Science of the Total Environment 619–620 (2018) 1–8

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*

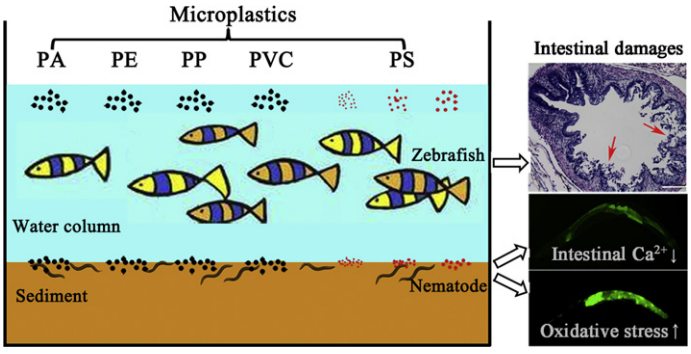
Lili Lei [a], Siyu Wu [a], Shibo Lu [a], Mengting Liu [a], Yang Song [a], Zhenhuan Fu [a], Huahong Shi [b], Kathleen M. Raley-Susman [c], Defu He [a,d,*]

[a] Lab of Toxicology, School of Ecological and Environmental Sciences, East China Normal University, 500# DongChuan RD, Shanghai 200241, China
[b] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200062, China
[c] Department of Biology, Vassar College, Poughkeepsie, NY 12604, USA
[d] Shanghai Key Laboratory for Urban Ecological Processes and Eco-Restoration, East China Normal University, Shanghai 200241, China

HIGHLIGHTS

- Toxicity was comparatively studied on five common types of microplastics (MPs).
- MPs with similar size induced intestine enterocyte damages in *Danio rerio*.
- MPs reduced Ca^{2+} but increased *gst-4* expression in intestine of *Caenorhabditis elegans*.
- 1.0 μm MPs caused stronger toxicity than 0.1 or 5.0 μm MPs in *Caenorhabditis elegans*.
- Intestine damages are key effects of pristine MPs mostly dependent on their sizes.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 September 2017
Received in revised form 8 November 2017
Accepted 8 November 2017
Available online 11 November 2017

Editor: D. Barcelo

Keywords:
Microplastics
Intestinal damages
Caenorhabditis elegans
Danio rerio
Oxidative stress
Ecotoxicity

ABSTRACT

Microplastics have been frequently detected in aquatic environments, and there are increasing concerns about potential effects on biota. In this study, zebrafish *Danio rerio* and nematode *Caenorhabditis elegans* were used as model organisms for microplastic exposure in freshwater pelagic (*i.e.* water column) and benthic (*i.e.* sediment) environments. We investigated the toxic effects of five common types of microplastics: polyamides (PA), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and polystyrene (PS) particles. Results showed no or low lethality in *D. rerio* after exposure for 10 d at 0.001–10.0 mg L^{-1} microplastics. The PA, PE, PP and/or PVC microplastics with ~70 μm size caused intestinal damage including cracking of villi and splitting of enterocytes. Exposure to 5.0 mg m^{-2} microplastics for 2 d significantly inhibited survival rates, body length and reproduction of *C. elegans*. Moreover, exposure to microplastics reduced calcium levels but increased expression of the glutathione S-transferase 4 enzyme in the intestine, which indicates intestinal damage and oxidative stress are major effects of microplastic exposure. Among 0.1, 1.0 and 5.0 μm sizes of fluorescently labeled PS, 1.0 μm particles caused the highest lethality, the maximum accumulation, the lowest Ca^{2+} level in the intestine and the highest expression of glutathione S-transferase 4 in nematodes. Taken together, these findings suggest that intestinal damage is a key effect of microplastics; and that the toxicity of microplastics is closely dependent on their size, rather than their composition.

* Corresponding author at: Lab of Toxicology, School of Ecological and Environmental Sciences, East China Normal University, 500# DongChuan RD, Shanghai 200241, China.
E-mail address: dfhe@des.ecnu.edu.cn (D. He).

https://doi.org/10.1016/j.scitotenv.2017.11.103
0048-9697/

1. Introduction

In the past 60 years, plastic production has rapidly risen with the result of an accumulation of plastic debris all over our planet (Bouwmeester et al., 2015). Thus, microplastics are so ubiquitous that they can be frequently detected in aquatic environments including oceans, lakes and rivers, even in sediments and marine products such as seafood and salts (Van Cauwenberghe et al., 2013; Eerkes-Medrano et al., 2015; Li et al., 2015; Paço et al., 2017; Wright and Kelly, 2017). Initially, scientific and public attention mostly focused on large plastic debris. During the last few years, microplastics have received an increasing attention and now have become an emerging area of research (Duis and Coors, 2016; Horton et al., 2017; Wang et al., 2017; Wright and Kelly, 2017). Microplastics are plastic debris smaller than 5 mm (GESAMP, 2015), and are often defined as solid synthetic organic polymers with sizes between 100 nm and 5 mm (Duis and Coors, 2016; Chen et al., 2017; Wright and Kelly, 2017). Primary microplastics come from human materials including cosmetic products, synthetic clothes, drug vectors and car tires (Browne et al., 2011; Paço et al., 2017). Another key source of microplastics arises from the breakdown of large plastic debris (Ivar do Sul and Costa, 2014; Lambert and Wagner, 2016; W.C. Li et al., 2016).

