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Biomarker responses in zebrafish (*Danio rerio*) larvae exposed to pristine low-density polyethylene fragments☆

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ABSTRACT

There are serious concerns over the adverse impacts of microplastics (MPs) on living organisms. The main objective of this study was to test the effects of MPs on the total length, weight, condition factor (CF), transcriptional level of antioxidant, anti and pro-apoptotic, and neurotransmitter genes, and the histopathology of the gill, liver, brain, kidney, and intestine in the larvae of zebrafish (*Danio rerio*). Fish were exposed to one of three levels of pristine low-density polyethylene (LDPE) fragments (5, 50, or 500 μg/L) for 10 or 20 days. No significant changes were observed in any of the selected biomarkers across MP concentrations at days 10 or 20. The expression of *casp9* (caspase 9, apoptosis-related cysteine protease), *casp3a* (caspase 3, apoptosis-related cysteine protease a) and *cat* (catalase), however, were significantly lower in the larvae sampled at day 20 than day 10. We provide evidence that virgin short-term exposure to LDPE fragments has minimal impact on biomarker responses in *D. rerio* larvae.

1. Introduction

Plastics are an indispensable part of modern life. The low production cost, high elasticity and durability, and the low degradation rate of plastics are among the main factors for their applicability to a variety of industries. Plastic materials, however, that enter waterways as waste are continuously fragmented by natural and synthetic processes into small particles (1–1000 μm) called microplastics (MPs; Karami et al., 2016b). The presence of MPs in freshwater (Eerkes-Medrano et al., 2015) and marine ecosystems (Cole et al., 2011) has been reported by previous studies.

Due to their size, shape, and colour resemblance as prey or food (Shaw and Day, 1994), plastic particles are deliberately or inadvertently ingested by a range of species. This may block their alimentary tract (Moore, 2008), resulting in accumulation within the body, or can even translocate to other organs (Farrell and Nelson, 2013). Despite the relatively large number of studies on MP loads in aquatic environments, their biological impact on organisms are largely unclear. Low-density polyethylene (LDPE) is one of the most widely-used plastic polymers (PlasticsEurope, 2015) and have been found in different environmental compartments (Yu et al., 2016). Depending on their origins, MPs are categorized into primary and secondary sources. Primary MPs are deliberately manufactured (e.g. microbeads) while secondary MPs are generated by the fragmentation of macroplastics. Fragments are one the major types of MPs widely detected in different environmental compartments (e.g., Ballent et al., 2016). Hence, to mimic environmental exposure to MPs, non-uniformly shaped MP fragments were employed in this study. Earlier studies have shown virgin MPs could contain persistent organic pollutants (POPs, Rochman et al., 2013b). Therefore, to control the toxicity posed by other contaminants loaded on MPs, the level of major environmental contaminants on virgin LDPE were tested before the exposure experiment.

Zebrafish (*Danio rerio*) is a commonly used vertebrate in a wide variety of toxicological studies. This is primarily due to their small size as well as ease of breeding and rearing in captivity (Spitsbergen

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and Kent, 2003). Since early life stages are sensitive to environmental stressors (Cara et al., 2005), exposure to MPs could interfere with their ontogenesis and survival. Full absorption of yolk-sac (i.e., end of the yolk-sac larval stage) in *D. rerio* occurs within 7–8 days of post-fertilization. The larval stage ends at the age of 30-day post-fertilization (dpf, see the ZFIN webpage at https://zfin.org/zf_info/zfbook/stages/).

Some contaminants can induce the production of reactive oxygen species (ROS) in aquatic organisms, leading to oxidative stress (Livingstone, 2003). Teleosts possess defence systems such as free radical scavengers and specific antioxidant enzymes to counteract the adverse impacts of ROS (Halliwell and Gutteridge, 2015). Copper/zinc superoxide dismutase (SOD1) is an important antioxidant enzyme neutralizing oxygen radical-mediated toxicity. Other enzymes such as GPx (glutathione peroxidase), CAT (catalase), GST (glutathione *S*-transferase) are among the major antioxidant enzymes and are commonly used as biomarkers of exposure to contaminants (Karami et al., 2015). Virgin and unplasticized plastic polymers are believed to be inert, having no impacts on the organism's health. Lu et al. (2016), however, showed the potential of polystyrene (PS) MPs to induce oxidative stress in adult *D. rerio*. So far, no study has investigated changes in antioxidant biomarkers in organisms following the exposure to LDPE.

Other important biomarkers include B-cell leukaemia/lymphoma 2 (Bcl-2), which is an important anti-apoptotic protein (Adams and Cory, 2007), and is a major component of chemoresistance in humans (Amundson et al., 2000). Tumour protein p53 (*tp53*) is a tumour suppressor gene (Levine et al., 1991). Aspartic specific cysteine proteases (caspases) are a family of enzymes playing a major role in apoptosis (Fan et al., 2005). Oxidative stress, inflammation, and apoptotic responses to nanoplastics and MPs have been shown in earlier studies (Wang et al., 2013; Lu et al., 2016).

