Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of *Xenopus tropicalis*

Lingling Hu, Lei Su, Yingang Xue, Jingli Mu, Jingmin Zhu, Jiang Xu, Huahong Shi

State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200062, China
Key Laboratory of Environmental Protection of Water Environmental Biological Monitoring of Jiangsu Province, Changzhou Environmental Monitoring Centre, Jiangsu 213001, China
Division of Marine Chemistry, National Marine Environmental Monitoring Center, Dalian 116023, China

**Highlights**
- The ingestion of polystyrene microspheres was determined in *Xenopus tropicalis* tadpoles.
- Microspheres presented in gills and digestive tract of tadpoles after 1 h exposure.
- The number of microspheres in tadpoles greatly decreased 1 d after clearance.
- Food decreased the absorption and increased the elimination of microspheres.
- Microspheres were likely to be quickly ingested and egested by tadpoles.

**Graphical Abstract**

**Abstract**

Microplastic is an emerging contaminant affecting freshwater and marine ecosystem across the globe. In the present study, the filter feeding tadpoles of *Xenopus tropicalis* were exposed to polystyrene microspheres (1 and 10 μm) for 48 h. Microspheres were observed in gills and digestive tract of tadpoles within 1 h after exposure as well as in feces 6 h after exposure. The accumulation of microspheres in the tadpoles were concentration dependent (Univariate ANOVA, *p* < 0.001), but no time dependent accumulation of microspheres was observed in tadpoles 48 h after exposure (Univariate ANOVA, *p* > 0.05). After the exposed tadpoles were transferred to clean water, the number of microspheres in the tadpoles decreased dramatically after 1 d and continued to decrease gradually afterwards. The absorbed polystyrene particles in unfed tadpoles was significantly higher than those in the fed tadpoles at 12 and 24 h after exposure. After transfer to clean water, the fed tadpoles showed a significant decrease in the amount of absorbed polystyrene particles, while the unfed tadpoles showed no significant change in the amount of absorbed polystyrene particles. Our results suggested that microspheres were likely to be ingested and egested relatively fast by tadpoles. Our results indicated that aquatic vertebrate organisms might ingest more microplastics if the abundance of microplastics continues to increase while the available food becomes less.

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1. Introduction

Plastics are widely used in our daily life because of their unique properties, such as their light weight, strength, durability, and corrosion-resistance (Thompson et al., 2009; Cole et al., 2011). Globally, plastic production has risen rapidly over the past 60 years and was over 311 million tons per annum in 2014 (PlasticsEurope, 2015). Larger plastic items can degrade to millions of microplastics (O’Brien and Thompson, 2010; Lambert and Wagner, 2016). Microplastics may also come from primary plastics, which are intentionally used as resin pellets or as ingredients of personal care products (Fendall and Sewell, 2009).

A wealth of studies demonstrate that microplastics are ubiquitously distributed in the marine environments, including the polar regions, mid-ocean islands and the deep sea (Barnes et al., 2009; Ivar do Sul et al., 2013; Van Cauwenberghe et al., 2013). Microplastics have even been found in marine products such as seafood and sea salts (Li et al., 2015; Yang et al., 2015). In recent years, microplastics have also been found in freshwater ecosystems including rivers and lakes (Moore et al., 2011; Erik森 et al., 2013; Lechner et al., 2014; Zhang et al., 2015). Increasingly, studies of plastics pollution in these waters support that microplastic pollution can also be a serious issue in freshwater environments (Wagner et al., 2014; Erkes-Medrano et al., 2015).

Ingestion of microplastics by organisms may lead to gut blockages, reduce appetite, or facilitate the transfer of persistent organic pollutants or toxic additives to the organism (Von Moos et al., 2012; Wagner et al., 2013b; Bakir et al., 2014; Hamlin et al., 2015). In addition, microplastics can also accumulate throughout the aquatic food web (Farrell and Nelson, 2013). To date, accumulation of microplastics has been documented in many of marine invertebrates (Supplementary Table 1), and only a few studies have focused on the accumulation of microplastics in freshwater organisms, especially in vertebrates (Table 1).

Filter feeders are one of the most valuable groups because their extensive feeding activity exposes them directly to microplastics present in the environment. Microplastics have been found in many filter feeders including sea cucumbers (Thyonella gemmata), blue mussels (Mytilus edulis), Atlantic mackerel (Scomber scombrus), basking sharks (Cetorhinus maximus) and fin whales (Balaenoptera physalus) (Graham and Thompson, 2009; Fossi et al., 2014; Li et al., 2016; Rummel et al., 2016). Most of these organisms are marine species. With exception of those whole-life filter feeders, some organisms have features of filter feeders during their early life stages. For example, the tadpoles of amphibians filter feed until they finish metamorphosis.

