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Size-Dependent Effects of Micro Polystyrene Particles in the Marine Copepod *Tigriopus japonicus*

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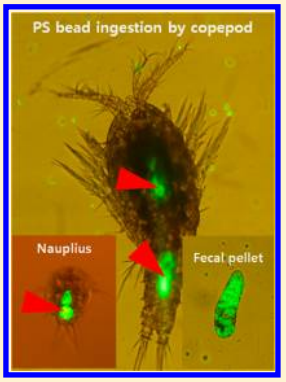
Supporting Information

ABSTRACT: We investigated the effects of three sizes of polystyrene (PS) microbeads (0.05, 0.5, and 6-μm diameter) on the survival, development, and fecundity of the copepod *Tigriopus japonicus* using acute and chronic toxicity tests. *T. japonicus* ingested and egested all three sizes of PS beads used and exhibited no selective feeding when phytoplankton were added. The copepods (nauplius and adult females) survived all sizes of PS beads and the various concentrations tested in the acute toxicity test for 96 h. In the two-generation chronic toxicity test, 0.05-μm PS beads at a concentration greater than 12.5 μg/mL caused the mortality of nauplii and copepodites in the F_0 generation and even triggered mortality at a concentration of 1.25 μg/mL in the next generation. In the 0.5-μm PS bead treatment, despite there being no significant effect on the F_0 generation, the highest concentration (25 μg/mL) induced a significant decrease in survival compared with the control population in the F_1 generation. The 6-μm PS beads did not affect the survival of *T. japonicus* over two generations. The 0.5- and 6-μm PS beads caused a significant decrease in fecundity at all concentrations. These results suggest that microplastics such as micro- or nanosized PS beads may have negative impacts on marine copepods.



INTRODUCTION

Plastics debris is accumulating in marine environments with the increase in plastics production.[1] Plastics undergo degradation by UV radiation and hydrolysis in the marine environment and are gradually split into micro- or nanoscopic plastic fragments and fibers referred to as "microplastics".[2,3] The concern over microplastics in the marine environment, including those not visible to the naked eye, has increased over the past decade due to the potential negative effects caused by their bioavailability to marine organisms, particularly filter feeders at the base of the food web.[3−5] Most studies have focused on proving the ingestion and accumulation of microplastics by aquatic organisms,[1,6−11] and a few studies have been conducted to investigate the adverse impact of microplastics on these organisms. For instance, Bhattacharya et al.[12] reported that 20-nm polystyrene beads hindered photosynthesis and promoted reactive oxygen species (ROS) production in the freshwater algae *Chlorella* and freshwater/saltwater algae *Scenedesmus*. Similarly, in the blue mussel *Mytilus edulis*, von Moos et al.[13] demonstrated that synthetic high-density polyethylene (0−80 μm in size) caused a strong inflammatory response, and Wegner et al.[14] reported that the filtering activity of the organism was reduced by the present of nanopolystyrene (diameter 30 nm). Furthermore, it is known that styrene, a monomer of polystyrene, affects viability and leads to DNA damage in both hemolymphs (*M. edulis* cells) and blood (*Symphodus mellops* cells),[15] and that the leachates of plastics composed of hazardous monomer including PVC and epoxy acutely affected *Daphnia magna*.[16]

As primary consumers and the most numerous metazoans in the marine ecosystem, copepods play an important role in the transportation not only of energy but also of aquatic pollutants across the marine food chain.[17,18] The harpacticoid copepod *Tigriopus japonicus* has been proposed as a suitable model species for assessing environmental risk in Western Pacific coastal regions.[17,19,20] The copepod is both an omnivore and a filter-feeding organism,[21,22] and it may prove useful as a test organism for estimating the influence of microplastics in the marine environment. Additionally, *T. japonicus* has an advantage as a test species in that the toxicity tests used to investigate the generational effects of several toxicants are well established.[20]

Microsized plastics over 1 μm in diameter, particularly polystyrene (PS) beads, have been used previously to study microplastic ingestion or feeding response of copepods because these small beads are available to copepods.[23−26] However, little is known about the ingestion of nanoplastics less than 1 μm in diameter on copepods.

