Marine Environmental Research 130 (2017) 69–76

Contents lists available at ScienceDirect

Marine Environmental Research

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

The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins

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ARTICLE INFO

Article history:
Received 14 December 2016
Received in revised form
8 May 2017
Accepted 26 June 2017
Available online 30 June 2017

1. Introduction

The accumulation and fragmentation of plastic debris in marine environments have become a global issue and a growing concern (GESAMP, 2015; Galloway and Lewis, 2016). Plastic debris can be regarded as a complex cocktail of contaminants, including chemical additives, residual monomers and ambient chemical substances sorbed on a polymer (Mato et al., 2001; GESAMP, 2015). Although the impact of larger plastic items, such as plastics bags, fishing nets and plastic fragments, on birds, turtles and marine mammals due primarily to entanglement and ingestion is well documented (Gall and Thompson, 2015), the adverse effects of microplastics (particles < 5 mm in diameter) on marine wildlife is less well understood. In recent years, considerable research has been conducted using laboratory experiments to quantify the physical and chemical impacts of microplastics on marine organisms, including phyto- and zooplankton, corals, echinoderms, bivalves, and fish (Cole et al., 2013; Wright et al., 2013; Hall et al., 2015; Lönnstedt and Eklöv, 2016; Sjollema et al., 2016). These laboratory studies show that microplastics can impact an organism at many levels of biological organisation including changes in gene expression, inflammation, behaviour, growth and breeding success (for reviews, see Cole et al., 2013; Wright et al., 2013; GESAMP, 2015). Evidence of the trophic transfer of microplastics along the food chain has recently been demonstrated from mussels to crabs (Farrell and Nelson, 2013) and from mesozooplankton to macrozooplankton (Setälä et al., 2014). It is well documented that early developmental stages of organisms exhibit a higher sensitivity to toxicants than other life stages, which may consequently adversely affect population levels (Beiras et al., 2012). There is previous evidence that pelagic larval stages, such as pluteus, trochophora and rotifer, are able to ingest and egest different sizes of plastic spheres (1.7, 2.7, 25, 32 μm) (Strathmann et al., 1972). The structures of marine communities depend, among other factors, on the ability of planktonic larvae to enter into adult populations, and there is the potential for the adverse impact of microplastics on eggs and embryos to threaten this. Therefore, developing embryos and marine larvae deserve special attention.

Polystyrene (PS) and polyethylene (PE) belong to the most commonly used plastics in the world and consequently to the most encountered plastics in marine environments (Andrady, 2011). PS is used in a variety of consumer and construction products, including food packaging and structures such as floating docks and buoys. HDPE is used in a wide variety of applications, such as plastic liquid food bottles and containers, plastic grocery sacks, housewares, sporting goods, piping, and plastic lumber (GESAMP, 2015). Many laboratory studies investigating the potential effects of microplastics use polymeric particles obtained from industry companies (Von Moos et al., 2012; Avio et al., 2015) or from commercial brands (Farrell and Nelson, 2013; Della Torre et al., 2014). Research on the particle toxicity of microplastics includes *in vivo* tests based on exposure to virgin microplastics, which are supposed to be free from any additives and/or residual monomers. Virgin fluorescent labelled plastic microspheres are frequently used as priority materials in laboratory exposure studies because they enable easy assessment of ingestion, bioaccumulation, tissue translocation and egestion processes (Besseling et al., 2013; Kaposi et al., 2014; Watts et al., 2014). However, the toxicity caused by co-contaminants leaching from the polymeric materials used in laboratory studies has received less attention and requires further research. Further, it has been found that unknown additives can leach from ingested microplastics (PVC) into the bodies of worms, reducing their feeding activity (Browne et al., 2013). The toxicological response

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http://dx.doi.org/10.1016/j.marenvres.2017.06.016

resulting from the ingestion of microplastics may thus be due to cumulative or synergetic effects caused by inert particle and various leaching chemicals.

Our previous experience suggested that PS microspheres cause an effect on the pelagic fertilization and larval development of the sea urchin *Paracentrotus lividus* (Lamarck, 1816). In the present study we tested the working hypothesis that the observed and reported effects of virgin PS microspheres and HDPE fluff might be, at least in part, due to the leaching of co-contaminants. For this purpose, we investigated potential toxic effects on the pelagic fertilization and larval development of the sea urchin *Paracentrotus lividus* after exposure to virgin and experimentally aged PS and HDPE materials and their leachates.

2. Material and methods

2.1. Model microplastics

Commercial synthetic polymers of polystyrene (PS) microspheres (Fluoresbrite™ Polychromatic Red 6.0 Microspheres) and high density polyethylene (HPDE) fluff (Abifor 1300/20, Abifor Zürich, Switzerland) were used as proxies for microplastics in marine systems. The PS microspheres were purchased from Polysciences Europe GmbH (Germany): they had a diameter of 6 μm and were uncharged. According to the technical specifications of the supplier, the PS microspheres are internally dyed using solvent swelling/dye entrapment. Further, the highly hydrophobic dyes (coumarin, fluorescein, rodhamine and phycoerythrin) remain trapped in the beads in aqueous environments, and some leaching of rodhamine may occur but only with aggressive washing. According to Abifor AG (Pers. comm. Marco Hilhorst, Abifor AG), the polymers used in this study were free of any additives. The HPDE fluff used was non-uniformly shaped grains ranging >0–80 μm in size (Gaussian distribution) and was free of additives. The grain size distribution according to EN-ISO 4610 standards is as follows: < 50 μm = 35–45%, < 63 μm = 60–80%, < 80 μm = 98–100% (Von Moos et al., 2012).