Microplastics are heterogeneous groups of particles differing in size, shape, and chemical composition which may be linked to their toxicity (Jeong et al., 2016; McDevitt et al., 2017). According to the monomer structure of the polymer's backbone, major microplastics can be classified as polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC) and polyamides (PA) (Bouwmeester et al., 2015; W.C. Li et al., 2016). Studies have demonstrated that microplastics and conjoint contaminants can be ingested and accumulated in aquatic organisms (Watts et al., 2014; Cole and Galloway, 2015) and terrestrial invertebrates (Wardrop et al., 2016; Hodson et al., 2017; Nelms et al., 2017), but the toxicity effects of different types of microplastics are largely unknown (Bakir et al., 2014; Hamlin et al., 2015; Hu et al., 2016). One recent study by Karami et al. (2017) reported no significant histological alterations and biomarker changes in zebrafish after exposure to PE fragments. In contrast, Lu et al. (2016) demonstrated signs of inflammation, lipid accumulation in the liver, as well as higher superoxide dismutase and catalase levels in *Danio rerio* after exposure to PS microspheres, indicative of oxidative stress.

Zebrafish *Danio rerio* is a small tropical freshwater fish used as a vertebrate model for toxicological studies due to its small size as well as ease of breeding and rearing (Spitsbergen and Kent, 2003). Additionally, the free-living nematode, *Caenorhabditis elegans* is the most abundant animal in soil ecosystems and is also found in aquatic environments. Hence *C. elegans* has emerged as an important animal model for benthic sediment toxicity assays (J. Li et al., 2016; Xu et al., 2017b). Furthermore, fluorescently-labeled transgenic strains of nematodes are available for investigating toxicological mechanisms (Xu et al., 2017a, 2017b). In this study, we examined *D. rerio* and *C. elegans* for toxicity assessment in pelagic and benthic environments.

We selected five common types of unplasticized and virgin microplastics: polyamides (PA), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and polystyrene (PS). A series of concentrations of microplastic particles of nearly the same size (PA, PE, PP, PVC) or different sizes (PS) were exposed to *D. rerio* and *C. elegans*. The survival rates and histopathological changes were investigated in *D. rerio*. Using fluorescently labeled transgenic nematodes, lethality, body size and reproductive toxicity, oxidative damage and calcium levels in the intestine were further examined after chronic exposure. The aim of this study was to investigate and compare toxic effects among the five types of microplastics in these two organisms.

2. Materials and methods

2.1. Microplastic particles and their characterization

Five types of microplastics, PA, PE, PP, PVC and PS, were used in the present study (Table S1). The PA, PE, PP and PVC microplastics were ground into particles with similar sizes using a mortar, and then sifted through a 200 mesh sieve. The morphology of these microplastics particles was observed and photographed using a Motic microscope and Images Advanced 3.2 software (Motic Electric Group, Xiamen, China). The mean diameter of PA, PE, PP and PVC particles was ~70 μm (Fig. 1a–h). The PS particles included three nominal sizes of 0.1 μm, 1.0 μm and 5.0 μm (Fig. 1i–k). Two kinds of PS particles were selected for study. One was virgin PS particles for the toxicity test. The other was red-fluorescently-labeled PS particles (with excitation/emission wavelengths of 565/670 nm), which were used to observe the distribution of microplastic particles in *C. elegans*. The distributions of PS diameters were detected using Zetasizer Nano ZS90 (Malvern Instruments, UK) (Fig. 1l–n). The composition of virgin PA, PE, PP, PVC and PS microplastics were assessed and confirmed by Fourier-transform infrared spectroscopy (FTIR) (Thermo Fisher Scientific, USA) (Fig. S1).

2.2. Zebrafish and exposure

Adult healthy zebrafish (*Danio rerio*; 2.5 ± 0.5 cm in body length) were maintained at 23 ± 1 °C with a 12 h light/dark photoperiod in culture water. Ultraviolet-sterilized and well-aerated water was used. The pH value, dissolved oxygen (DO) and water hardness ($CaCO_3$) were controlled at 7.2 ± 0.4, 6.6 ± 0.5 mg L^{-1} and 185 ± 10 mg L^{-1}, respectively. Fish were acclimated in 15 L glass tanks for 1 week before the experiment. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The suspensions of PA, PE, PP, PVC and PS particles were prepared using Ultraviolet-treated dechlorinated tap water and sonicated prior to exposure (Lu et al., 2016; Karami et al., 2017). The concentrations of suspensions were diluted into a series of 0.001, 0.01, 0.1, 1.0 and 10.0 mg L^{-1} for each type of microplastics. The dechlorinated tap water was used as the control group. Sixteen zebrafish were randomly added to 1.6 L exposure water for each concentration group. Three replicates were used for each exposure group (n = 48). A total of 1728 healthy fish were chosen and used in the experiments. The DO, pH value and temperature were recorded daily during the experiment. *D. rerio* were exposed to five types of microplastics for 10 days. The test solutions were completely replaced with fresh microplastic suspensions every 2 days. Fish were fed with commercial food (Inch-gold, Huizhou, China) twice a day. During exposure, dead fish were taken out immediately. The survival rates were calculated at the end of the exposure period.

2.3. Histopathology investigations in zebrafish

After exposure, 15 surviving fish were randomly chosen for histopathology investigation in each microplastics group at the exposure concentration of 1.0 mg L^{-1}. After fixing in 10% formalin, these fish were embedded in paraffin wax, sectioned at 5 μm thickness, and then stained with hematoxylin and eosin for microscopic observation. Coronal sections were cut in the abdomen; gill, liver, kidneys and intestine of each individual before being observed for histological changes. Intestinal damage, observed as inflammation or broken tissue, was assessed by assigning a score value ranging from 0 to 4 (0 normal; 1 slight; 2 moderate; 3 pronounced; 4 severe damage) as previously described (Table S2) (Bernet et al., 1999; Pereira et al., 2017). Additionally, intestinal fold architecture disruption and enterocyte damage were assessed as previously described (Fig. S2; Brugman et al., 2009).

