers resulted in a decreased reproductive output (Figures 1B and 2). A positive correlations between depletion of energy reserves and reduced reproduction rate in *D. magna* has been reported after exposure to nanopolystyrene.[23]

Abnormal swimming behavior was only observed in *C. dubia* exposed to polyester fibers, with their movement often inhibited as a result of entanglement in twisted fibers. While ingestion of fibers was not observed in the gut of *C. dubia* using the stereo microscope, the reduced reproduction and growth seen during chronic exposure to fibers is likely to be associated with inability to tolerate fibers as a stressor in the environment and loss of energy as a response to physical contact with fibers and damage to body. The potential for physical damage was investigated further in Section 3.5 below.

While microplastic beads and fibers are negatively affecting growth and reproduction of *C. dubia*, the mode of action of microplastics, particularly fibers, and effects on the cellular function of *C. dubia* are unknown. Jeong et al.[44] has recently provided first evidence regarding the mode of action of nano-sized microplastics in a marine copepod (*P. nana*). This study showed permeation of nano-sized polystyrene microbeads (0.05 μm) to the cell membrane and induction of the oxidative

409 stress response, leading to cell damage and reduction of growth rate and reproduction output.[44]
410 Further work is required to understand the mode of action of larger microplastics.
411
412 **3.5. Visual and morphological analysis**
413 The gut content of all surviving *C. dubia* after acute and chronic exposure was visually
414 analyzed using a stereo microscope. White PE beads were observed in the gut of *C. dubia* after 48 h
415 exposure to all concentrations (0.5 to 16 mg/L) (Figure 3B). The level of gut fullness increased with
416 test concentrations. For example, the percentage of gut fullness increased from <50% at
417 concentrations of 0.5 and 1 mg/L to 100% at concentration of 4 mg/L (Figure S3). However at
418 concentrations above 4 mg/L the gut of all examined organisms was observed as 100% full.
419 The test organisms exposed to fibers were inspected under both stereo and fluorescent
420 microscope. While no fibers were observed in the gut of *C. dubia*, small bubbles were observed
421 under the carapace of exposed organisms to fibers (Figure 3A), which increased with increasing
422 exposure concentrations and is likely a response to environmental stress. A similar phenomenon has
423 previously reported for *D. magna* exposed to silver nanowires.[45] PE beads were also found in the
424 gut of surviving *C. dubia* after chronic exposure, which was correlated to the test concentration. For
425 instance, higher level of gut fullness was observed at the highest concentrations while the gut of *C.*
426 *dubia* exposed to the lower concentrations only showed spots of microplastics (Figure S5).
427 Apart from reduced body size of *C. dubia* after exposure to polyester fibers and PE beads
428 (Figure 2), we also observed deformations in the body of *C. dubia* after 8 d exposure to polyester
429 fibers using scanning electron microscopy. Deformities, such as carapace and antenna deformities,
430 were observed at polyester fiber concentrations of 500 μg/L (4.3×10^3 particle/L) and 1000 μg/L
431 (8.6×10^3 particle/L), with completely deformed carapaces (Figure 4 A) compared to the negative
432 controls (MHW) (Figure 4 C). Moreover, the seta of the antenna displayed an abnormal shape and
433 were split (Figure 4 B) compared to the negative control (Figure 4 D), which could be due to
434 physical contact with the fibers. Although damage to antenna may potentially be caused by

handling during sample preparation for SEM, we did not find the same damage in the negative control organisms, nor in *C. dubia* exposed to the lower concentration of fibers (Figure S6). Interestingly, we did not observe any noticeable deformations in *C. dubia* exposed to PE beads. This observation may explain the greater adverse effects in *C. dubia* after exposure to polyester fibers.

