



Combined effects of microplastics and chemical contaminants on the organ toxicity of zebrafish (*Danio rerio*)

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ABSTRACT

Microplastics contamination of the aquatic environment is considered a growing problem. The ingestion of microplastics has been documented for a variety of aquatic animals. Studies have shown the potential of microplastics to affect the bioavailability and uptake route of sorbed co-contaminants of different nature in living organisms. Persistent organic pollutants and metals have been the co-contaminants majorly investigated in this field. The combined effect of microplastics and sorbed co-contaminants in aquatic organisms still needs to be properly understood. To address this, we have subjected zebrafish to four different feeds: A) untreated feed; B) feed supplemented with microplastics (LD-PE 125–250 µm of diameter); C) feed supplemented with 2% microplastics to which a mixture of PCBs, BFRs, PFCs and methylmercury were sorbed; and D) feed supplemented with the mixture of contaminants only. After 3 weeks of exposure fish were dissected and liver, intestine, muscular tissue and brain were extracted. After visual observation, evaluation of differential gene expression of some selected biomarker genes in liver, intestine and brain were carried out. Additionally, quantification of perfluorinated compounds in liver, brain, muscular tissue and intestine of some selected samples were performed. The feed supplemented with microplastics with sorbed contaminants produced the most evident effects especially on the liver. The results indicate that microplastics alone does not produce relevant effects on zebrafish in the experimental conditions tested; on the contrary, the combined effect of microplastics and sorbed contaminants altered significantly their organs homeostasis in a greater manner than the contaminants alone.

1. Introduction

Microplastics represent one of the most recent global concerns for environmental and human health. The major concern is for the aquatic environment; the massive production and extremely widespread use of plastic have recently turned this compound and its degradation products into one of the prevalent marine litter (Wright et al., 2013).

The presence of microplastics in the guts and tissues of aquatic species including some commercially important bivalve mollusks, crustaceans and fish is well documented. This phenomenon has been detected worldwide. Santillo et al. (2017) as well as Jovanović (2017) have reviewed the studies on the detection of microplastics in commercially important species. In their works it emerges that, even though, methods for detection and measurement have not yet been harmonized (EFSA, 2016; Peng et al., 2017), microplastics in marine organisms can be found from Indonesian and US waters to Portugal, Spain, North Atlantic, North and Baltic Sea as well as central Mediterranean.

The effects of microplastics on the health of aquatic animals have been investigated in a variety of species. Effects can be: *i*) physical (due to the shape, color and dimension of the particles) and *ii*) chemical (due to the presence of additives and / or of sorbed chemical contaminants).

Physical effects of microplastics are primarily internal abrasions, blockage of the intestine (Wright et al., 2013), reduction of animal feeding and energy assimilation (Besseling et al., 2013) that can lead to reducing energy and fertility (Duis and Coors, 2016). The size of the particles is also an important factor for aquatic animal health. Particles greater than 150 µm are generally not absorbed and produce only local inflammation. However, smaller particles might induce systemic exposure and even penetrate organs (EFSA, 2016). In this perspective nanoplastics represent the most concerning problem as pointed out by Bouwmeester et al. (2015).

Chemical effects are due to the release of plastic components such as vinyl chloride or styrene and of plastic additives such as alkylphenols, brominated flame retardants (BFRs) and bisphenol A, for example. According to Teuten et al. (2009) toxicity studies on microplastics

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should be focused on the release of those additives rather than on plastic itself. Another important issue, that is currently a matter of debate, is the toxicity of chemical contaminants that can adhere to the plastic particles. Microplastics can concentrate persistent organic pollutants (POPs) which have a great affinity for the hydrophobic surface of plastic compared to water. For this reason, plastic can become heavily contaminated (Wright et al., 2013). However, the uptake of chemicals released from plastic by biota does not seem to be relevant (Rochman et al., 2014). In spite of bioaccumulation and biomagnification of some POPs released from microplastics have been demonstrated (EFSA, 2016 and references therein), the risk of ingesting contaminants from plastic is probably the same as ingesting environmentally contaminated preys, or the contaminants dispersed in the water (Koelmans et al., 2016). In other words, the contribution of microplastics to the dispersion of POPs in the environment, where they already are abundantly present, does not seem to be relevant (Lohmann, 2017). Furthermore, the presence of microplastics in aquatic animal digestive tracts seems to be transient and for this reason it has a low potential for bioaccumulation and biomagnification (Güven et al., 2017; Grigorakis et al., 2017). Despite this, microplastics can become a food safety issue in the case of bivalves (mussels, clams and oysters) and of some crustaceans (brown shrimps and lobsters, for example) of which the guts are generally eaten. Nevertheless, exposure studies performed on mussels contaminated with microplastics and sorbed chemical contaminants have ruled out a realistic risk for consumers due to the insignificant increase in the concentration of PCBs and PAHs that consuming such food would produce (Li et al., 2015).

