Environmental Pollution 235 (2018) 918–930

Contents lists available at ScienceDirect

Environmental Pollution

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

Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (*Danio rerio*) gills and zebrafish embryos☆

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ARTICLE INFO

Article history:
Received 1 September 2017
Received in revised form
11 January 2018
Accepted 11 January 2018

Keywords:
Microplastics
Zebrafish gills
Zebrafish embryos
POP transfer
benzo[a]pyrene

ABSTRACT

Since only a few studies have investigated effects of microplastics (MPs) by routes other than ingestion, this study was designed to analyze the accumulation patterns and transfer of toxic substances associated with microplastic exposure by simple attachment to (1) adult zebrafish (*Danio rerio*) gills and (2) zebrafish embryos. Two sizes of fluorescently labelled polymers (1–5 and 10–20 μm) loaded with the model polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (BaP) were used to analyze fate, accumulation and transfer of microplastic-associated persistent organic pollutants (POPs) on gills and embryos.

Results indicate that microplastics did not permanently accumulate at high amounts in adult zebrafish gills after 6 nor 24 h of incubation: Most particles only superficially adhered to the mucus layer on the filaments, which is constantly being excreted. In contrast, the smaller and heavier MPs (1–5 μm) accumulated in high numbers on the surface of zebrafish egg chorions. In both exposure scenarios, transfer of BaP could be visualized with fluorescence microscopy: A prominent BaP signal was visible both in gill filaments and arches after 6 and 24 h incubation and in zebrafish embryos after exposure to BaP-spiked microplastics. Furthermore, the gill EROD (Ethoxyresorufin-O-deethylase) assay showed a clear trend to CYP 1A (Cytochrom P450 1 A) induction *via* exposure to BaP-spiked microplastics. However, BaP from spiked microplastics did not reach sufficiently high concentrations to be able to induce morphological effects in the fish embryo toxicity test (FET). In contrast, control exposure to waterborne BaP did induce effects in the FET.

As a conclusion, microplastics can also transfer POPs not only *via* ingestion, but also by simple attachment to epithelia or *via* the water column. However, further studies are needed to clarify if these interactions are of environmental concern relative to waterborne exposure to toxic substances.

1. Introduction

Microplastics, plastic particles smaller than 5 mm (Andrady, 2011; Barnes et al., 2009; Cole et al., 2011) or 1 mm (Galloway and Lewis, 2016) – depending on definition – represent an increasingly widely discussed issue with respect to environmental pollution (Galloway and Lewis, 2016; Barboza and Gimenez, 2015; Thompson, 2015; Thompson et al., 2004; Eerkes-Medrano et al., 2015; do Sul and Costa, 2014). Recently, along with plastic debris, microplastics were even generally referred to as a 'planetary boundary threat' (Galloway and Lewis, 2016). Deriving from larger plastic items or produced as such small particles (Cole et al., 2011), microplastics are thought to have a variety of impacts on the environment (Thompson, 2015; Eerkes-Medrano et al., 2015). Numerous studies have shown interactions between these newly introduced particles and aquatic organisms (Wright et al., 2013). Detritus feeders, filter feeders as well as predators and even corals were shown to ingest and be effected by different types and sizes of microplastics, depending on natural feeding habits (Browne et al., 2008, 2013; Carlos de Sa et al., 2015; Cole et al., 2013; Hall et al., 2015; Lusher et al., 2013; Rochman et al., 2013; Setala et al., 2014; Sussarellu et al., 2016). Thus, there is no doubt that microplastics are being ingested by a wide variety of aquatic organisms.

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.

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https://doi.org/10.1016/j.envpol.2018.01.028
0269-7491/

Furthermore, since environmental microplastics found worldwide on beaches have been shown to carry considerable amounts of different highly toxic substances (Frias et al., 2010; Fries et al., 2013; Rios et al., 2007), it is of special importance to know if such substances might be transferred to organisms in interaction with microplastics. Numerous recent studies have made attempts to evaluate if these substances desorb from microplastics following ingestion and if this might pose an additional threat to organisms in aquatic environments. In addition to mathematically-based hypotheses, various experimental approaches have been used, from chemical analyses of whole tissues to more sensitive assays such as enzymatic tests, histological analyses and fluorescence tracking (Rochman et al., 2013; Batel et al., 2016; Chua et al., 2014; Gouin et al., 2011; Koelmans et al., 2016).

As a matter of fact, in all of these studies the major focus was set on oral ingestion of microplastics, and only a few studies included other pathways for microplastic exposure. Watts et al. (2014) reported that, upon exposure, clean microspheres were not only found in the intestinal tract, but also in the gills of the shore crab (*Carcinus maenas*) until 21 days post exposure (Watts et al., 2014). Lu et al. (2016) exposed zebrafish (*Danio rerio*) to very high amounts of nano- (70 nm) and micro- (5 and 20 μm) polystyrene particles and found 5 μm particles in gills, gut and liver after 7 days of exposure, while 20 μm particles were found in gills and gut only (Lu et al., 2016). Furthermore, among others, they found that microplastics induced an inflammatory response, lipid accumulation in the liver and modified metabolic profiles. Nobre et al. (2015) analyzed the leaching of pollutants from plastic pellets to embryos of the sea urchin (*Lytechinus variegatus*) *via* the water column and found that especially plastic additives from virgin pellets caused anomalous embryonic development (Nobre et al., 2015). Plastic pellets from beach samples did not induce any toxic effects. In most cases, however, in contrast to intestinal uptake of microplastics, alternative pathways of microplastic exposure and subsequent effects on aquatic organisms have been neglected so far.