3.6. Implications and outlook

The results from this study demonstrated dose-dependent effects after acute and chronic exposure to both PE beads and polyester fibers, with fibers consistently showing greater negative effects than PE beads. Further, the microplastic fibers caused a 50% reduction in reproductive output of *C. dubia* at concentrations approximately six times higher than the reported environmental concentrations. Consequently, more subtle effects may occur at lower concentrations. Unlike previous studies, we did not observe any ingested fibers in *C. dubia*. However, malformations were observed in the carapace of organisms exposed to polyester fibers. This demonstrates that the adverse impact of microplastic fibers on exposed aquatic organisms is not solely due to ingestion but also external physical damage, and that the latter can significantly affect survival, growth and fecundity of *C. dubia.* We have also evaluated the short-term effect of a binary mixture of PE beads and polyester fibers, which is the first of its kind to be reported for microplastics. The results showed less than additive effects after acute exposure to a mixture of PE bead and polyester fiber microplastics, with the effect of the mixture similar to the predicted effect based on the model of independent action. It should be noted that applying conventional toxicity testing methods to microplastics, as well as mixture toxicity models developed for chemicals, may have limitations and requires further validation. Therefore, it is important to identify the mode of action and to develop new approaches for microplastic toxicity testing in the future. We also suggest more studies on the acute and chronic effects of binary mixture with different types of microplastics to provide better predictions on mixture effects.

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Supporting Information: Further information about microplastic counting and preliminary bioassays, as well as additional bioassay data, is provided.

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588

589 **Table 1**: *C. dubia* 48 h (acute) lethal concentrations (LC_{50} and LC_{10}) for PE beads and polyester
590 fibers in mg/L (95% confidence interval (CI)) based on survival (Figure 1A), with number of
591 particles/L at each effect concentration.

Test material	LC_{50}		LC_{10}		Slope	df	R^2	SS	Sy.x
	mg/L	Number of particles	mg/L	Number of particles					
Polyester fibers	1.5 (1.3-1.7)	1.3×10^4	0.6 (0.4-0.9)	5.5×10^3	-2.4	26	0.94	4618	9.9
PE beads	2.2 (1.9-2.6)	7.4×10^4	1.1 (0.7-1.8)	3.9×10^4	-3.5	22	0.90	2592	14.4

592 df: degrees of freedom; SS: absolute sum of squares; Sy.x: standard error of the estimate

593

594 **Table 2:** *C. dubia* 8 d (chronic) effect concentrations (EC_{50} and EC_{10}) of PE beads and polyester
595 fibers (95% confidence interval (CI)) based on reproduction output (Figure 1B), with number of
596 particles/L at each effect concentration.

Test material	EC_{50}		EC_{10}		Slope	df	R^2	Sy.x
	µg/L	Number of particles	µg/L	Number of particles				
Polyester fibers	429 (345-539)	3.5×10^3	208 (136-325)	2.4×10^3	-3.1	58	0.75	28
PE beads	958 (760-1353)	3.2×10^4	84.3 (29.1-244)	2.7×10^3	-0.8	58	0.58	16

597 df: degrees of freedom; Sy.x: standard error of the estimate.

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599

600

601 **Table 3:** Survival and reproduction of *C. dubia* exposed to PE beads during chronic bioassays.

Concentration (µg/L)	Adult Survival (%)	Number of neonates in each brood (mean ± S.D.)		602
		First brood	Second brood	Third brood
Negative Control	100	3.0 ± 0.7	6.8 ± 2.3	11.2 ± 4.1
62.5	100	3.2 ± 0.7	5.7 ± 1.0	9.6 ± 4.3
125	100	2.4 ± 0.9	4.9 ± 1.1	7.5 ± 3.2
250	100	2.5 ± 1.1	4.0 ± 1.9	9.1 ± 4.7
500	100	2.7 ± 0.4	5.0 ± 0.8	7.1 ± 2.9
1000	100	1.5 ± 1.4	4.9 ± 2.9	5.5 ± 1.5*
2000	60	0.9 ± 0.9	3. ± 1.9*	3.5 ± 2.1*

603 Note: * shows significant difference ($p < 0.05$); Negative Control represents MHW.

604

605 **Table 4:** Survival and reproduction of *C. dubia* exposed to polyester fibers during chronic