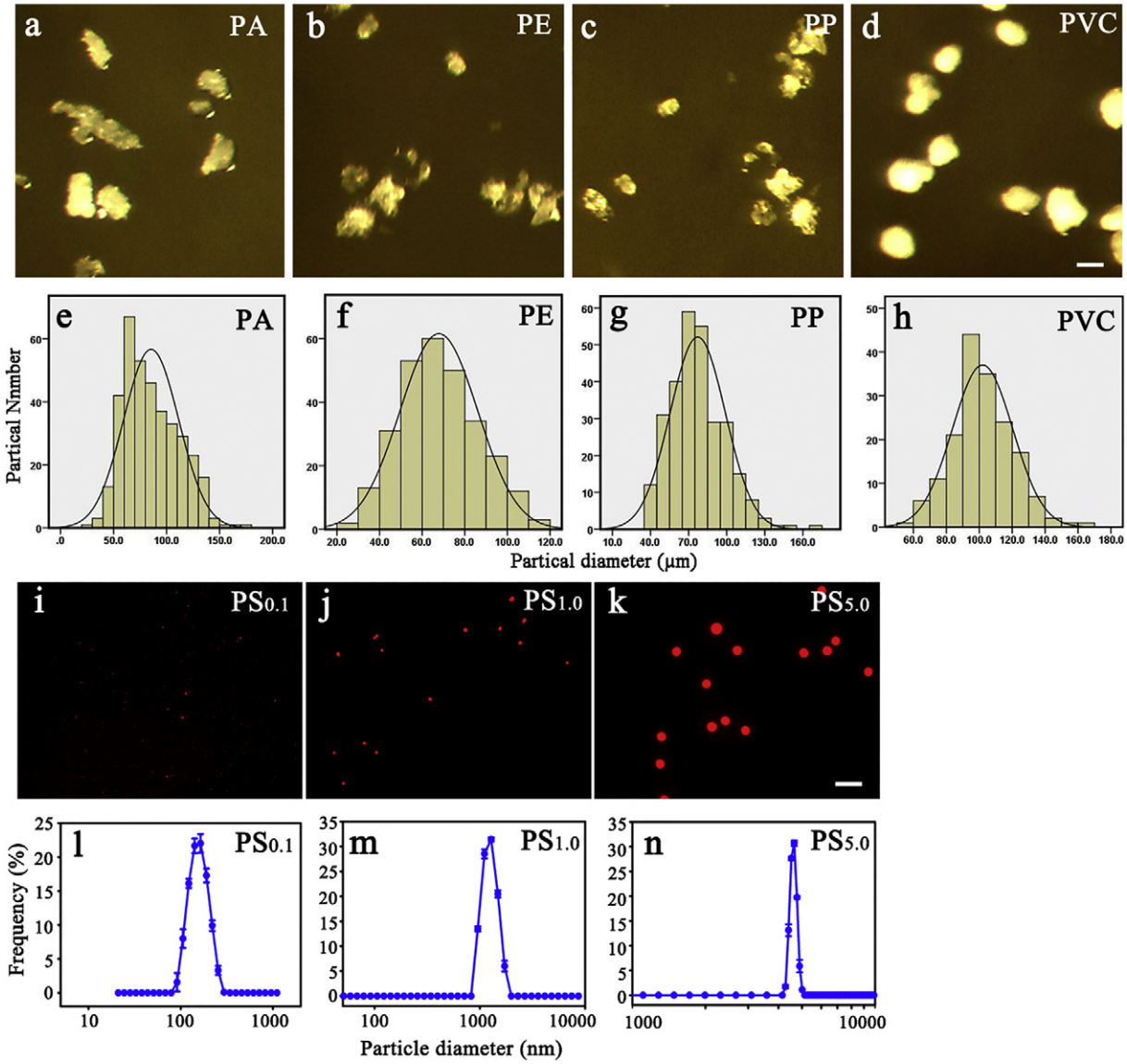


Fig. 1. Photomicrographs (a–d, i–k) and particle size distribution (e–h, l–n) of microplastic particles in the present study. Bar = 40 μm for a–d, or 10 μm for i–k.

2.4. C. elegans strains and exposure

All strains of *C. elegans* were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA) and maintained according to standard techniques as previously described (Brenner, 1974). Wild-type (Bristol, N2) and transgenic strains were used, including KWN190 (*nhx-2p*::*D3cpv* + *pha-1*(+)) (Zhang et al., 2016) and CL2166 ((*pAF15*)*gst-4p*::*GFP*::*NLS*) (Hunt et al., 2011). KWN190 and CL2166 transgenic strains were used to investigate intestinal calcium levels and oxidative damages in worms (Hunt et al., 2011; Zhang et al., 2016). Nematodes were maintained on nematode growth medium (NGM) petri plates, seeded with *Escherichia coli OP50* at 20 °C (Xu et al., 2017a). Using previous methods described in Xu et al. (2017a, 2017b), brood stage nematodes were collected into 1.5 mL centrifuge tubes by washing plates with M9 buffers and dissolving with bleaching solutions (0.45 M NaOH, 2% HClO). After centrifugation, eggs were washed three times with M9 buffers in order to remove the bleaching solutions. The eggs were hatched on a new plate coated with food and used for toxicity experiments.

Five types of microplastic suspensions were prepared in K-medium (32 mM L^{-1} KCl, 51 mM L^{-1} NaCl). The microplastic suspensions were added to the surface of NGM agar and the control group was seeded with *Escherichia coli OP50*. Exposure concentrations were designed as a series of 0.5, 1.0, 5.0 and 10.0 mg m^{-2}. Each well contained about 30 age-synchronized nematodes. L2 stage nematodes were exposed for 2 days with four parallel experiments. After exposure, the number of dead nematodes was recorded; the survival rates were calculated (Xu et al., 2017a, 2017b).