The West African clawed frog (Xenopus tropicalis) is an emerging model animal in developmental biology and ecotoxicology (Berg et al., 2009; Gao et al., 2015; Hu et al., 2015). As a vertebrate, X. tropicalis is closely related to humans. It features a small size, has a short generation time, produces thousands of eggs, and has abundant background information. Recently, Xenopus embryos and tadpoles have been used in studying the ecotoxicology of nanoparticles (Mouchet et al., 2008; Russellino et al., 2015; Webster et al., 2016). Amphibians are thought to be indicator species of overall environmental health, and global declining amphibian populations have drawn special attention since the early 1990s (Stuart et al., 2004). However, there are few reports about microplastics uptake in amphibians. Therefore, the early developmental stages of X. tropicalis are an ideal freshwater vertebrate model to study microplastics.

In this study, we exposed X. tropicalis tadpoles to uncoated polystyrene (PS) plastic particles, one of the most abundant polymer types in samples collected from environments and organisms (Van et al., 2012; Vianello et al., 2013; Zhang et al., 2015). Our aims were to study the uptake, accumulation and elimination of microplastics in freshwater tadpoles as well as the effects of feeding on these processes.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO) and 3-aino-benzoic acid ethyl ester (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human chorionic gonadotrophin (HCG) was purchased from Ningbo second hormone factory (Ningbo, China). The remaining chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Plastic particles preparation

Standard and fluorescently labeled 1 and 10 μm polystyrene microspheres (green 468 nm excitation/508 nm emission and 1.05 g cm$^{-3}$ in density) were purchased from Duke Scientific Corporation. The water was filtered through 0.45 μm filter paper and maintained at 25 °C and pH 6.4–6.7 for the exposure experiments. Microplastic stocks were sonicated at 40 kHz for 1 min prior to quantification and use for exposures.

2.3. Husbandry of frog adults and tadpoles

X. tropicalis adults were obtained from Nasco (Fort Atkinson, WI, USA). The husbandry of X. tropicalis adults was performed as described previously (Guo et al., 2010). In brief, tadpoles at stage 45 were fed on Sera Micron powder (Fort Atkinson, WI, USA) four times each day (Nieuwkoop and Faber, 1994). Approximately 2 g of Sera Micron powder was mixed into 40 mL of distilled water and stored at 4 °C. The tadpoles were fed approximately 0.6 mL stored solution per liter of tank water, and food levels were adjusted as larvae grew. Half of the water in each tank was changed daily to remove waste and uneaten food (Showell and Conlon, 2009).

Table 1

Comparison of accumulation and elimination of microplastics in vertebrates.

<table>
<thead>
<tr>
<th>Perca fluviatilis</th>
<th>Danio rerio</th>
<th>Xenopus laevis</th>
<th>X. tropicalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat of organisms</td>
<td>brackish</td>
<td>freshwater</td>
<td>freshwater</td>
</tr>
<tr>
<td>Exposure stages</td>
<td>larve</td>
<td>adult</td>
<td>embryo</td>
</tr>
<tr>
<td>Type of microplastics</td>
<td>polystyrene</td>
<td>polystyrene</td>
<td>polystyrene</td>
</tr>
<tr>
<td>Size of microplastics (μm)</td>
<td>0.07–20</td>
<td>0.05</td>
<td>1, 10</td>
</tr>
<tr>
<td>Exposure concentrations</td>
<td>0.01–0.08 particles mL$^{-1}$</td>
<td>4.5 × 10$^3$–2.9 × 10$^4$ particles mL$^{-1}$</td>
<td>4.5–18 mg L$^{-1}$</td>
</tr>
<tr>
<td>Absorption time</td>
<td>14 d</td>
<td>4 h</td>
<td>52–545</td>
</tr>
<tr>
<td>Retention time</td>
<td>-</td>
<td>-</td>
<td>&gt;6 d</td>
</tr>
<tr>
<td>Location of accumulation</td>
<td>stomach</td>
<td>gill, liver, gut</td>
<td>cytoplasm, nucleus, periphery of digestive gut cells</td>
</tr>
<tr>
<td>References</td>
<td>Lonnsfedt and Eklöv, 2016</td>
<td>Lu et al., 2016</td>
<td>Tussellino et al., 2015</td>
</tr>
</tbody>
</table>
2.4. Exposure experiments