Therefore, the present study was designed to focus on the accumulation, behavior and effects of microplastics on epithelia or outer surfaces of organisms in general. Due to their great surface, fish gills carry a high potential for microplastic accumulation and attachment in all aquatic organisms. Due to the constant filtration of water, high microplastic concentrations might also pose an additional threat to the gills of aquatic organisms, e.g. if microplastics accumulate and transfer harmful substances to the gills. On the other hand, fish eggs with their lipophilic chorion might also represent a potential surface for increased deposition and accumulation of microplastics. Therefore, both adult zebrafish gills and zebrafish eggs with fish embryos were studied as alternative pathways for the potential transfer of POPs *via* microplastics.

Benzo[a]pyrene was used as a model polynuclear aromatic hydrocarbon (PAH), since it has been shown (1) to induce EROD activity in zebrafish gills (Jönsson et al., 2009), (2) to cause morphological effects in the fish embryo toxicity test (FET) (Huang et al., 2014; Weigt et al., 2011) and, given its autofluorescence properties, (3) to be traceable by means of epifluorescence microscopy (Batel et al., 2016).

2. Material and methods

2.1. Material

Adult zebrafish aged 24 months were obtained from the breeding and maintenance facilities of the Aquatic Ecology and Toxicology Group at the Center for Organismal Studies Heidelberg (licensed by regional animal welfare authorities under 35–9185.64/BH Braunbeck). Temperature was maintained at 26.0 ± 1.0 °C, and fish were kept under a constant artificial dark/light regimen of 8/16hrs. Constant filtration in combination with permanent flow-through conditions (two-fold water exchange per day) guaranteed that ammonia, nitrite, and nitrate concentrations were kept below detection limits (5, 1 and 140 mg/L, respectively). Fish were fed commercially available artificial diets (TetraMin™ flakes; Tetra, Melle, Germany) twice daily *ad libitum*, supplemented with *Artemia* nauplii. Zebrafish spawning groups consisted of 15–20 zebrafish, and zebrafish eggs were collected according to OECD guideline 236 (OECD TG 236, 2013).

Fluorescent microplastic particles with sizes of 1–5 μm (proprietary polymer of undisclosed composition) and 10–20 μm (polyethylene) were purchased from Cospheric LLC (Santa Barbara, CA, USA). Densities were 0.99–1.01 g/cm^3 for 10–20 μm particles and 1.3 g/cm^3 for 1–5 μm particles. The microplastic particles were labelled with green fluorescence (505 nm peak) with excitation wavelengths of 365 nm and 470 nm. Benzo[a]pyrene, resorufin and resorufin ethyl ether (ethoxyresorufin) were obtained from Sigma-Aldrich (Deisenhofen, Germany). The anesthetic tricaine consisted of 400 mg/L tricaine mesylate (ethyl-3-aminobenzoate methanesulfonic acid, Sigma Aldrich) and 480 mg/L sodium bicarbonate (Sigma Aldrich). Phosphate buffer consisted of 3.2 g/L $NaH_2PO_4 \times H_2O$, 13.75 g/L $Na_2HPO_4 \times 2\ H_2O$ and 250 mg/L $MgCl_2 \times 7\ H_2O$ (AppliChem, Darmstadt, Germany).

2.2. Methods

2.2.1. Zebrafish gills

2.2.1.1. Microplastic accumulation in zebrafish gills and histological analyses. Four fish per group were exposed in 1 L tanks under static conditions (no flow-through) to either pure water (no microparticles) or microplastics (1–5 or 10–20 μm) dissolved in water. Three mg of each microplastic preparation, corresponding to roughly 5×10^6 of 1–5 μm and 1.2×10^6 of 10–20 μm particles were thoroughly suspended in 5 ml Aqua bidest (purified water) immediately before adding to the exposure tanks. A constant airflow assured mixing of microplastics. After 6 or 24 h, respectively, the fish of each tank were euthanized with 400 mg/L tricaine (MS-222) and decapitated behind the gill opercle. Heads were immediately transferred for fixation to Davidson's fixative medium (330 ml 96% EtOH, 220 ml 37–40% formaldehyde, 115 ml glacial acetic acid in 335 ml Aqua bidest.) for 3 day at 4 °C. Using entire heads allowed examination of not only the gills themselves, but also surrounding tissues (e.g. gill chamber) with regard to microplastic particle accumulation. After fixation, specimens were dehydrated by a graded series of ethanol in a TP1020 tissue processor (Leica, Wetzlar, Germany), embedded in paraffin wax (heated paraffin embedding module EG1140 H; Leica, Germany), rapidly hardened on a EG1140 C cold plate (Leica, Germany) for several minutes and stored overnight at −22 °C. Embedded samples were cut at 4 μm thickness with a HN40 microtome (Reichert-Jung, Nussloch, Germany), transferred to glass slides covered with glycerol albumen solution (Serva, Heidelberg, Germany), dried overnight at room temperature and stained with standard hematoxylin-eosin (HE) according to (Romeis, 1989). Slides were analyzed with epifluorescence at an excitation wavelength of 465–495 nm and an emission wavelength of 515–555 nm (Nikon, Düsseldorf, Germany).

2.2.1.2. The transfer of benzo[a]pyrene from microplastics to gills. The potential of microplastics to function as vectors of toxic hydrophobic compounds was investigated by spiking the particles with benzo[a]pyrene (BaP) before exposure. Loading was performed after Batel et al. (2016): In brief, 3 mg of 1–5 μm and 10–20 μm plastic particles were pre-incubated with 20 μl of 12.6 mg/ml BaP in 10 ml Aqua bidest (= 1 μmol, 252 μg BaP) in a