2.5. Body length, number of eggs and brood size in C. elegans

The L2 stage nematodes were exposed to 5.0 mg m^{-2} microplastics of PA, PE, PP, PVC or PS particles until they reached the adult stage. After that, worms were transferred to agar-padded slides and sealed with coverslips. The body length of nematodes and number of eggs per worm were respectively determined. Body length and egg numbers were examined from at least 20 nematodes in each group. For brood size assay, nematodes were individually transferred to new NGM plates. The newborn nematodes were counted until the completion of the egg laying. All experiments were performed with four replicates (Xu et al., 2017a, 2017b).

2.6. Distribution of fluorescently labeled PS microplastic in C. elegans

After *C. elegans* were exposed to fluorescently-labeled PS particles with nominal sizes of 0.1, 1.0 and 5.0 μm, the washed nematodes were transferred to agar-padded slides, immobilized with 100 mM sodium azide and then sealed with coverslips. Images of nematodes were captured to assay fluorescence intensity using a fluorescence microscope (LEICA DM400 B, Leica Microsystems Company, Shanghai, China). The intensity of fluorescence was quantified using the Image-Pro Plus 6.0 software (Media Cybernetics, America). The average fluorescence intensity was calculated for a quantitative analysis.

2.7. Calcium level assay in intestine of C. elegans

In the KWN190 strain of *C. elegans*, the calcium indicator protein D3cpv was expressed throughout the cytoplasm of intestinal cells (Palmer and Tsien, 2006). After exposure, nematodes were washed and then transferred to agar-padded slides. *In vivo* calcium levels were visualized through the calcium indicator D3cpv which expressed from the intestine-limited promoter *Pnhx-2* in transgenic (rnyEx109) nematodes (Geng et al., 2012; Coburn et al., 2013). Images of immobilized worms were captured to examine Ca^{2+} levels by a fluorescence microscope. Fluorescence intensity of green fluorescence protein (GFP) was measured through the Image-Pro Plus software. The intensity of fluorescent puncta was examined for at least 30 nematodes in each group.

2.8. Expression of gst-4 in transgenic C. elegans

In the CL2166 strain of *C. elegans*, *gst-4*::*GFP* was co-expressed in nematodes (Hunt et al., 2011). Observable GFP was a marker of the expression level of *gst-4*, which reflected oxidative damage. After exposure to microplastics, nematodes were washed and transferred to slides. Images of immobilized worms were captured to assay expression levels of *gst-4* by a fluorescence microscope. Fluorescence intensity was examined in at least 30 nematodes in each group (J. Li et al., 2016).

2.9. Data analysis

Mean differences between treated groups and controls were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's test. Probability levels (p-values) of <0.05 were considered statistically significant.

3. Results

3.1. Lethal effects of microplastic particles in D. rerio

After exposure to PA, PE, PP, PVC and/or PS particles with the same concentration series of 0.001–10.0 mg L^{-1}, the survival percentages of *D. rerio* were highlighted in Table 1. After 10 days exposure, 59 fishes died: PA (n = 3), PE (n = 5), PP (n = 26) and PVC (n = 25). Compared to the control, there were no significant differences ($p > 0.05$) in survival rates in all exposure groups except 10.0 mg L^{-1} PP group ($p < 0.05$, Table 1). 10.0 mg L^{-1} PP caused a mean reduction of 27.1% in survival rates. However, under the same concentration series of 0.001–10.0 mg L^{-1}, PS particles with 0.1 μm, 1.0 μm and 5.0 μm sizes did not induce any death in zebrafish (Table 1).

3.2. Morphological and histopathological changes in D. rerio

After exposure, differences in morphology between the surviving and dead fish were observed. According to our observations, the surviving fish presented normal overall body morphology similar with the control (Fig. 2a); however, the dead fish showed swollen abdomens after microplastics exposure. In surviving fish, histological alterations in the intestine were discovered in PA, PE, PP and PVC groups in comparison with the control group (Fig. 2b–f). The main intestinal damage included cracking of villi and splitting of enterocytes (Fig. 2). Results showed that 73.3–86.7% individuals presented significant damage in the intestine after exposure to PA, PE, PP and/or PVC microplastics (Table S2). Additionally there were no obvious differences among PA, PE, PP and PVC groups. Nevertheless, after exposure to PS particles with three sizes of 0.1, 1.0 and 5.0 μm, the intestine exhibited normal morphology, which was similar to that in the control (Fig. 2b, g–i). The semi-quantitative evaluation also demonstrated that intestinal fold disruption and enterocyte damage appeared in PA, PE, PP and PVC groups, rather than in 0.1, 1.0 and 5.0 μm size PS groups (Fig. S2). Additionally, there was no histological damage to the gill, liver and kidneys of zebrafish after microplastics exposure (Fig. S3).

3.3. Lethal effects of microplastic particles in C. elegans

After exposure to the same concentrations (0.5–10.0 mg m^{-2}) of PA, PE, PP, PVC and/or PS microplastic particles, we assayed nematode survival and found that PA, PE, PP and PVC microplastics had significant deleterious effects on the survival of nematodes ($p < 0.05$) except for PVC at 0.5 mg m^{-2} and for 0.1 μm PS at 1.0–10.0 mg m^{-2} (Fig. 3a–d). The effects were not dose-dependent. We found a significant size effect of PS particles. Namely, 0.1 μm PS particles caused a slight reduction in the survival rate ($p > 0.05$, mg m^{-2}); whereas 1.0 μm particles caused strong lethality in nematodes in a dose dependent manner ($p < 0.001$); and 5.0 μm PS particles possessed moderate lethality ($p < 0.001$, Fig. 3e).