In brief, there were triplicates for each control and treatment group in an experimental series. In the accumulation experiment, 30 tadpoles at stage 45/46 were randomly transferred into a 200 mL glass Petri dish. The tadpoles were exposed to polystyrene microspheres at three concentrations of each particle size: 10, 103 and 105 particles mL−1 (1 μm) and 0.1, 10 and 102 particles mL−1 (10 μm), and exposure time was 1–48 h. In the elimination experiment, two hundred tadpoles at stage 45/46 were transferred into a 5 L glass tank and exposed to 103 particles mL−1 microspheres (1 and 10 μm) for 48 h. After exposure, approximately 150 healthy, live tadpoles were rinsed with filtered water three times and transferred to a clean tank with flowing water. The elimination time of the tadpoles was 1–6 d. In the feeding experiment, 30 tadpoles were allowed to feed or remain unfed and each were exposed to 103 particles mL−1 1 μm microspheres for 24 h and then transferred to clean water for elimination. Following previously described procedures, only fed groups were provided with food during the 24 h elimination time. Five tadpoles were collected at each time point in each group for analysis.

2.5. Observations and measurement

The collected tadpoles were washed with filtered water and anesthetized with 100 mg L−1 MS-222 immediately. Five tadpoles and subsamples of fecal material from each group were photographed under an Olympus BX53 fluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan), and images were taken with an Olympus DP 80 camera.

2.6. Quantification of microspheres in water, tadpoles and feces

After observation, all the tadpoles, solutions and feces were collected separately and emptied into 250 mL glass bottles 15 cm in height. Approximately 30 mL of 30% H2O2 was added to each bottle to digest the tadpoles or feces. Bottles were covered at 25 °C for one week. After digestion, 200 mL of filtered water was added to each bottle. Each solution was mixed and sonicated for 2 min. Then they were transferred onto 0.45 μm pore size, 25 mm diameter cellulose nitrate grid membrane filters (Whatman WME) using a vacuum with a pump. Microspheres on the filter paper were observed and counted under a fluorescence microscope (Olympus BX53). Fifteen squares (3.1 × 3.1 mm) on the filter were randomly chosen for the calculation of microsphere number. The average abundance of microspheres in fifteen squares was determined and multiplied by 51 to estimate the total microsphere abundance on the filter paper (Supplementary Fig. 1).

2.7. Data analysis

Statistical analyses were performed using SPSS17.0 software. Student’s t-tests were used for comparisons of two groups. One-way analysis of variance (ANOVA) with Tukey’s HSD (homogeneous variances) or Dunnett T3 (heterogeneous variances) post-hoc test was performed to compare the various treatments. Formal comparisons were made using univariate ANOVA and a Student-Newman-Keuls (S-N-K) post-hoc test to determine the relationship of concentration- and time-effects. Pearson’s rank correlation coefficient test was used to assess the correlation between size and number of polystyrene microspheres consumed by tadpoles. P < 0.05 was treated as significant in all tests.

3. Results

3.1. Uptake of polystyrene microspheres in tadpoles

Microspheres were not found in control tadpoles, but they were clearly observed in tadpoles only 1 h after exposure to 1 and 10 μm polystyrene microspheres. They were observed in gills, alimentary canal, stomach and intestine of exposed tadpoles (Fig. 1A–D). Microspheres were also found in feces 6 h after exposure (Fig. 1E, F).

Quantitative analysis suggested that the accumulation of microspheres was not significant after exposure for longer times to either 1 μm (F = 1.03, p = 0.447) or 10 μm (F = 1.01, p = 0.459) microspheres (Fig. 2). However, the amount of microspheres in tadpoles significantly increased in a concentration dependent manner at the same time point after exposure to 1 μm (F = 30.21, p < 0.0001) and 10 μm (F = 54, p < 0.0001) microspheres (Fig. 2). The maximum concentration of 1 μm microspheres in the tadpoles was 4.9 × 106 particles individual−1. In addition, no significant difference in accumulation was observed between 1 and 10 μm polystyrene particles (Pearson, p > 0.05).

3.2. Elimination of polystyrene microspheres in tadpoles

After the exposed tadpoles were transferred to clean water, the number of microspheres in the tadpoles decreased greatly after 1 d and continued to decrease gradually afterwards (Fig. 3). The depuration rate was 58% (1 μm) and 78% (10 μm) 1 d after clearance. The average numbers of microspheres were 257 (1 μm) and 42 (10 μm) particles individual−1 6 d after clearance. The final depuration rate was more than 95% for 1 and 10 μm microspheres.

