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Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (*Cyprinodon variegatus*)

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ABSTRACT

The increasing global contamination of plastics in marine environments is raising public concerns about the potential hazards of microplastics to environmental and human health. Microplastics formed by the breakdown of larger plastics are typically irregular in shape. The objective of this study was to compare the effects of spherical or irregular shapes of microplastics on changes in organ distribution, swimming behaviors, gene expression, and enzyme activities in sheepshead minnow (*Cyprinodon variegatus*). Both types of microplastics accumulated in the digestive system, causing intestinal distention. However, when compared to spherical microplastics, irregular microplastics decreased swimming behavior (i.e., total distance travelled and maximum velocity) of sheepshead minnow. Both microplastics generated cellular reactive oxygen species (ROS), while ROS-related molecular changes (i.e., transcriptional and enzymatic characteristics) differed. This study provides toxicological insights into the impacts of environmentally relevant (fragmented) microplastics on fish and improves our understanding of the environmental effects of microplastics in the ecosystem.

1. Introduction

Although the extensive use of plastics in commercial, industrial and medicinal applications is convenient for modern life, there is increasing awareness of their deleterious environmental effects (Wright et al., 2013; Cole et al., 2011). A particular concern is pollution arising from plastic items that are imprudently discarded and eventually enter the sea (Cole et al., 2011). In the marine environment, ultraviolet radiation, wave action, and other physical, chemical and biological processes cause the successive fragmentation of these materials into micro-sized particles, referred to as secondary microplastics (Cole et al., 2011; Fendall and Sewell, 2009). Other important sources of microplastic pollution are cosmetics, exfoliating materials used in facial and hand washes (Cole et al., 2011; Fendall and Sewell, 2009), plastic resins (Barnes et al., 2009), and air-blasting equipment (Cole et al., 2011). These microplastics have accumulated in various environments, with maximum concentrations reaching 100,000 particles m^{-3} (Noren and Naustvoll, 2010).

The small size of microplastics means that they are readily ingested by marine organisms, causing problems in marine ecosystems (Cole et al., 2013; Lu et al., 2016). Microplastics have been detected in fish and birds as well as in zooplanktonic organisms such as copepods (Derraik, 2002; Fendall and Sewell, 2009; Moore et al., 2001). One study suggests that fish confuse microplastics with prey (de Sa et al., 2015). Recent studies that investigated the impact of microplastics on several fish species observed that microplastics accumulated in the gills, gut and liver (Avio et al., 2015b; Greven et al., 2016; Lu et al., 2016; Güven et al., 2017; Jovanović, 2017). The distribution of microplastics among organs differed according to their size (Lu et al., 2016). Ingestion of microplastics by marine organisms may be fatal (by causing starvation) or may cause sub-lethal symptoms that affect digestion or locomotion (i.e., impairing escape from predators or migration) (Avio et al., 2016; Pawar et al., 2016). Recently, risks of ingested microplastics were reported, including obstruction, physical damage, and histological changes in the intestines; behavioral changes; changes in lipid metabolism; and liver metastasis (Jovanović, 2017). Microplastics are also known to cause growth and developmental inhibition, endocrine disruption, energy disturbance, oxidative stress, immune and neurotransmission dysfunction, and genotoxicity (Avio et al., 2015a; Besseling et al., 2014; Della Torre et al., 2014; Lee et al., 2013; Rochman et al., 2014). Microplastics have been reported to induce oxidative stress by activating p-JNK and p-P38 in a monogonont rotifer (Jeong et al., 2016) and to reduce functions linked to health and biodiversity in worms (Browne et al., 2013). Contaminated polyethylene

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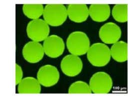
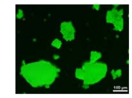
Table 1
Characteristics of tested microplastics.

	Size (μm)	Shape	Polymer type	Density (g/cm^3)	Image
Spherical microplastics	150–180 (Particles in size range > 90%)	Sphere (Spherical: > 90%)[b]	Polyethylene	1.025	
Irregular microplastics	6–350[a] (Fragmented from 300 to 355 μm microplastics)	Irregular (Spherical: 0.7%)[c]	Polyethylene	1.025	

[a] Minor axis: 39 ± 40 (mean ± standard deviation (S.D.)), Major axis: 55 ± 56 (mean ± S.D.).
[b] Manufacture supplied.
[c] Particles with circularity > 0.9, analyzed from 1281 samples were indicated as spherical shape.

plastic debris caused down-regulation of vitellogenin, choriogenin, and estrogen receptor gene expression in Japanese medaka, indicating potential endocrine disruption (Rochman et al., 2014) and polycarbonate and polystyrene plastic particles stressed the innate immune system of fathead minnows (Greven et al., 2016). In addition, Chen et al. (2017) reported that microplastics caused up-regulation of *zfrho* visual gene expression in zebrafish.