3.4. Developmental and reproductive toxicity of microplastics in C. elegans

We also observed significant inhibition effects on body length in nematodes after exposure to 5.0 mg m^{-2} of PA, PE, PP, PVC and/or PS particles with different sizes ($p < 0.05$, Fig. 4a). These microplastics caused 4.89–11.44% reductions in mean of body length. Further, microplastic particles significantly reduced both embryo number and brood size. All five types of microplastics possessed similar inhibition effects on reproduction; the means of the inhibition rates in embryo number and brood size were 14.40–25.22% and 2.44–28.02%, respectively (Fig. 4b, c).

3.5. Changes in calcium levels and microplastic accumulation in the intestine of C. elegans

Exposure to PA, PE, PP or PVC microplastic particles with similar sizes led to significantly decreased intestinal calcium levels, as

Table 1
Survival percentages of zebrafish after exposed to different types of microplastic particles with various concentrations.

Concentration (mg L^{-1})	PA	PE	PP	PVC	$PS_{0.1}$	$PS_{1.0}$	$PS_{5.0}$
0.001	100 ± 0	96 + 9	92 + 9	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.01	100 ± 0	98 ± 9	94 ± 16	85 ± 32	100 ± 0	100 ± 0	100 ± 0
0.1	98 ± 9	96 ± 18	94 ± 16	88 ± 41	100 ± 0	100 ± 0	100 ± 0
1	98 ± 9	100 ± 0	94 ± 0	92 ± 18	100 ± 0	100 ± 0	100 ± 0
10	98 ± 9	100 ± 0	73 ± 24*	83 ± 24	100 ± 0	100 ± 0	100 ± 0

Control data is 100 ± 0%. *$p < 0.05$, compared to the control. Data are means ± SE of three parallel experiments (n = 48).

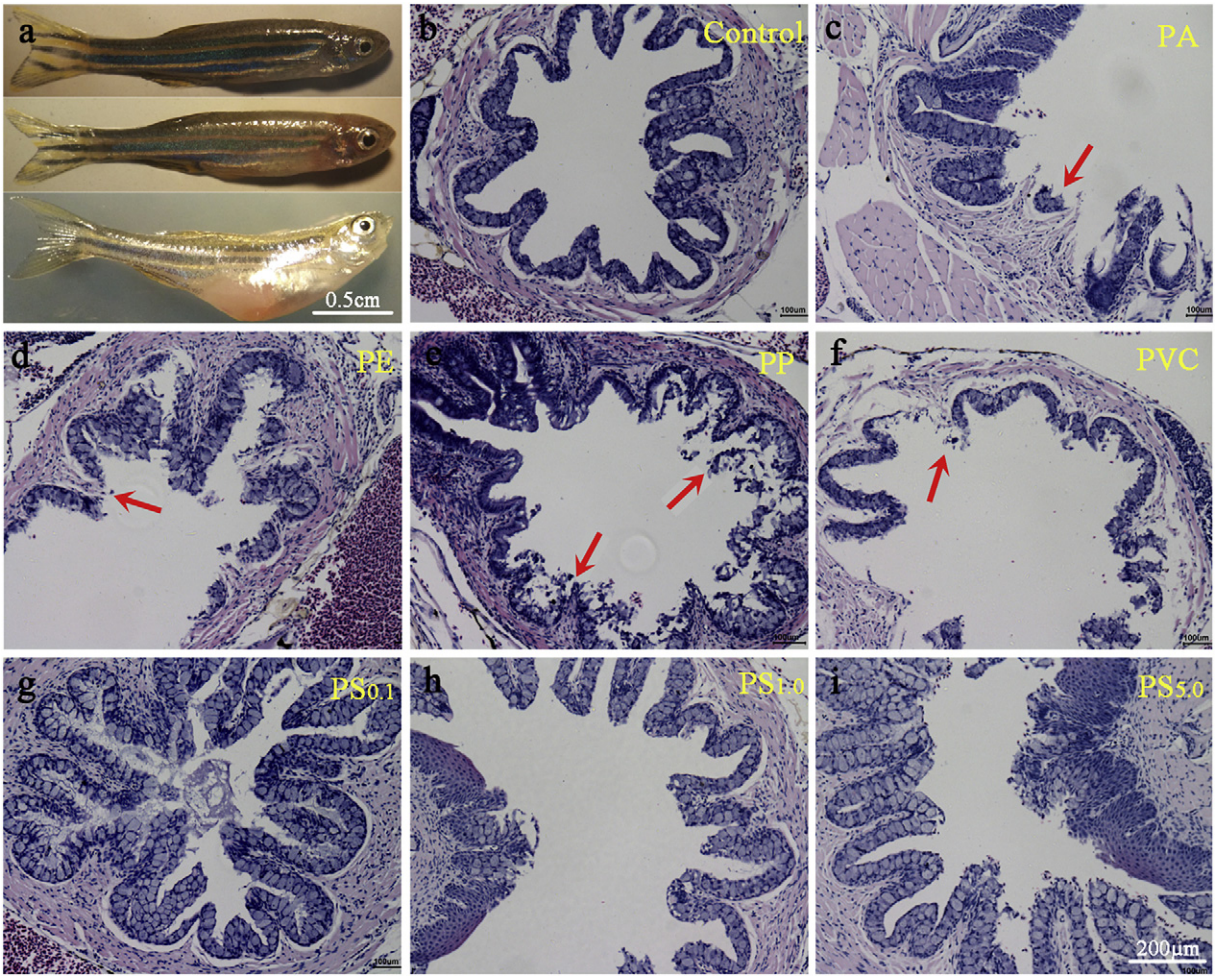


Fig. 2. Outside view and intestinal photomicrographs in *D. rerio* after microplastics exposure. a: Outside view of representative zebrafish in the control (up), the survival (middle) and the dead (down) after exposure. b–i: intestinal photomicrographs in the control (b), exposed to 1.0 mg L^{-1} microplastics of PA (c), PE (d), PP (e), PVC particles (f) with similar size, and PS particles with the size of 0.1 μm (g), 1.0 μm (h) and 5.0 μm (i). Red arrows show damages in the intestine. Bar = 100 μm for b–i.