The purpose of this study was not only to examine ingestion of nano- or microplastics by copepods in terms of bioavailability

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but also to determine the toxicity of three different sizes of commercially available PS microbeads as model micro- or nanoplastics. These beads were selected to measure the effect of different sizes of microplastics on the survival, development, and fecundity of the copepod *T. japonicas* using acute and chronic toxicity tests.

MATERIALS AND METHODS

Copepod Maintenance. Individuals of the harpacticoid copepod *Tigriopus japonicus* were obtained from the Faculty of Marine Bioscience and Technology, College of Life Sciences, Gangneung-Wonju National University, Gangneung, South Korea, and were maintained in the Korea Institute of Ocean Science and Technology (KIOST) from 2010 onward. These copepods were cultured in filtered (0.2 μm) seawater (32 psu) in an incubator kept at 20 ± 1 °C under a 12 h light:12 h dark cycle using *Tetraselmis suecica* as a food source. The pH, dissolved oxygen (DO), and salinity of the seawater were 7.8, 8.5 mg/L, and 32 psu, respectively. The alga was cultured at 20 ± 1 °C with 24-h light exposure (4000 lx) in a Conwy medium.[27]

Tested Microplastics. In this study, we tested three different sizes of polystyrene microbeads (2.5% solids suspension; Polyscience, USA) with diameters of 0.05, 0.5, and 6 μm. We used two types of beads, fluorescently labeled (for ingestion tests, with an excitation of 441 nm/emission 486 nm) and plain (for toxicity tests) PS microbeads (Supporting Information (SI) Table S1).

Polystyrene Bead Ingestion by *T. japonicus*. The first experiment was conducted to determine whether the copepods were able to ingest micro- and nanoplastics. Ten *T. japonicus* adults starved for 24 h were added to each of 12 wells in tissue culture plates (SPL Life Sciences, Seoul, South Korea), each containing 4 mL of test solution with 250 μg of fluorescently labeled PS microbeads (9.1×10^{11} particles/mL for 0.05-μm PS bead; 9.1×10^{8} particles/mL for 0.5-μm PS bead; 5.25×10^{5} particles/mL for 6-μm PS bead). Nauplii were treated the same way as for the adults, starved for 24 h and 10 individuals placed in each of 12 well in tissue culture plates. The test solutions were sonicated for 30 min immediately prior to use in each experiment. Each treatment or control (seawater) was run in triplicate. The plates were incubated at 20 ± 1 °C under a 12 h light:12 h dark cycle. After 24 h, the copepods were observed under a fluorescent microscope (Axioplan 2, Zeiss, 50−400× magnification). During the test period, no food was provided. The seawater used in this study was filtered with a 0.2-μm mesh (fiber filter; Millipore, Billerica, MA, USA).

A second experiment was conducted to establish whether the copepods ingest micro- and nanoplastics when phytoplankton were added to the seawater medium. For this experiment, the 6-μm fluorescent PS beads (2.5×10^{5} particles/mL) similar in size to live food organisms (e.g., *Tetraselmis suecica*, 7 μm ESD[28]) were used as model plastics, and microalgae were supplied at the same concentration (2.5×10^{5} cells/mL) with the bead. After 24 h of incubation, the number of PS beads and *T. suecica* in the vessels were counted to calculate the ingestion rate using the method of Frost.[29] Other conditions and processes were the same as those specified above.