Microplastics found in marine environments are mostly irregular in shape as a result of weathering and fragmentation (Eriksen et al., 2014; Hidalgo-Ruz et al., 2012; Kärrman et al., 2016). Cole et al. (2014) reported that most microplastics present on the seashore were irregular particles, and only a small proportion of these microplastics were spherical. Microplastics found in the digestive tracts of fish sampled from marine environments are highly variable in terms of shape and roughness (Mazurais et al., 2015). Boerger et al. (2010) reported that 94% of plastics ingested by organisms in the North Pacific central gyre were fragmented plastics. Avio et al. (2015b) observed that approximately 90% of microplastics (fragmented 57%; fibrous 23%; films 11%) found in the gastrointestinal tracts of fish in the Adriatic Sea were irregular type. A spherical shape might cause less injury and a weaker gut inflammatory reaction than would irregular shapes (Mazurais et al., 2015). However, most experimental studies on microplastics have used spherical particles, ignoring the potential toxic effects caused from exposure to irregular microplastics (Cole et al., 2013; Lu et al., 2016; Wardrop et al., 2016). Recently, the effects of irregular microplastics have been reported (Von Moos et al., 2012; Karami et al., 2016; Ogonowski et al., 2016), but the number of studies is smaller than the number of studies that have dealt with spherical microplastics. This indicates that toxicities derived from spherical particles may not accurately reflect the actual toxicity in the environment.

In the present study, we investigated the toxicological effects of irregularly shaped microplastics, representing those commonly found in the environment, and compared their effects with those caused by exposure to spherical microplastics; specifically, swimming behavior, distribution in the organs, ROS generation, and changes at the transcriptional and enzymatic levels were evaluated in sheepshead minnow (*Cyprinodon variegatus*) larvae. The sheepshead minnow is used as a standard laboratory test organism for studying marine pollution levels in effluents released into marine and estuarine waters by the United States Environmental Protection Agency (US EPA, 2002), and the species' sensitivity to chemicals and easy breeding make it a suitable model to test the effect of microplastics in the marine environment (Hendon et al., 2008). This study provides an environmentally relevant identification and assessment of the risks associated with microplastic exposure in marine ecosystems.

2. Materials and methods

2.1. Test materials and characterization

Two sizes of green polyethylene microspheres with similar properties, such as density and polymer type, were purchased as dry powders (Cospheric, Santa Barbara, CA, USA). Microspheres with diameters 150–180 μm were used to examine the effects of spherical microplastics. Irregular microplastics were produced by fragmenting 300–355 μm microspheres using a homogenizer (TissueLyser LT, Qiagen, Hilden, Germany). The fragments were soaked in 0.01% Tween-80, and the suspension was filtered through a 10-μm polycarbonate membrane. The fragmented microplastics retained on the filter were washed with distilled water, dried for 3 d at room temperature and collected in a glass vial. The size-distribution, circularity and particle numbers of fragmented microplastics were analyzed using a fluorescence microscope (IX51, Olympus, Tokyo, Japan) with Java-based image processing and analysis software (ImageJ 1.50i, National Institute of Health, Bethesda, MD, USA). The number of spherical microplastic particles was calculated based on their density, size, and weight. The physicochemical characteristics of the microplastics are shown in Table 1.

2.2. Test organism maintenance

Sheepshead minnows (*Cyprinodon variegatus*) were obtained from NLP, Co., Ltd., Busan, Korea and cultured for one year in our laboratory. Approximately 10 to 15 healthy adult sheepshead minnows were raised in a glass tank (20 L) filled with 18 L of artificial seawater (ASTM, 2013) under a 16 L: 8D photoperiod and constant temperature (26 ± 1 °C). Water parameters were measured daily: salinity was adjusted to 26‰; pH was 7.6 ± 0.5; dissolved oxygen was 7.2 ± 0.3 mg/L. The water was renewed twice a week. Fish were fed 1.0% body weight twice daily with *Artemia* sp. nauplii and commercial fish food (TetraMin, Tetra Werke, Melle, Germany). For experiments, larval fish (three-week-old larvae, 8 ± 1 mm) were obtained from fertilized eggs.

2.3. Test regime

The microplastics were weighed and suspended in 0.01% Tween-80 to prepare the stock microplastics colloidal suspensions (500 and 2500 mg/L). Tween-80 solution, diluted in deionized water, was utilized as a surfactant to obtain the dispersibility of microplastics in artificial seawater. The nonionic surfactant Tween-80 has frequently been included as an ingredient in dosing vehicles for in vivo studies (Zhang et al., 2003; Khan et al., 2015). Stock suspensions were diluted 10 times with artificial seawater to make test concentrations (50 and 250 mg/L) of microplastic test suspensions (final concentrations of Tween-80 were adjusted to 0.001%, v/v).