measured by fluorescence intensity ($p < 0.05$; Fig. 5a–e). After exposure to PS particles with 0.1, 1.0 and 5.0 μm sizes, different effects on intestinal calcium levels were found. Among them, 1.0 μm PS particles caused a significant decrease in intestinal calcium levels ($p < 0.05$); however, 0.1 or 5.0 μm PS particles resulted in a slight reduction without a significant difference ($p > 0.05$, Fig. 5g–i, m). Furthermore, statistics showed that 1.0 μm PS particles resulted in stronger inhibition effects in intestinal calcium levels than 0.1 or 5.0 μm PS particles ($p < 0.05$, Fig. 5m).

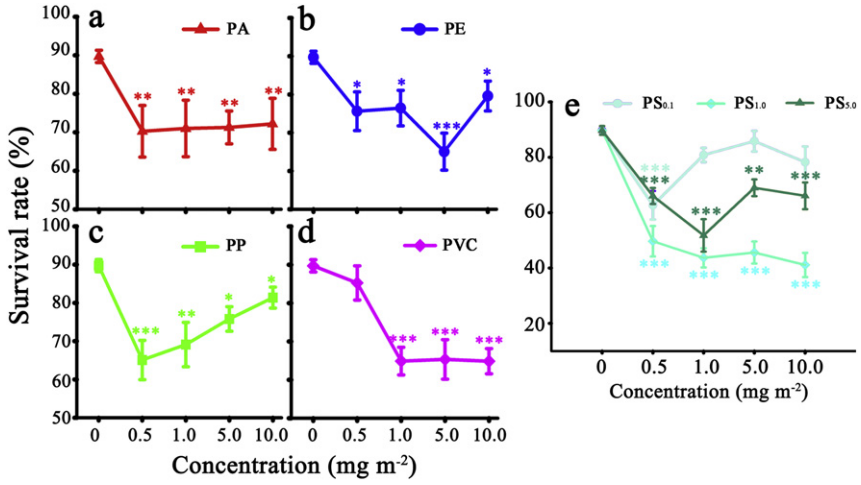


Fig. 3. Survival percentages of *C. elegans* after exposure to (a) PA, (b) PE, (c) PP, (d) PVC particles with similar sizes, and (e) PS microplastics with different sizes. Data are means ± SE of four parallel experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the control.

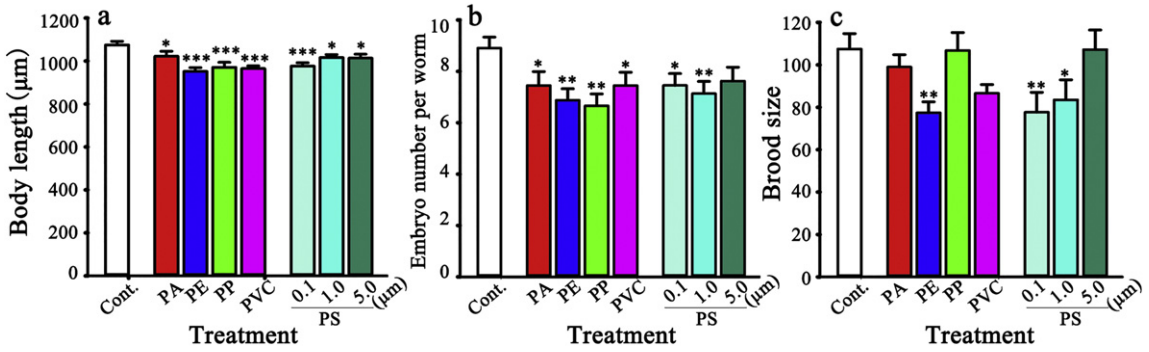


Fig. 4. (a) Body length, (b) embryo number, and (c) brood size of *C. elegans* after exposure to PA, PE, PP, PVC particles with similar sizes, and PS microplastics with different sizes. Nematodes were exposed to 5 mg m^{-2} particles for 2 d. Data are means ± SE of four parallel experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the control.

Microplastic distributions in nematode body tissues were analyzed by assaying fluorescently-labeled PS particles. After 2 days exposure, PS particles were clearly visible throughout the digestive system, from the lumen of the pharynx to the gut lumen and rectum (Fig. 5j–l). Among three sizes of PS microplastics, 1.0 μm PS particles showed the strongest fluorescence intensity in the intestine of nematodes; however, 0.1 μm or 5.0 μm PS particles presented relatively weak fluorescence intensity, which indicated differences of microplastics accumulation in the intestine (Fig. 5j–m). Moreover, results demonstrated an inverse relationship between microplastics accumulation and intestinal calcium levels among three size-dependent PS groups (Fig. 5m).

3.6. Gst-4 expression of nematodes responded to microplastic particles

Treatment with 5.0 mg m^{-2} of PA, PE, PP and/or PVC microplastic particles caused a significant increase in the expression of *gst-4* in the intestine of CL2166 transgenic nematodes ($p < 0.05$; Fig. 6a–e, i). After exposure to PS particles with sizes of 0.1, 1.0 or 5.0 μm, the increase fluorescence intensity of *gst-4*::*GFP* was also found in nematodes ($p < 0.05$, Fig. 6f–h, i). Among them, 1.0 μm PS particles caused significantly stronger expression of *gst-4* than 0.1 μm or 5.0 μm PS particles ($p < 0.05$, Fig. 6f–h).