Acute Toxicity Test. Semistatic 96-h acute toxicity tests were conducted using adult females following the methods described by Lee et al.[19] In addition, nauplii (<24 h old) were tested with the same methods above. Briefly, 10 copepods were added randomly to each of 12 wells in tissue culture plates, each containing 4 mL of test solution. The plates were incubated at 20 ± 1 °C with a 12 h light:12 h dark cycle. Each treatment or control (seawater) was run in triplicate. Copepods were not fed during the test period. Test solutions in culture plates were renewed (~90%) after 48 h. The three sizes of polystyrene beads were tested at concentrations of 0, 6, 13, 31, 63, 187, 250, and 313 μg/mL (1.14×10^{12} particles/mL for 0.05-μm PS bead; 1.14×10^{9} particles/mL for 0.5-μm PS bead; 6.57×10^{5} particles/mL for 6-μm PS bead) for the copepod acute toxicity tests. The mortality rates of *T. japonicus* were examined following 96 h of exposure under a stereomicroscope using scattered light (SZX9; Olympus, Tokyo, Japan) following the method described by Kwok and Leung.[30]

Chronic Toxicity Test. We conducted chronic toxicity tests using a slightly modified version of the two-generation toxicity tests described by Lee et al.[20] Briefly, 10 newly hatched nauplii (<24 h old) were transferred to each well in 12-well tissue culture plates at each concentration range with a 4 mL working volume with three replicates. These nauplii were cultured under the conditions described above until adult females developed egg sacs. Test solutions were renewed (~50% of the working volume for the nauplius stage and ~90% of the working volume for the copepodite stage) daily, and *T. suecica* was added at a density of approximately 1×10^{5} cells/mL immediately after the renewing. The developmental stages were observed daily under the stereomicroscope and recorded to calculate the time of development, such as from nauplii to copepodite (N−C) and from nauplii to adults with egg sacs (N−A). At the same time dead animals were removed from the culture vessel. The sex ratio and survival (%) were determined after the maturation of all copepods. The maturation period of the controls was 14 days on average but varied in the exposed groups. The development of the egg sac was considered to be the time of maturation. To measure the fecundity (the number of nauplii from the first clutch) of an adult female, six egg-sac-bearing females per concentration range were individually transferred to a new 12-well culture dish. These females were cultured under the conditions described above until the first offspring hatched. *T. suecica* was provided at 2×10^{4} cells/copepods/day. Test solutions were renewed daily, and the resulting nauplii were counted under the stereomicroscope. For the experiment with the second generation (F_1), 10 nauplii (F_1) produced by each female (F_0) in the first brood per concentration range were randomly transferred to 12-well tissue culture plates. The experimental and exposure conditions were the same as those used for the F_0 generation test.

The bead concentrations to be used were chosen by the concentration level of the diet (*T. suecica*) provided. For instance, in the case of the 6-μm beads, which are of a size similar to *T. suecica*, 12.5 μg/mL of the beads equates to 105 000 beads. Therefore, the three sizes of polystyrene beads were tested at concentrations of 0, 0.125, 1.25, 12.5, and 25 μg/mL (2.1×10^{5} particles/mL).

Statistical Analysis. In the ingestion experiment, data were statistically analyzed for significant differences using an unpaired Student's *t*-test to compare the *T. suecica* and bead treatment groups. Measurements of the survival, developmental time, and fecundity of copepods were statistically analyzed using Dunnett's *post hoc* test to compare the exposed and control groups in the two-generation toxicity test. Differences were considered significant at $P < 0.05$. Data are presented as the means ± standard error (SE), and all statistical analyses were conducted using SPSS version 17.0 (SPSS, Inc., Chicago,

IL, USA). Toxicity curves were adjusted to four-parameters logistic curves using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA), which was used to calculate the EC_{50} values.

RESULTS

Water Characteristics. The dissolved oxygen, pH, temperature, and salinity of all test solutions before each water change were 8.25 ± 0.07 mg/L, 7.8 ± 0.03, 20 ± 0.2 °C and 32 psu, respectively. The aggregates were observed in the test solution of 0.05-μm PS bead but not 0.5- and 6-μm PS beads.