Even though the role of microplastics in POP dissemination and biomagnification can be considered negligible, its role on the toxicity of aquatic animals is still a matter of debate and needs to be understood further. A preliminary study of Pedá et al. (2016) performed on seabass highlighted the fact that the combination of microplastics and chemical contaminants can increase the adverse effects produced by either plastic or contaminants considered individually. Studies performed on juvenile marine species (*Pomatoschistus microps*) showed that the effect of microplastics on contaminants could vary depending on the chemical and on the endpoint evaluated. For example, microplastics did not affect the toxicity of gold nanoparticles (Ferreira et al., 2016) but did affect the acute toxicity of chromium (VI) (Luís et al., 2015). In other words, microplastics might potentiate the toxic effect of chemical contaminants.

In this context, the objective of this work was to explore further the effect of microplastics on environmental contaminants to ascertain whether we are underestimating the risk of microplastics and especially contaminated microplastics for human and environmental health.

Our hypothesis was that microplastics significantly increases the toxic effects of chemical contaminants. To test it, we used the zebrafish (*Danio rerio*) animal model due to its relevance for eco- and human toxicology. On this model we compared the effects produced at organ level by three contaminated feeds; one containing only microplastics, one containing microplastics with sorbed chemical contaminants and one containing chemical contaminants without microplastics. The results obtained confirmed our hypothesis and altogether they highlight the need to consider the potentiating effect of microplastics on the toxicity of chemical contaminants in risk assessment evaluations.

2. Materials and methods

2.1. Chemicals used for the exposures and analytical quantification

Standards used for the spiking of the feed were: perfluorooctanoic acid (PFOA)(CAS 335-67-1); perfluorononanoic acid (PFNA)(375-95-1); perfluorooctanesulphonamide (PFOSA)(754-91-6); heptadecafluorooctane sulfonic acid (PFOS) (1763-23-1); 2,4,6-tribromophenol (2,4,6-TBP) (118-79-6); α -hexabromocyclododecane (α -HBCD) (3194-55-6) all from Sigma-Aldrich (St. Louis, MI, USA); methylmercury was

from Alfa Aesar (Haverhill, MA, USA) 9 #33553 (CAS 115-09-3); a technical mixture (DE71) of polybrominated biphenyl ethers (PBDEs) containing a congener composition as described by La Guardia et al. (2006) was kindly provided by Martha Axelstad, National Food Institute, Technical University of Denmark (Denmark); and polychlorinated biphenyl (PCB) congeners 28, 52, 101, 118, 138, 153, 180 were kindly provided by Michiel Kotterman, Wageningen Marine Research, Wageningen University (The Netherlands). The analytical standards and the C13- labelled internal standards of the halogenated compounds were from Wellington laboratories (Guelph, Ontario, Canada).

Acetone and methanol were from Rathburn (Walkerburn, UK); hexane, ethanol, acetic acid, concentrated sulphuric acid were from Merck (Darmstadt, Germany); anhydrous sodium sulphate was from VWR (Radnor, US-PA). Water was from a Milli-Q Gradient A10 system (Millipore, Bedford, US-MA).

The microplastics used in this work consisted of low density polyethylene (LD-PE) plastic particles (125–250 μ m) which were used to mimic the composition of marine litter. The size and shape of the plastic particles were chosen to not harm the fish; particle size was in fact too small to cause obstruction of the gut system and rounded so they would not damage the epithelial surface. The LD-PE plastic particles were made from RIBLON 30 milled to plastic particles size < 400 μ m, which were sieved to the size range 125–250 μ m. A “pre-wash” with octane and pentane was carried out to extract any solvent-soluble plastic monomers as well as to remove very fine attached plastic particles.

2.2. Feed preparation

All the feeds used in this work were produced by the Danish Technological Institute (DTI, Kolding, Denmark), their detailed composition and manufacturing is described elsewhere (Granby et al., 2018). In summary, the feed formulation consisted of a mix of fishmeal, fish oil, wheat and antioxidants. Four feeds were originally produced: A) basic feed without any added contaminant but with background contaminant levels; B) basic feed to which 4% of clean plastic (125–250 μ m diameter of LD-PE) was added; C) basic feed with 2% of plastic that had previously been immersed overnight in a solution of chemical contaminants to simulate environmental conditions where the contaminants are bound to the LD-PE microplastics due to their high affinity to plastic. The addition of the contaminants in this case was carried out by first solubilising the contaminants in ethanol, then mixing the contaminants solution with plastic particles overnight at room temperature allowing the ethanol to evaporate and the contaminants to adhere to the plastic; D) basic feed to which chemical contaminants with double concentration with respect to C were added. In this case the contaminants were added directly to the fish oil which was then spray-coated on the extruded feed pellets. The contaminants and their respective concentrations measured after the production of the feed formulations are shown in Table 1. Feed composition and characteristics are summarized in Table 2.

2.3. Animals and feeding trials

Groups of 15 wild type zebrafish of 10 months of age were placed in 4 different fish tanks in the aquatic facility of AZTI (REGA ES489010006105). Independent tanks were filled with 3 L of water taken from the main aquatic facility. No filtering systems were used in the experimental tanks. Before the experiment fish were acclimatised for two weeks. During this time fish were kept in the tanks where the experiment took place and maintained under experimental conditions (at a temperature of 26 ± 1 °C, on a 12-h light/12-h dark cycle). 75% of the water was changed every 3 days and replaced with new water. During the acclimatisation period fish were fed with the control feed (Feed A). For the experiment, in each tank, fish were fed with a different type of feed (0.2 g/day divided into two portions given in the

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