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Effects of polystyrene microbeads in marine planktonic crustaceans

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ABSTRACT

Plastic debris accumulates in the marine environment, fragmenting into microplastics (MP), causing concern about their potential toxic effects when ingested by marine organisms. The aim of this study was to verify whether 0.1 μm polystyrene beads are likely to trigger lethal and sub-lethal responses in marine planktonic crustaceans. MP build-up, mortality, swimming speed alteration and enzyme activity (cholinesterases, catalase) were investigated in the larval stages of *Amphibalanus amphitrite* barnacle and of *Artemia franciscana* brine shrimp exposed to a wide range of MP concentrations (from 0.001 to 10 mg L^{-1}) for 24 and 48 h. The results show that MP were accumulated in crustaceans, without affecting mortality. Swimming activity was significantly altered in crustaceans exposed to high MP concentrations (> 1 mg L^{-1}) after 48 h. Enzyme activities were significantly affected in all organisms exposed to all the above MP concentrations, indicating that neurotoxic effects and oxidative stress were induced after MP treatment. These findings provide new insight into sub-lethal MP effects on marine crustaceans.

1. Introduction

Global plastic production has consistently increased over the last few years and currently stands at about 300 million tons (Plastics Europe, 2015). Due to its production and high durability, plastic rapidly accumulates in the environment, being the most common type of marine litter worldwide (Bhattacharya et al., 2010). Since plastic debris tends to end up in waterways, aquatic habitats are mostly concerned, where several degradation processes break up plastic litter into a wide array of particle size fractions (Gewert et al., 2015), ranging from macroscopic (> 5 mm) to microscopic (< 1 μm). Microplastics (MP) include particles less than 5 mm in diameter, which can be readily ingested by biota, thus accumulating across the marine food chain (Setälä et al., 2014). Their presence is considered as an emerging threat for the marine ecosystem, more than larger plastic items (i.e. entanglement, GESAMP, 2015). In this regard, and in the light of the Marine Strategy Framework Directive, MP distribution, and impact should be further monitored in order to achieve good environmental status by 2020 (MSFD 2008/56/EC).

MP ingestion has been documented for several marine species (Avio et al., 2015; Hall et al., 2015; Jeong et al., 2016; Oliveira et al., 2013; Sun et al., 2017; Van Cauwenberghe et al., 2015). In marine invertebrates, most research refers to controlled laboratory experiments (Ivar do Sul and Costa, 2014), where plastic microspheres (Ø < 5 mm) are commonly used in laboratory-based feeding experiments, since they have a similar size to algal prey, the likelihood of MP ingestion is emphasized (Wright et al., 2013). Therefore, MP can be prey analogues for planktonic organisms, being handled and ingested in a similar manner (Brillant and MacDonald, 2000), as demonstrated for crustaceans, polychaetes, echinoderms (Batel et al., 2016; Della Torre et al., 2014; Nobre et al., 2015; Setälä et al., 2014). These studies have been conducted by exposing planktonic organisms to polyethylene and polystyrene MP. These polymers are the most persistent and commonly used plastics worldwide and are buoyant in water. Unlike polystyrene MP, polyethylene MP do not seem to significantly affect marine planktonic invertebrates (Kaposi et al., 2013; Nobre et al., 2015). However, polystyrene MP may pose a hazard to marine organisms, for styrene monomers are known to be carcinogenic and endocrine disruptors (Lithner et al., 2011). Data regarding the effects and toxicity of > 1 μm polystyrene microbeads in marine planktonic invertebrates are still scarce and limited to few species. For instance, micro-sized polystyrene particles have been shown to negatively affect microalgal growth (Sjollema et al., 2016), sea urchin development and gene expression (Della Torre et al., 2014), crustacean survival, reproduction and feeding (Bergami et al., 2016; Cole et al., 2015; Lee et al., 2013). Crustaceans are primary consumers and the most abundant metazoans in the marine

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ecosystem. Therefore, they are widely used as bioindicators in determining ecosystem quality with respect to environmental contaminants (Yarsan and Yipel, 2013). Most studies on polystyrene microbeads in crustaceans have been focused on different species of copepods, demonstrating that their survival is affected by MP (Cole et al., 2015; Lee et al., 2013). Recently, Bergami et al. (2016) reported that polystyrene MP might pose a risk to other planktonic crustaceans, such as larvae of *Artemia franciscana* brine shrimp, by impairing feeding, motility, and physiology, but not survival. Thus, only in some crustaceans does survival seem to be affected by > 1 μm polystyrene plastics, while all authors reported sub-lethal effects (i.e. feeding, behavior, physiology) induced by such particles in all investigated marine crustaceans.

The aim of this study was to expand knowledge on lethal and sublethal effects caused by micro-sized plastics at environmental and high concentrations in two marine crustaceans. To achieve this goal, we assessed ingestion effects of 0.1 μm commercially available polystyrene beads in the planktonic stages of *Amphibalanus amphitrite* cyrriped and of *A. franciscana* brine shrimp. We further assessed mortality, behavioral (swimming speed alteration) and biochemical responses (i.e. cholinesterase and catalase activities) at concentrations below the highest MP concentration estimated for marine water (< 0.5 mg L^{-1}, Koelmans et al., 2015) and at high concentrations (up to 10 mg L^{-1}). Mortality was evaluated in order to see whether such MP rates may have toxic effects on the selected crustacean species. In addition, swimming activity, known to be a more sensitive end-point than mortality, was measured with an automated recording system. MP effects were investigated on cholinesterase and catalase which are biochemical biomarkers of damage and defence. In particular, acetylcholinesterase (AChE, E.C. 3.1.1.7) and propionylcholinesterase (PChE, E.C. 3.1.1.8) were selected since they catalyze acetylcholine hydrolysis (ACh) in the cholinergic system of both crustaceans (Braun and Mulloney, 1994). Catalase was monitored as an oxidative stress indicator in barnacle nauplii and brine shrimp larvae (Desai and Prakash, 2009; Gambardella et al., 2014).