Polystyrene Bead Ingestion by *T. japonicus*. All three sizes of PS beads were observed in the guts of all tested adults and nauplii of *T. japonicus* under the no-food conditions (Figure 1). Furthermore, all copepods exhibited feeding on

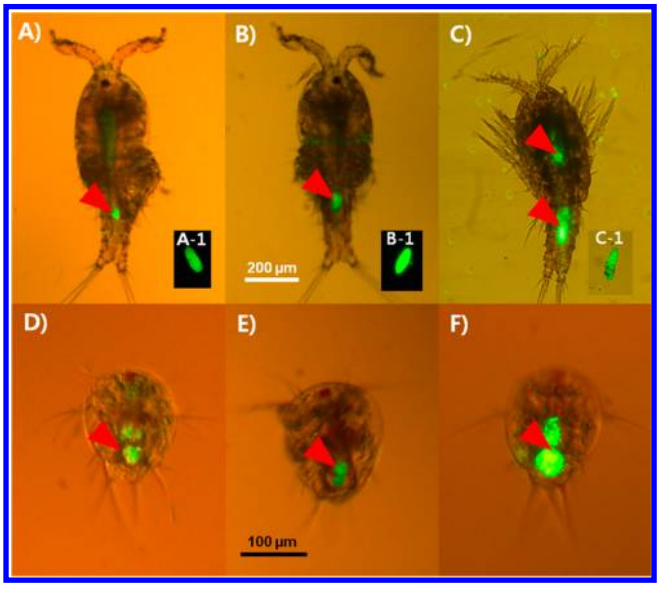

Figure 1. Fluorescently labeled polystyrene beads (arrows) of 0.05-μm (A, D), 0.5-μm (B, E), and 6-μm (C, F) diameter ingested by *Tigriopus japonicus* including adults (A, B, C) and nauplii (D, E, F) exposed to several concentrations (9.1×10^{11} particles/mL for 0.05-μm PS bead; 9.1×10^{8} particles/mL for 0.5-μm PS bead; 5.25×10^{5} particles/mL for 6-μm PS bead) for 24 h. A-1, B-1, and C-1 are fecal pellets egested from adults.

plastics even when phytoplankton *T. suecica* was added to the medium as a food source (Figure 2). In this experiment, there was a significant difference in the ingestion rate of the nauplii between the *T. suecica* and 6-μm PS bead ($p < 0.05$; SI Figure S2).

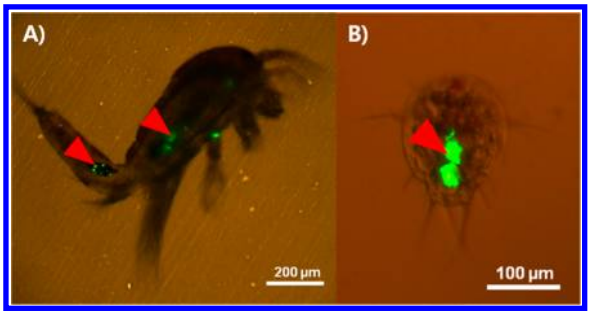

Figure 2. Fluorescently labeled polystyrene beads (6-μm; arrows) and *Tetraselmis suecica* ingested in *Tigriopus japonicus* exposed to the beads (2.5×10^{5} particles/mL) and the algae (2.5×10^{5} cells/mL) for 24 h.

Acute Toxicity Test. After 96 h, both nauplii (N1−2) and adult females of *T. japonicus* survived when exposed to the highest concentration (313 μg/mL) of the three sizes of PS beads (SI Table S2). Therefore, LC_{50} levels for the copepods were not calculated.