2.2. Bioassays and test solutions

About twenty mature sea urchin adults (*Paracentrotus lividus*) were sampled in March and April (reproductive period of the species) from the reference site Cala Reona (Murcia, SE Spain) and transported immediately to the laboratory. Gametes were obtained following methodology described in Saco-Álvarez et al. (2010) and Beiras et al. (2012), with minor modifications. Specimens were stimulated to spawn by osmotic-shock, injecting 2 mL of 0.5 M KCl through the peristomal membrane into the coelom. The organisms were allowed to spam for up to a maximum of 10 min. Animals providing relatively little or dilute gametes were excluded for gamete sampling. Sperm was aspirated “dry” from the gonopore of selected males using a Pasteur pipette and samples were separately stored in beakers and held on ice (< 15 min) until its posterior use. Females were inverted over a 200 mL beaker containing dilution FSW and left to release eggs. Prior to fertilization, gamete viability was assessed under a microscope (zoom x100) by placing by placing a subsample of gametes from each specimen in a drop of FSW. Eggs from each batch were checked for size and roundness, and they considered viable when more than 90% of the eggs were round, free of germinal vesicles and with a diameter ranging from 90 to 100 μm. Selected egg batches from three females were then filtered through a 150 μm screen to remove pellets and pooled in a beaker containing 400 mL FSW. Eggs were washed three times by decantation, removing supernatant and adding dilution FSW and the final density determined. Sperm with highest motility and density (qualitatively assessed) was selected to conduct the bioassays. A standard sperm solution was prepared by adding about 500 μL of the selected sperm to 24.5 mL of dilution FSW. Pre-trial testings were always conducted with the selected gametes in order to make sure the fecundation ratio was higher than 91% (Ghirardini et al., 2001). Sperm density was not calculated in our study as the control treatment in fertilization bioassay gives essential information regarding biological quality of the test organisms.

Exposure concentrations were chosen on the basis of trial experiments. All the test solutions for fertilization and embryotoxicity bioassay were prepared with filtered clean seawater (FSW) (offshore seawater from 150 m depth; 0.45 μm; HA Millipore; salinity = 38.0‰; pH = 8.02; dissolved oxygen = 8.1 mg/L). Physicochemical parameters were recorded in all test chambers before and after each bioassay and adjusted when necessary to maintain optimum test conditions (7.0 < pH < 8.5; 35.0 < salinity < 38.1; dissolved oxygen > 2 mg/L; H2S < 0.1 mg/L; NH3 < 40 mg/L) (Saco-Álvarez et al., 2010). All the fertilization and embryotoxicity bioassays were performed at a controlled temperature (20 °C).

2.2.1. Fertilization bioassays

Sea urchin eggs were mixed (stirring gently) with PS microspheres for 10 min in four test solutions (0 microspheres PS/mL FSW (control) and nominal concentrations of 10^3, 10^4 and 10^5 microspheres PS/mL FSW). Sixty mL of test solutions (2700 eggs/mL FSW) were added to each test chamber (consisting of sterilized 100 mL flat-bottomed glass jars) before sperm addition. Then, a standard sperm solution was prepared as described above and 50 μL of it was added to each test chamber (control and treatments). Solutions were stirred gently using a thin glass bar during 15 min and then the fecundation process was stopped by adding two drops of 40% buffered formalin in each test chamber. Three replicates were prepared per concentration (control and treatments). The fertilization rate (%) in each test chamber was calculated by determining under the microscope the percentage of resulting zygotes (fertilized eggs identified by the fertilization membrane) in three subsamples of 20 μL; Total n > 300). In addition, the normal zygotes obtained after exposing sea urchin gametes to PS microspheres were subsequently used to test potential late effects on embryo and larval development.

2.2.2. Embryotoxicity bioassays

The sea urchin embryotoxicity test (SET) was performed following the methodology described by Durán and Beiras (2010) and Beiras et al. (2012). Briefly, approximately 400 zygotes of *Paracentrotus lividus* were added straight after fertilization to the test chambers, being the developmental stage homogenous for all the treatments/replicates at the beginning of the test. Zygotes were added to each test chamber (10 mL) and incubated for 48 h at the control temperature (±20 °C) and natural photoperiod. After that time, embryogenesis was stopped by adding two drops of 40% buffered formalin in each test chamber. Larvae were not fed during the bioassay. Five replicates were prepared per treatment. All samples were examined under optical and fluorescence microscopes after incubation (Olympus BX43; Software cellSens), and two toxicity parameters were calculated: percentage of pluteus larval abnormality (n > 150) (larvae were considered as morphologically normal when they exhibited four separated arms after 48 h incubation) and larval growth (n > 30) (expressed as the percentage of net response or PNR). The PNR value is the response in the treatments (increase in length of the larvae) divided by the control, calculated according to Thain (1991).

The SETs included four experimental conditions: i) treated zygotes (zygotes obtained from exposed gametes from previous