3.3. Effects of feeding on uptake and elimination of polystyrene microspheres in tadpoles

The number of absorbed polystyrene particles in unfed tadpoles was significantly higher than those in the fed tadpoles at 12 and 24 h after exposure (Fig. 4A). Microplastics were found in the feces of fed tadpoles but not in the unfed tadpoles during the exposure periods. After transfer to clean water, the fed tadpoles showed a significant decrease in the amount of absorbed polystyrene microspheres (p < 0.001), while the unfed tadpoles showed no significant change in the amount of absorbed polystyrene microspheres (Fig. 4B).

4. Discussion

4.1. Uptake and accumulation of microspheres in tadpoles

Generally, the ingestion of plastic microspheres by animals is the result of particle concentration, feeding mode and encounter rate in a patchy environment (Setälä et al., 2016). In the present study, the uptake of microspheres took no more than 1 h after exposure in tadpoles, and the excretion of microspheres occurred after no more than 6 h. The recorded uptake and excretion time of microspheres differs in species and sizes in previous studies. The uptake of 20 and 1000 nm particles was observed 30 min after exposure in Daphnia magna (Rosenkranz et al., 2009). Microspheres (10 μm) were found within the foregut of crabs 6 h after exposure (Watts et al., 2014). 100 nm polystyrene microspheres were detected in the feces of mussels (M. edulis) and oysters (Crassostrea virginica) 24 h after clearance (Ward and Kac, 2009). Our results suggest that microspheres were likely ingested and egested relatively fast by tadpoles.

During the exposures of this study, the accumulation of microspheres in tadpoles did not show a time-dependent relationship. These results are in accordance to the accumulation in water fleas.
(D. magna) and mussels (M. edulis) (Rosenkranz et al., 2009; Von Moos et al., 2012). However, the quantity of microspheres ingested by sea urchin larvae (Tripneustes gratilla) declined over time (Kaposi et al., 2014); the number of microspheres increased in fish during the first 48 h and in haemolymph of crabs up to 24 h after exposure (Farrell and Nelson, 2013; Lu et al., 2016).

The accumulation of microspheres in the tadpoles showed a significant concentration-dependent relationship. These results are in accordance to those in adults of polychaetes (Arenicola marina) and larvae of sea urchin (T. gratilla) (Besseling et al., 2013; Kaposi et al., 2014). The highest concentration of microspheres is up to 1.1 items ml⁻¹ in freshwater environment (Moore et al., 2011), indicating that the lower concentrations used in the present study are environmentally relevant. Our results suggest that aquatic organisms will ingest and accumulate more microspheres if the concentrations of particles continue to increase in the future. The high accumulation of microplastics within organisms may have many impacts such as gut blockage, physical injury, altered feeding behavior and reduced energy allocation (Wegner et al., 2012; Wright et al., 2013a; Cole et al., 2015; Watts et al., 2015).

4.2. Elimination of microspheres from the tadpoles

In the present study, the absorbed microspheres were retained in the tadpoles for at least 6 d, though they were egested quickly on the first day of clearance. Xenopus tadpoles egested most of microspheres from their digestive system within 6 days, and the remaining particles were likely retained in the gills. Microspheres could be retained longer in the gills than in the digestive system because gills have many arborizations, providing a larger surface area and a greater likelihood of adherence to particles. For example, microspheres were still present in haemolymph of crabs up to 21 d after transfer to a tank with flow-through seawater (Farrell and Nelson, 2013). Similarly, polystyrene microspheres translocated from the gut to the circulatory system of mussels within 3 d and persisted for over 48 d (Browne et al., 2008). Prolonged residence time of plastics implies that they will lead to more severe effects on the organisms (Wright et al., 2013a).

Ward and Kach (2009) suggested that smaller polystyrene microspheres (0.1 μm) have a longer gut retention time than larger ones (10 μm) in mussels (M. edulis) and oysters (C. virginica). Rosenkranz et al. (2009) also found that the depuration rate of 1000 nm carboxylated polystyrene microspheres reached 90% while the rate of 20 nm microspheres was only 40% in D. magna 4 h after clearance. In the present study, however, there was no significant difference in the depuration rate between large and small particles 1 or 6 d after clearance (p > 0.05). This might be due to only one order difference in diameter between two types of particles.
4.3. Effect of feeding