Chronic Toxicity Test. In terms of the survival responses of the F_0 (A) and F_1 (B) generations of *T. japonicus* exposed to three sizes of PS beads at various concentrations (Figures 3 and SI S4), copepod survival was significantly affected compared with the control (survivability in F_0 and F_1 were average 90% and 87%, respectively) when exposed to 0.05-μm PS beads at 1.25 μg/mL ($p < 0.05$). Additionally, beads of >12.5 μg/mL in the F_0 generation and those of >1.25 μg/mL in the next generation caused mortality in both the nauplii and copepodites ($p < 0.001$). In the 0.5-μm PS bead treatment, although there was no significant effect on the F_0 generation ($p > 0.05$), the highest concentration (25 μg/mL) induced a significant decrease in the survival of the F_1 generation compared with the control ($p < 0.01$). The 6-μm PS beads did not affect the survival of *T. japonicus* over two generations ($p > 0.05$). The length of nauplius phase (N−C) and the copepod generation time (N−A) showed similar patterns in terms of survival (Figures 4 and SI S5). However, the length of the nauplius phase was significantly longer with 1.25 μg/mL of 0.05-μm PS beads compared with that of the controls of either generation ($p < 0.05$). In the 0.5-μm PS bead treatment, the concentration of 25 μg/mL caused a significant developmental delay in the F_1 generation ($p < 0.01$). There were no effects of 6-μm PS beads over two generations. There was no significant difference in sex ratio over two generations ($p > 0.05$; SI Figure S3). The smallest beads (0.05-μm) did not affect the fecundity of copepods ($p > 0.05$). However, 0.5- and 6-μm PS beads caused significant decreases in fecundity at all concentrations ($p < 0.05$; Figures 5 and SI S6).

DISCUSSION

Microplastics have been found in marine zooplankton such as copepods, arrowworms, fish larvae, and salps.[31,32] The ingestion of plastic beads, which are similar in size to copepods' phytoplankton diet, has been confirmed through several laboratory studies on the feeding responses of copepods.[23−25,33,34] Plastic ingestion depends on the species, with selective or nonselective feeding behavior in calanoid copepods when exposed to plastic beads only. For instance, *Acartia tonsa* and *Eurytemora affinis* have been found to ingest various sizes of microplastics (7−70 μm) and ~15-μm latex beads,[24,35] whereas *A. clausi* and *Eucalanus pileatus* did not feed on the 15.7- and 20-μm PS beads used here, respectively.[25,33] In addition, it is known that calanoid copepods discriminate between live food such as phytoplankton and nonliving particles such as polystyrene beads by using rejection or ingestion.[23,25] In our study, *T. japonica,* including adults and nauplii, ingested microsized PS beads (6-μm diameter) and even nanosized particles (0.05- and 0.5-μm beads). It is difficult to find a report on the ingestion and egestion of nanoplastics of less than 1 μm diameter in copepods up to now. This may be the first report of nanoplastics ingestion by copepods. Furthermore, when *T. suecica* was added as a live feed source in the present study, not only were a number of 6-μm-diameter PS beads detected in fecal pellets and the gut of *T. japonicas*, but also selective feeding was not found in the adults, and even the nauplii preferred the beads to the phytoplankton (SI Figure S2). The result that the nauplii, which mainly spend time on

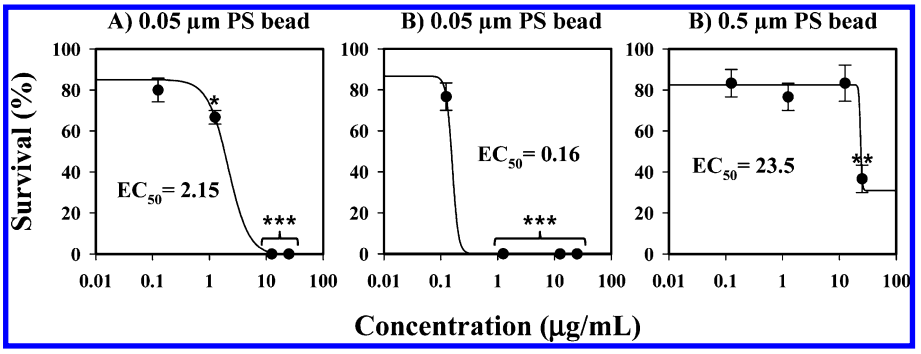

Figure 3. Effect on the survival of the F_0 (A) and F_1 (B) generations of *Tigriopus japonicus* exposed to different polystyrene beads at differing concentrations. Symbols *, **, *** on data bars indicate significant difference over the controls, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