4. Discussion

This study elucidated the toxic effects of microplastic particles of different chemical composition (PA, PE, PP, PVC and PS) and different sizes (0.1 μm, 1.0 μm and 5.0 μm) on a vertebrate fish, *D. rerio*, and a benthic nematode, *C. elegans*. Intestinal damage was a primary effect in both organisms. In *D. rerio*, microplastics caused cracking of villi and splitting of enterocytes. In *C. elegans*, microplastics caused oxidative stress and changes in intestinal calcium levels. The effects were more dependent on microplastic size than on chemical composition.

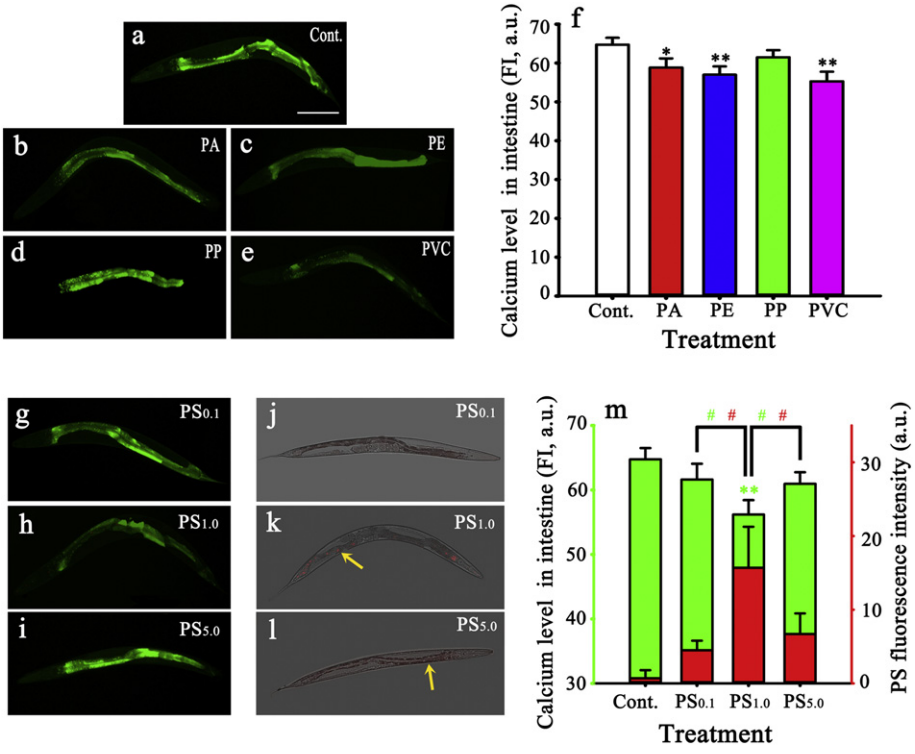


Fig. 5. Changes in calcium levels and microplastic accumulation in *C. elegans*. a–f: Expression of the calcium indicator d3cpv in the intestine of control or microplastics-exposed nematodes (a–e), and the quantified values (f). g–l: Calcium levels in the intestine after exposure to PS microplastics with different sizes. j–l: Accumulation of PS microplastics with different sizes in the intestine (yellow arrows). m: Quantified values of calcium levels and microplastics accumulation in the intestine after PS exposure. Data are means ± SE of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the control. $^{\#}p < 0.05$, when compared between groups. Bar = 200 μm.

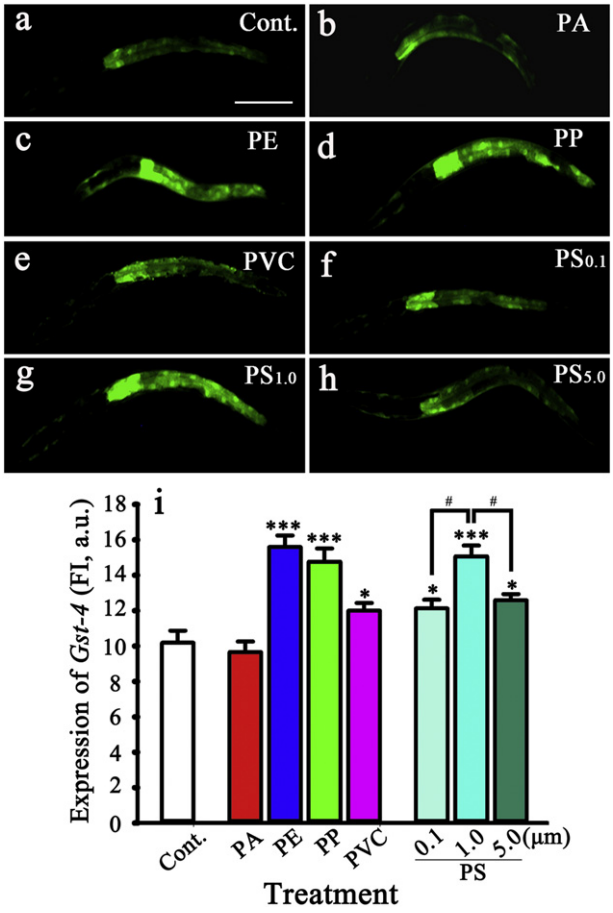


Fig. 6. Effects of microplastics exposure on expression of *gst-4*::*GFP* in *CL2166 C. elegans*. a–h: Expression of *gst-4*::*GFP* in control and nematodes exposed to different types of microplastics. i: Fluorescence intensity of *gst-4*::*GFP* in control and microplastics-exposed nematodes. Data are means ± SE of four parallel experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the control. $^{\#}p < 0.05$, when compared between groups. Bar = 200 μm.