A. amphitrite nauplii and *A. franciscana* Instar I larvae were selected since they are an established model species in ecotoxicological studies (Costa et al., 2016; Huang et al., 2016; Libralato, 2014; Manfra et al., 2015). Moreover, *A. amphitrite* was chosen due to the little information about the effects of MP on this species (Li et al., 2016).

2. Materials and methods

2.1. Polystyrene microbeads

Visiblex blue-dyed and fluorescent polystyrene particles (0.1 μm nominal diameter) were purchased from Phosphorex (cat. ns. 1100B, 2002), supplied as a 10 mg mL^{-1} in deionised water suspension. Visible blue-dyed MP were used for chemical characterization and toxicity bioassays, while fluorescently labelled (345 nm excitation/435 nm emission) particles were employed for uptake evaluation in planktonic invertebrates.

Both MP were sonicated for 1 min using Branson 2510 bath sonicator (Branson Ultrasonic, Danbury, CT, USA) and then suspended in 0.22 μm filtered natural seawater (FSW, supplied from the Aquarium of Genova, Italy; **salinity 37‰**) up to 100 mg L^{-1} concentration. This stock concentration was used to bring MP to the various concentrations used in the tests (0.001–0.01–0.1–1–10 mg L^{-1}). The tests were performed immediately after MP suspension preparation.

2.2. Chemical characterization

Visiblex blue-dyed MP were characterized by size and effective surface charge (ζ-potential). Prior to each measurement, MP were resuspended in ultrafiltered (0.22 μm Teflon filter) seawater (37‰ salinity) for size characterization and in distilled water for measuring the effective surface charge at all tested concentrations. They were then sonicated for 1 min using Branson 2510 bath sonicator (Branson Ultrasonic, Danbury, CT, USA). The size of MP dispersed in natural FSW was determined for each concentration by Dynamic Light Scattering (DLS) using a Malvern Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Measurements were conducted at 25 °C by transferring 1 mL of stock solution to a square cuvette for DLS analysis. A 50 mW laser with 638.2 nm wavelength was used as light source. For each concentration, measurements were recorded at 173° (backscatter) detection angle and performed in triplicate, each containing 11 runs. The same measurements were also repeated after 24 and 48 h, in order to detect any agglomerates in FSW over time.

MP ζ-potential was measured using a Malvern Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Measurements were conducted at 25 °C by transferring 1 mL of stock solution to a square cuvette for ζ-potential measurements.

2.3. Organisms

II stage nauplii of the barnacle *Amphibalanus amphitrite* and Instar I larvae of the brine shrimp *Artemia franciscana* were exposed to MP. During the bioassays organisms were not fed.

2.3.1. A. amphitrite

Nauplii of *A. amphitrite* were obtained from laboratory cultures of adult brood stock at CNR ISMAR (Genoa, Italy) according to the method described by Piazza et al. (2016). Twenty to thirty adult barnacles were reared in 700 mL beakers containing aerated 0.45 μm FSW at 20 ± 1 °C, with a 16:8 h light:dark cycle. They were fed every other day with 50–100 mL of *Artemia salina* at a density of 20 larvae mL^{-1}, and 200–400 mL of *Tetraselmis suecica* at a concentration of 2 × 10^6 cells mL^{-1}. Seawater was changed three times a week, and barnacles were periodically rinsed with clean water to remove epibionts or debris. Nauplii were collected and maintained in 500 mL gently aerated beakers with 0.22 μm FSW in a final concentration of 10–15 larvae mL^{-1}, until they were used for toxicity tests.

2.3.2. A. franciscana

Certified dehydrated cysts of *A. franciscana* were purchased from the company MicroBioTests Inc. (Belgium) and used for the experiments (Batch n. AF/F2015). Instar I stage larvae were obtained as described by Garaventa et al. (2010), by incubating 500 mg of cysts for 24 h at 28 °C under light source (3000–4000 lx) and continuous aeration of the cyst suspension in seawater (37‰ salinity). The newly hatched larvae were separated from non hatched cysts based on their phototaxis and then transferred with a Pasteur pipette into a beaker containing 0.22 μm FSW in a final concentration of 15–20 larvae mL^{-1}.

2.4. Acute toxicity test

Organisms were transferred from the beakers into each well of 24 multi-well plates containing 1 mL of different MP concentrations using a small 80 μm mesh filter. They were incubated in the dark, for 24 and 48 h, at 20 °C for *A. amphitrite* nauplii and at 25 °C for *A. franciscana* larvae according to Gambardella et al. (2015a). After exposure, mortality analysis was performed under a stereomicroscope: completely motionless larvae were counted as dead organisms, and the percentage of mortality was compared to the controls. Organisms that do not change their own barycentre position and do not move their appendages in 5 s are referred to as 'motionless' (Garaventa et al., 2010).

In addition, bioassays were performed with reference toxicants. Cadmium nitrate and potassium dichromate were selected as reference toxicants for barnacle nauplii and brine shrimp larvae, according to Piazza et al. (2016) and APAT IRSA CNR (, 8070, 2003) protocol. All tests were performed in quadruplicates.

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