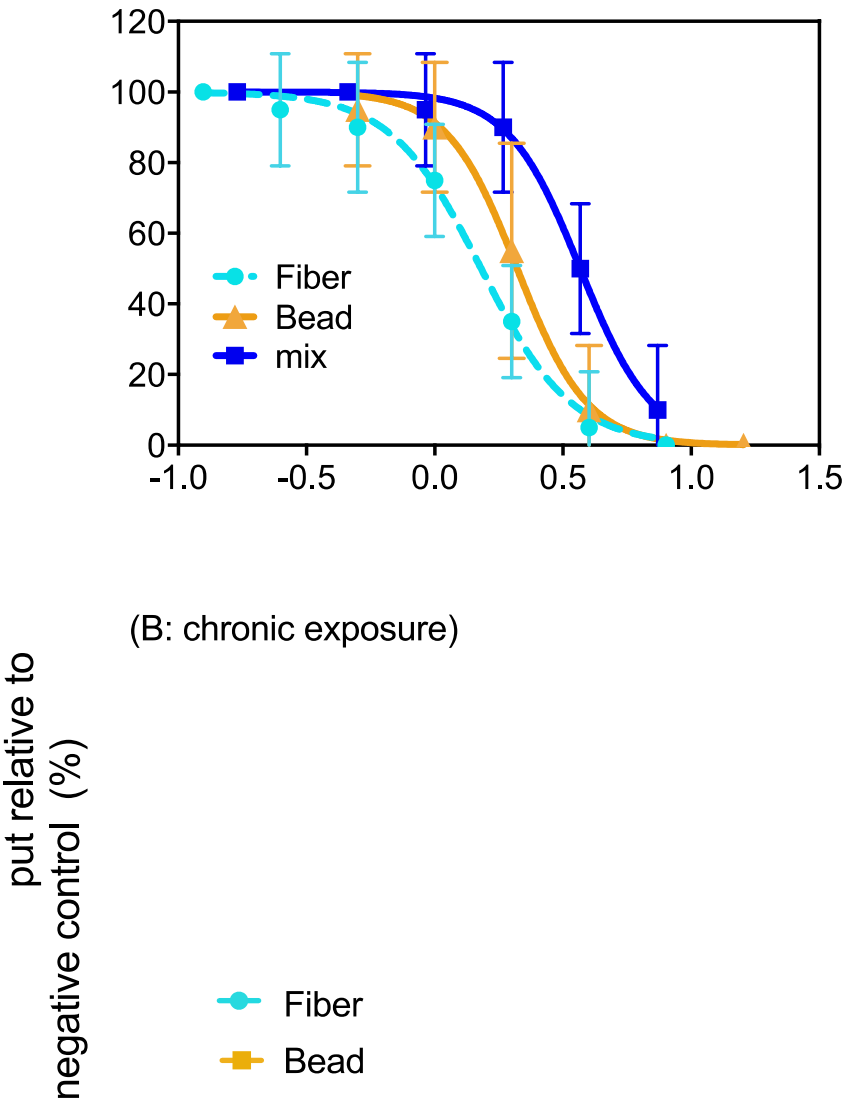
606 bioassays.

Concentration (µg/L)	Adult Survival (%)	Number of neonates in each brood (mean ± S.D)		
		First brood	Second brood	Third brood
Negative Control	100	3.0 ± 1.2	4.9 ± 1.3	16.3 ± 3.2
31.25	100	3.0 ± 0.6	6.1 ± 1.6	17.9 ± 0.9
62.5	100	2.8 ± 1.0	9.6 ± 4.3*	16.7 ± 7.0
125	100	2.7 ± 0.8	5.4 ± 0.8	20.3 ± 2.2*
250	100	2.9 ± 0.8	3.9 ± 1.8	12.5 ± 5.1*
500	90	1.8 ± 0.8	2.5 ± 1.6*	5.3 ± 3.5*
1000	60	1.8 ± 0.9	0.2 ± 0.4*	2.8 ± 2.6*