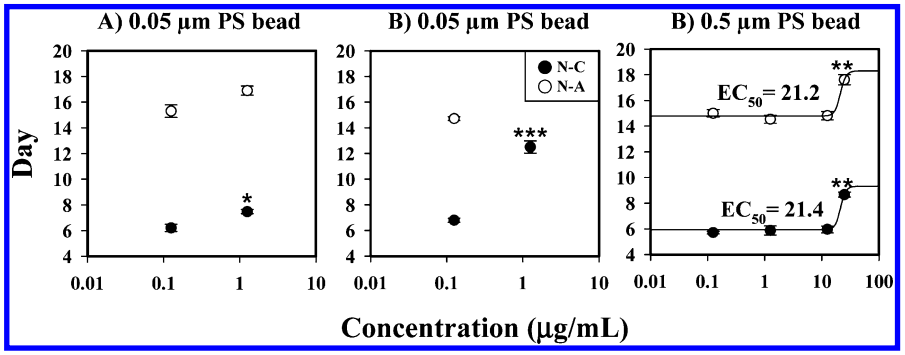

Figure 4. Effect on the nauplius phase (nauplius to copepodid, N−C) and development time (nauplius to adult, N−A) in the F_0 (A) and F_1 (B) generations of *Tigriopus japonicus* exposed to different polystyrene beads at differing concentrations. Symbols *, **, *** on data bars indicate significant difference over the controls, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

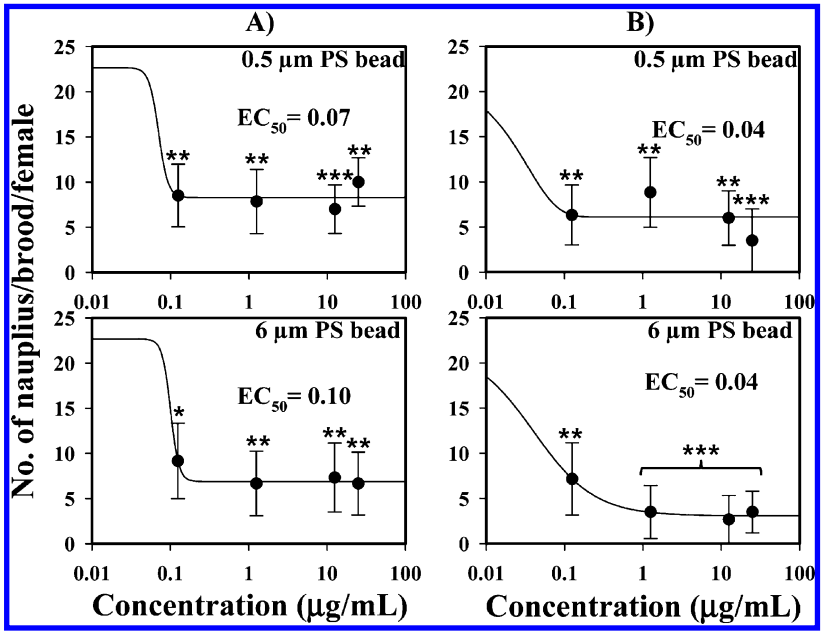

Figure 5. Effect on fecundity in the F_0 (A) and F_1 (B) generations of *Tigriopus japonicus* exposed to different polystyrene beads at differing concentrations. Symbols *, **, *** on data bars indicate significant difference over the controls, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

the bottom, have a preference for the bead may be due to sinking of the PS beads. As a result, *T. japonicus* may be an appropriate model organism to assess the effects of microplastics, and even nanoplastics, in marine environments.