The chief advantage of molecular biomarkers is their rapid response time but has poor ecological relevance (Thomas, 1990). In contrast, growth/reproductive biomarkers require a longer time to respond to stress (i.e. less sensitive to stress) while carrying high biological and ecological significance (Donaldson, 1990). These parameters, however, have been scarcely investigated in the organisms exposed to MPs. Histological endpoints stand between the molecular and growth/reproductive biomarkers (Teh et al., 1997). To better understand the biological impacts of MPs on *D. rerio* larvae, we employed a suite of biomarkers belonging to different levels of biological organization.

The general aim of this study was to gain a baseline understanding on whether pristine MPs could cause any adverse effects in fish larvae. The main objective of this study was to test if the exposure to different concentrations of virgin LDPE fragments for different durations would impact total length, weight, CF, gene expressions, and pathological features in *D. rerio*.

2. Materials and methods

2.1. Particle size, count, and morphology

Microplastics were purchased from Toxemerge Pty Ltd (Australia). The particle size distribution of the MP fragments (% volume) was determined in triplicate by laser diffraction using Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The morphology of gold-coated MP fragments was evaluated by scanning electron microscopy (SEM, Hitachi S-3400-II, USA). The model MPs were irregularly shaped virgin LDPE fragments (Fig. 1) with 90% of the particles sized below 17.6 μm [mass division diameter 90 (D90) = 17.6], 50% (D50) below 10.9 μm, and 10% (D10) below 4.64 μm. The number of particles per gram of powder was estimated using a hemocytometer. The concentrations tested in this study (i.e. 5, 50, 500 μg/L equivalent to 1040, 10,400, and 104,000 particle/L, respectively) were within the reported range in aquatic environments (Moore et al., 2011; Zhao et al., 2014).

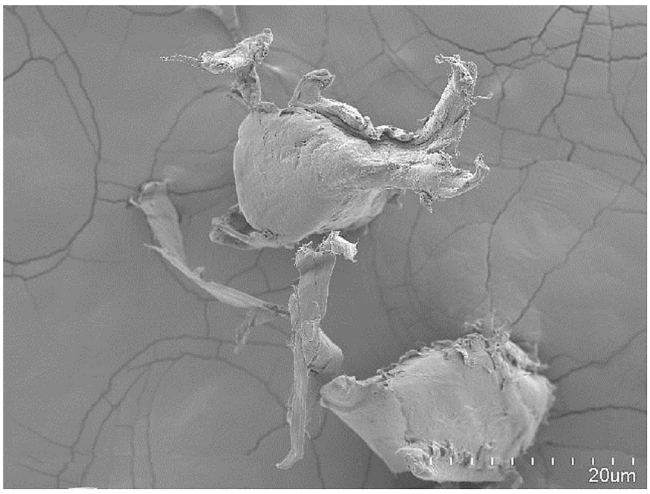

Fig. 1. Scanning electron microscope (SEM) image of the LDPE fragments.

2.2. Fish husbandry

Wild juvenile *D. rerio* were purchased from a local ornamental fish dealer in Selangor, Malaysia. Fish were fed with frozen bloodworms *ad libitum* twice a day for four months after which point they reached maturity. Spawning behavior was then induced by randomly selecting and placing 1 female and 2 males into a breeding chamber and left undisturbed overnight (Lyche et al., 2013). The following morning eggs were collected.

2.3. Exposure experiment

To cover the entire larval stage, we exposed 8-dpf *D. rerio* to MPs for 10 and 20 days. *Danio rerio* larvae (5 dph) were randomly distributed among 2 L glass beakers filled with 1.8 L UV-treated dechlorinated tap water (5 beakers/replicates per treatment, 6 fish per beaker, 50 beakers in total). Fish belong to each sampling points were kept in separate beakers. Fish were fed *ad-libitum* on commercial powder (Cargill, crude protein: 38–40%) twice daily for three days. Throughout the study, beakers were gently aerated with an airstone connected to a centralised pump.

A stock suspension of LDPE fragments (18 mg/mL) was prepared in HPLC–grade ethanol. Ethanol was used as the carrier solvent due to its lower density than the LDPE polymers in order to minimize particle aggregation, and hence reduce experimental error. Sufficient volumes of the stock solution were added to the beakers that contained 8-dpf larvae to obtain final concentrations of 5, 50 and 500 μg/L MPs. During the exposure, larvae were fed *ad libitum* twice a day once in the morning with newly-hatched *Artemia* nauplii (<5 h) and once in the afternoon with fish powder sprinkled on top of each beaker. This strategy ensured the uptake of MPs on the surface and within the water column. Negative and solvent control group (<0.01% ethanol v/v) were employed per sampling points. The experiment was performed under 12:12 light/dark cycles. The test solution was completely replaced with fresh spiked solution once daily. Water parameters were measured daily: temperature 28.42 ± 1.24 °C, pH 7.26 ± 0.53, dissolved oxygen 6.88 ± 0.19 mg/L, alkalinity 49.00 ± 3.96 mg $CaCO_3$, hardness 55.00 ± 2.09 mg $CaCO_3$,