607 Note: * shows significant difference ($p < 0.05$); Negative Control represents MHW.

List of figures

634 **Figure 1**



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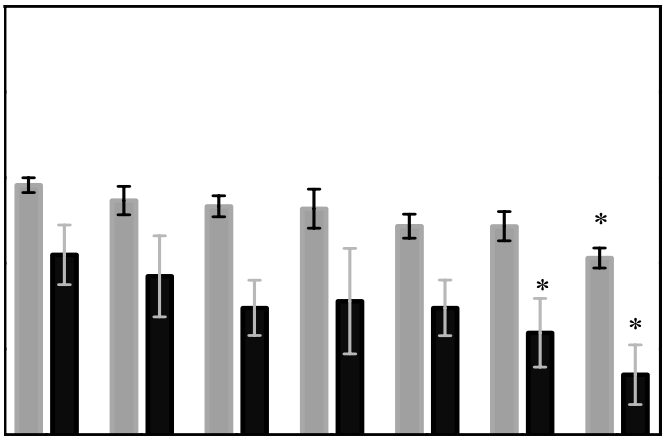
642 **Figure 2**



(B: polyester fibers)

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Figure 3

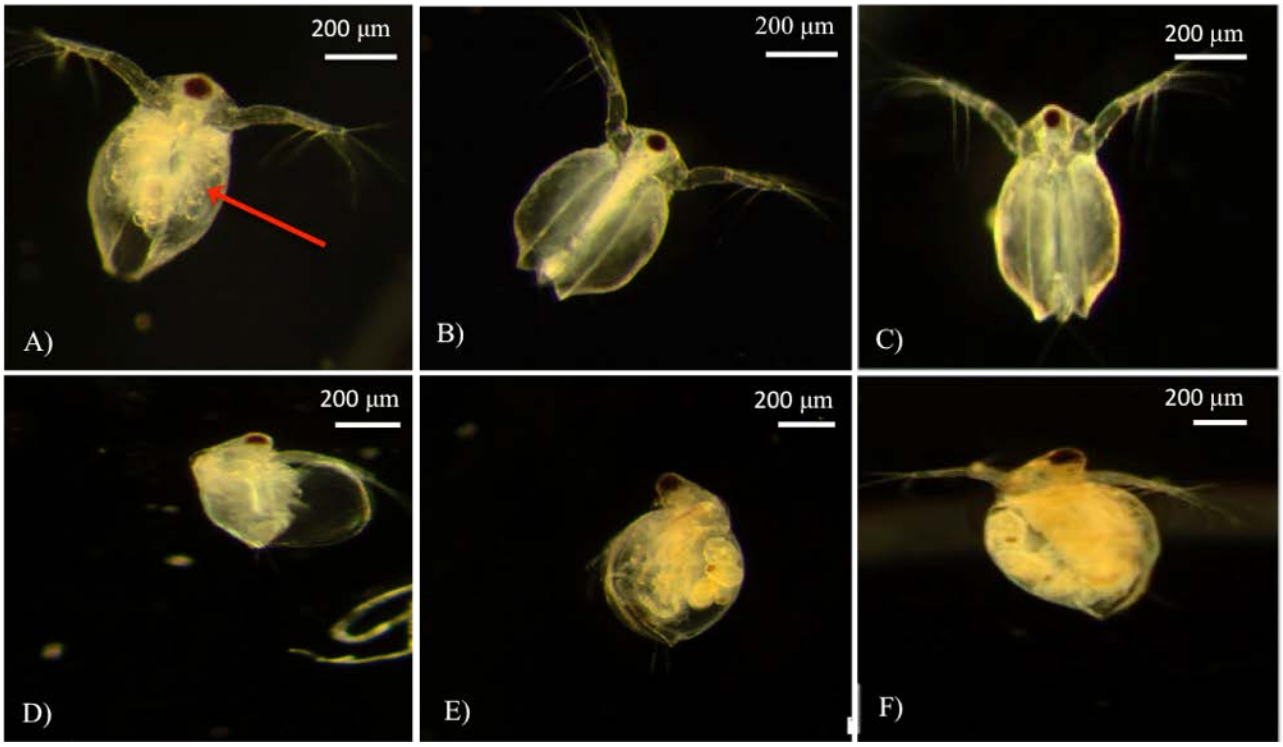
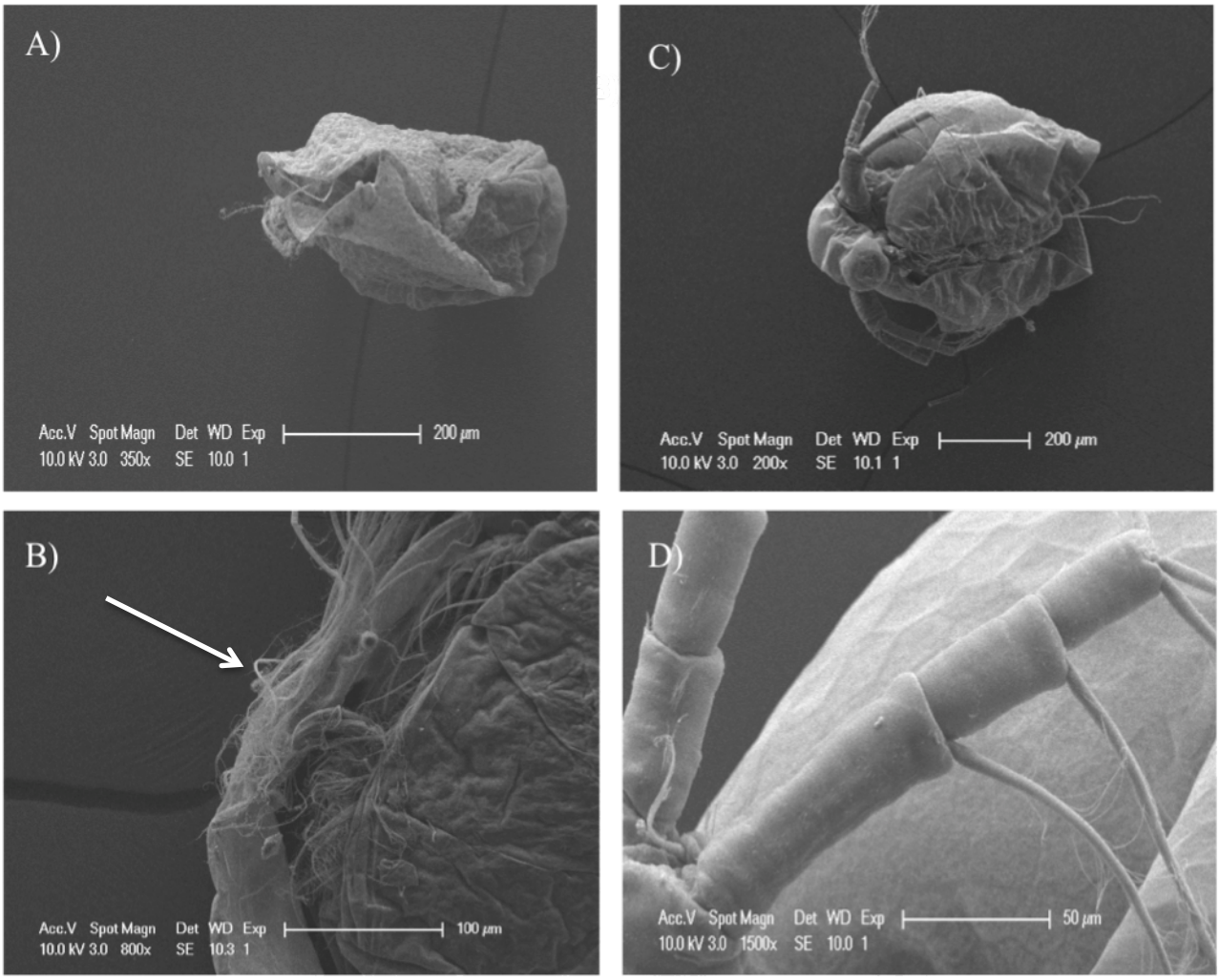


Figure 4


A)
Acc.V Spot Magn Det WD Exp 200 µm
10.0 kV 3.0 350x SE 10.0 1
C)
Acc.V Spot Magn Det WD Exp 200 µm
10.0 kV 3.0 200x SE 10.1 1
B)
Acc.V Spot Magn Det WD Exp 100 µm
10.0 kV 3.0 800x SE 10.3 1
D)
Acc.V Spot Magn Det WD Exp 50 µm
10.0 kV 3.0 1500x SE 10.0 1