In the 96-h acute toxicity test, survival of *T. japonicus* was not affected by any size or concentration of PS beads tested in this study. Generally, as nanosized particles (<200 nm) get smaller, they increase in toxicity owing to the facility of movement into cells, the increase in their surface area, and enhanced intrinsic toxicity.[36−38] In particular, it has been shown for mammalian cells that gold nanoparticle uptake by cells is dependent on size and that a nanoparticle size of 50 nm resulted in maximal uptake by a cell compared with smaller or larger sized particles.[39] Moreover, it is known that 20−60-nm carboxyl

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polystyrene particles may reach the vicinity of the endoplasmic reticulum and that particles are able to disturb the enzymatic activity of CYP450 in insect cells.[36] In contrast, Petersen et al.[40] found no clear evidence that carbon nanotubes (CNTs) were absorbed into cellular tissues despite the fact that accumulated CNTs remained in the gut of the water flea *Daphnia magna*. Although the 50-nm PS beads tested in this study were sonicated before use to avoid aggregation, aggregates were still found under this treatment. The aggregation/agglomeration of the beads may contribute to decreased toxicity due to an increase in aggregate particle size. Nevertheless, in the two-generation toxicity test, the nauplii exposed to PS beads at >12.5 μg/mL in the F_0 generation, and even >1.25 μg/mL in the F_1 generation, died within approximately 1 week, before the metamorphosis of a nauplii into copepodids. Thus, these results suggest that the chronic toxicity test, including the whole-life cycle test, is necessary to assess the toxicity of nanosized PS in *T. japonicus*.

In the two-generation test, high concentrations of 0.05- and 0.5-μm PS beads caused increased toxicity and impacts on survival and development of copepods in the F_1 generation. There is the potential for the ingested and accumulated PS beads in ovigerous females to be transferred to their offspring. However, the fluorescent beads were not observed in eggs of their ovisac under a fluorescent microscope. To date, there has been no clear evidence on the bioaccumulation and transfer to progeny of nanoparticles in marine organisms.[41] Although further study is required to find direct evidence that nanoplastics are passed from dam to offspring, these results showing increased toxicity may provide indirect evidence.

Large-sized PS beads of 0.5- and 6-μm diameter induced a decrease in fecundity in this study. First, this can be attributed to insufficient nutrition or the inhibition of digestion due to the large amount of microplastics ingested as prey. No filter feeder in the marine environment can digest and absorb ingested microplastics.[3] Moreover, many reports have shown that a limitation in food quantity can lead to low egg production in copepods.[42−44] Usually, copepods tend to ingest larger particle and indiscriminately intake unrecognizable small particles by filtering appendages.[24,45] Therefore, the 0.05-μm PS bead did not affect the fecundity of copepods in this study. Second, PS beads may physically inhibit the fertilization of copepods. A large number of unfertilized egg sacs that failed to develop[46] were found in this study (data not shown). Our report on this phenomenon is the first, and it is necessary to carefully prove this through a detailed histological study.

In summary, microplastics such as nano- or microsized PS bead may have negative effects including decrease of survivorship and retardation of development, especially diminution of reproduction on marine copepods.

For a more sensitive and precise assessment, biochemical or molecular biological traits such as a variations in RNA content and gene expression patterns related to stress and detoxification should be studied further. Such research could also use several genes and the mRNA expression of *T. japonicus*, which have been described previously.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 showing the difference of fluorescence between treatment and control; Figure S2 showing the ingestion rates of *T. japonicus* exposed to a mixture of *T. suecica* and 6-μm PS beads; Figures S3−S6 showing the effect on the sex ratios, survival, development time, and fecundity in the two generations of *Tigriopus japonicus* exposed to different polystyrene beads, respectively; Table S1 elucidating polystyrene beads used in this study; Table S2 showing the results of the 96-h acute toxicity test of three sized micro polystyrene beads. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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