4.1. Ecologically relevant toxic effects

The concentrations of microplastic particles used in the present study are in keeping with reported levels in many freshwater environments (Eerkes-Medrano et al., 2015; Horton et al., 2017; Sruthy and Ramasamy, 2017; Nel et al., 2018). We showed that these concentrations caused little lethality in *D. rerio*, which also agrees with a previous report exposing *D. rerio* to 2 mg L^{-1} PS for three weeks (Lu et al., 2016) or 0.5 mg L^{-1} for 20 days (Karami et al., 2017). While not acutely lethal, these concentrations of microplastic particles caused substantial histopathological damage to the intestine, likely the major locus of exposure. Those fish that did die showed obvious distension of the abdomen, perhaps indicating extensive accumulation of microplastic particles.

Microplastic particle exposure did decrease survival in *C. elegans*. The lethal effects were independent of the chemical composition of the microplastic particles, but did correlate with the size of the particles. In particular, 1 μm particles were the most lethal, caused the most extensive reproductive damage, and caused the largest effects in the intestines and also showed the largest accumulation. Sizes smaller and larger than 1 μm did not cause lethality and had much more moderate effects on measures of accumulation, oxidative stress, and calcium levels. These results are consistent with previous reports (Lee et al., 2013; Jeong et al., 2016) and show a strong association between particle size and toxicity. They also suggest that it is the ability of the intestine to accumulate the particles that leads to the toxic effects on reproduction and survival.

4.2. Microplastic particles exert oxidative damage to intestinal tissue

Our findings present compelling evidence that the intestinal damage resulting from exposure to microplastic particles is oxidative. *D. rerio* incurred histological damage specifically to intestine tissue and not to gill or liver, in agreement with other studies in *D. rerio* (Duis and Coors, 2016; Horton et al., 2017) and European sea bass *Dicentrarchus labrax* (Pedà et al., 2016). Microplastic particles accumulation in the intestine exerts functional damage, as revealed by reduced calcium levels in exposed *C. elegans*. Previous studies in *D. rerio* demonstrated PS microspheres induced oxidative stress and activated superoxide dismutatse and catalase activity at concentrations similar to those examined in our study (Lu et al., 2016). We showed that Glutathione S-transferase 4 (GST-4) is upregulated in response to microplastic particles exposure in *C. elegans*. GST-4 is one of the major cellular detoxification enzymes, and is considered a major participant in phase II detoxification of both endogenous products of oxidative stress and electrophilic xenobiotics (Kahn et al., 2008; Yu et al., 2014). Glutathione S-transferase binds with glutathione and catalyzes glutathione conjugation with target substrates in order to facilitate their excretion from the cell. Several scholars (*i.e.* Hasegawa et al., 2008; Paiva et al., 2015) have reported that *gst-4* expression is induced by oxidative stressors and phytochemicals. The detailed mechanisms by which microplastics induce oxidative damage in exposed intestinal tissue require further investigation.

4.3. Comparative effects of particle size and chemical composition

This is the first study to compare the toxic effects of both microplastic chemical composition and particle size. As plastics degrade in the environment, they decompose into particles of different sizes and composition (Kahn et al., 2008; Bouwmeester et al., 2015; Eerkes-Medrano et al., 2015). We discovered that the chemical composition was not the key factor in intestinal damage in *D. rerio* and *C. elegans*. Rather, it is the ability of the particles to enter the intestine and accumulate that caused toxicity. The *D. rerio* that died exhibited a large accumulation of microplastics and the degree of accumulation of 1 μm particles in *C. elegans* intestines corresponded with the degree of toxicity. The presence of microplastic particles of all sizes in *D. rerio* in intestinal tissue correlated with the similar degrees of histological damage. In contrast, gill and liver tissue did not show damage, likely because of the lack of particle access and accumulation. In *C. elegans*, the 1 μm size PS particles accumulated in the intestine, caused the highest lethality, the most reproductive toxicity, the lowest level of intestinal calcium and the highest expression of *gst-4*, indicating the largest degree of oxidative damage. Previously, it was generally speculated that smaller particles would be more toxic than larger engineered particles because of larger surface area (Choi and Hu, 2008; Lu et al., 2016; Horton et al., 2017). This idea was supported by experiments from Jeong et al. (2016) using *Brachionus koreanus*. However, our present study suggests that higher toxicity risks occur in nematodes exposed to particles with moderate size. This study indicates that toxic effects of microplastics may be size-dependent as a result of particles gaining entry and accumulating in organisms, which further induces tissue damage through mechanical injury or insufficient nutrition.

5. Conclusions

This study demonstrated that microplastic particles of PA, PE, PP, PS and PVC caused intestinal damage in *D. rerio*, including cracking of villi and splitting of enterocytes. We also found microplastic particle-induced lethality and reproductive dysfunction, reduction in intestinal calcium levels and increased expression of the oxidative stress gene *gst-4* were dependent on particle size in *C. elegans*. These results provide evidence of oxidative damage due to ingestion and accumulation of microplastic particles. Further, they demonstrate the toxicity of microplastics is closely dependent on their sizes, rather than their

chemical composition. These findings provide novel insights into ecological toxicity of microplastics on aquatic organisms.

Acknowledgements

This study was supported by the National Key Research and Development of China (NO. 2016YFC1402204) and a grant from the Natural Science Foundation of Shanghai (No. 16ZR1409300). Thanks for Prof. Yaping Zhao in the help of FTIR spectroscopy assay. Thanks for Caenorhabditis Genetics Center contributing transgenic strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.11.103.

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