In present study, when food was absent, there was a 2 (12 h) to 4 (24 h) fold increase in the quantity of microspheres in tadpoles, and the tadpoles egested less microspheres. Similarly, in the study of Kaposi et al. (2014), when sea urchin larvae were exposed to microspheres for 6 h, the quantity of larvae with microspheres in their stomachs increased by 10 folds without feeding compared to that with feeding. The filter apparatus of Xenopus tadpoles enables them to exploit a wide range of food sources such as phytoplankton and detritus from the water. It is very likely that microplastics are closely associated with the food of the tadpoles, and tadpoles might take these waterborne microplastics as a kind of nekton and ingest them. As some of the highest concentrations of microplastics are found in freshwater systems (Moore et al., 2011), ingestion of microplastics in tadpoles may be exacerbated in oligotrophic environments. Conversely, ingestion of microplastics in tadpoles may
be reduced when they have adequate food.

Plastics debris is accumulating in marine environments with the increase in plastics production. It is reported that the mass of plastic is approximately six times that of plankton in the north Pacific central gyre (Moore et al., 2001). If the level of plastic pollution in oceans continues to increase, plastic garbage will outweigh fish by 2050 (World Economic Forum, 2016). In the Goiana Estuary, the total density of microplastics (items 100 m$^{-3}$) represented half of the total fish larvae density and was comparable to fish eggs density (Lima et al., 2014). Our results indicate that aquatic organisms might ingest more microplastics if the abundance of microplastics continue to increase while the available food decreases, which will greatly increase the ecological risk of microplastics to organisms. Long-term exposure experiments have been conducted utilizing *Xenopus*, from egg and young larval stages through completion of metamorphosis. For example, 2 mg L$^{-1}$ nano-ZnO was found to inhibit metamorphosis of *X. laevis* (Nations et al., 2011). In the future, we will study the hydrodynamic behaviour of the particles and the interaction with food.

### 4.4. Pathways of microspheres in tadpoles

In the present study, we found that the major routes for uptake of microspheres by the *Xenopus* tadpoles were through the mouth and gills. *Xenopus* tadpoles have large branchial baskets, dense gill filters, and lack the keratinized mouthparts to graze on particles attached to a substrate. Generally, small particles are directly entrapped in mucous strands in the pharynx, and large particles are trapped by papillae in the buccal cavity as well as on gill filters or mucus in the pharynx. In the present study, microspheres could have been carried into the mouths of tadpoles from the surrounding water, transported through the esophagus into the stomach, passed on to the intestine and then egested through the cloaca (Fig. 5). The captured particles in the gills could also have been passed by cilia through the pharynx into the foregut.

Previous studies suggest that the accumulation of microplastics in specific locations in organisms greatly depends on the size of the particles and the species (Wright et al., 2013b). For example, 0.5 μm polystyrene microspheres accumulated in the hepatopancreas, haemolymph and ovaries of crabs, but 8–10 μm polystyrene microspheres mainly accumulated in gills and gut (Farrell and Nelson, 2013; Watts et al., 2014). In mussels, however, 3 and 9.6 μm polystyrene microspheres translocated from the gut cavity to the circulatory system (Browne et al., 2008). The accumulation of nanoplastics was detected in the digestive tract, eyes and pharynx, but not in the brain or blood in *X. laevis* tadpoles at stage 45/46 after exposure to 50 nm polystyrene nanoparticles (Tussellino et al., 2015).

*X. tropicalis* has been used as an important test species for assessing the impact of environmental toxins on amphibians, which are in decline in many areas of the world due to increased use of pesticides and other toxic chemicals (Collins and Storfer, 2003). *X. tropicalis* embryos and tadpoles have been proved very sensitive to chemical exposure (Shi et al., 2012). To date, microplastics pollution is a growing concern in the freshwater environment (Su et al., 2016). However, there are no reports of ingestion of microplastics in tadpoles of *X. tropicalis*. Therefore, our results are of particular interest for the biomonitoring of microplastics because the filter-feeding activity of tadpoles exposes them directly to microplastics in the aquatic environments.

### 5. Conclusion

In conclusion, we found that polystyrene microspheres were likely ingested and egested relatively fast by tadpoles. The accumulation of microspheres in the tadpoles was concentration dependent but not time dependent. The absorbed microspheres were retained in the tadpoles for at least 6 d, though they were egested quickly on the first day of clearance. The presence of food decreased the uptake and increased the elimination of microspheres in the tadpoles. The increasing input of plastic from the land to the sea will undoubtedly lead to the increase of microspheres and relative decrease of the proportion of food to small particles in the aquatic environment. The increase of microplastics and decrease of available food in the freshwater environments might have combined and adverse effects on the aquatic organisms.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.09.002.

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