Ecotoxicology and Environmental Safety 145 (2017) 250–257

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Effects of polystyrene microbeads in marine planktonic crustaceans

Chiara Gambardella[a,*], Silvia Morgana[a], Sara Ferrando[b], Mattia Bramini[c], Veronica Piazza[a], Elisa Costa[a], Francesca Garaventa[a], Marco Faimali[a]

[a] Institute of Marine Science (ISMAR), National Council of Researches (CNR), Via De Marini 6, 16149 Genova, Italy
[b] Department of Earth, Environment and Life Sciences (DISTAV), Università di Genova, Viale Benedetto XV 5, 16132 Genova, Italy
[c] IIT, Italian Institute of Technology, Via Morego 30, 16163 Genova, Italy

ARTICLE INFO

Keywords:
Crustaceans
Enzyme activity
Microplastics
Mortality
Swimming
Toxicity

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

* Corresponding author.
E-mail addresses: chiara.gambardella@ge.ismar.cnr.it, chiagamba@gmail.com (C. Gambardella).

http://dx.doi.org/10.1016/j.ecoenv.2017.07.036
Received 25 January 2017; Received in revised form 15 May 2017; Accepted 17 July 2017
Available online 24 July 2017
0147-6513/

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.

2.5. Swimming Speed Alteration (SSA) test

Swimming Speed Alteration (SSA) – a sub-lethal behavioral end-point – was also evaluated. The Swimming Behavioral Recorder System (e-magine IT, Genoa, Italy) was used to track swimming paths as described in detail in Faimali et al. (2006). Briefly, swimming behavior was monitored in dark conditions, under infrared light, for three seconds. The resulting digital images were analyzed using an advanced image processing software to reconstruct individual swimming paths and measure the average swimming speed (mm/s) for each test population organism (10–20 organisms). Data were expressed as percentages of swimming speed alteration (SSA) normalized to controls' swimming speed (S), as follows:

SSA (%) = [(S Treated Control/S Control) × 100].

2.6. MP accumulation

Fluorescently labelled particles were employed for evaluating MP accumulation in planktonic invertebrates. Tests were performed as described in the paragraph 2.4. Following exposure, the organisms were removed and washed with fresh FSW three times to remove MP bound to the exoskeleton: in this way, according to Nasser and Lynch (2015), only ingested particles would be assessed. Organisms were fixed in 4% paraformaldhehyde solution in phosphate-buffered saline (PBS, pH 7.4) and observed under a Leica DMRB light and epi-fluorescence microscope. Images were acquired using a DFC420C Leica CCD camera and Leica software (Leica Application Suite V3). The resulting images were stored and displayed with Leica software program, using TIFF image format.

2.7. Enzyme activity

Visiblex blue-dyed MP were employed for quantifying AChE, PChE and catalase activity in marine crustaceans. The activity of these enzymes is a good biomarker for *A. amphitrite* and *Artemia* sp. exposure to pesticides and nanomaterials (Faimali et al., 2003; Gambardella et al., 2014; Mesarič et al., 2015). Tests were performed as described for acute toxicity test. Enzyme activity was measured in controls and in organisms exposed to MP for 48 h.

2.7.1. Cholinesterase activity analyses

AChE and PChE activities were measured according to the Ellman's (1961) method, which was modified ad hoc for Shimadzu (UV-160) spectrophotometer. After 48 h MP exposure, the organisms were rinsed in PBS (pH 7.4), maintained for 2 weeks at − 20 °C and then homogenized and sonicated for 25 min in a bath sonicator (FALC, mod. LBS1, Italy). The organisms were passed through a syringe needle (Ultrafin 29G, 12.7 mm length) in the presence of 1% triton × 100, and centrifuged for 30 s at 18,363 × *g*. The kinetics of AChE and PChE activities were quantified by measuring the absorbance at 412 nm wavelength. Substrate cleavage speed was measured for 3 min and compared with the linear equation of a standard curve previously obtained by supplying known amounts of ChEs. Protein content in controls and in exposed organisms was measured according to the Bradford method, using Biorad reagent, and referred to a standard curve obtained using BSA (Sigma). Cholinesterase activities were expressed in nmoles of hydrolyzed acetylthiocholine or propyonilthiocoline chloride/min/mg protein (extinction coefficient, $\varepsilon 405 = 13{,}600\ M^{-1}\ cm^{-1}$).

2.7.2. Catalase activity analyses

Catalase activity was determined according to Aebi (1984) and Jemec et al. (2008). Here, 50 µL protein supernatant was combined with 950 µL hydrogen peroxide solution (10.8 mM) prepared in 50 mM potassium phosphate buffer (pH 7.0). The final hydrogen peroxide concentration was 10 mM. The reaction was followed spectrophotometrically for 2 min at 25 °C and 240 nm using a Shimadzu UV-160 spectrophotometer. Catalase activity is expressed as µmoles of degraded hydrogen peroxide/min/mg protein (extinction coefficient, $\varepsilon 240 = 43.6\ M^{-1}\ cm^{-1}$). The concentrations of the substrates used for all tested enzymes were saturated and it ensured a linear trend in the absorbance according to time and concentration of protein.

2.8. Statistical analyses

All data are expressed as means ± standard error of the 4 replicates. Lethal concentration (LC_{50}; MP concentration and reference toxicants resulting in 50% deaths of exposed organisms after 24 h and 48 h), effective SSA concentration (EC_{50}; MP concentration and reference toxicants resulting in 50% SSA effect in the exposed organisms after 24 h and 48 h) and related 95% confidence limits were calculated using Trimmed Spearman Karber analysis (Finney, 1978). Significant differences between controls and treated samples were determined using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the assumption of normality, non parametric Kruskal Wallis test and Mann Whitney test were used to compare individual treatments. For SSA test, statistical analysis has been performed using swimming speed data. Data were considered significantly different when $p < 0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

3. Results

3.1. MP behavior in Sea Water

DLS analysis confirmed 0.1 µm nominal size of MP in distilled water (Table 1 and Supplementary Fig. a). Results showed that MP size increased in FSW as soon as they are dispersed in this medium (Fig. 1a) and at each exposure time (Table 1 and Supplementary Fig. b). DLS analysis show multiple peaks, where a shift in apparent particle size after 24 and 48 h was observing, suggesting an increase in agglomeration, compared to the 0 h exposure. Further, it can be noted that the attenuator factor increased in the most diluted samples. These values reached 10 and 11, respectively, at 0.1 and 0.01 mg L^{-1} MP, revealing scarce measurement reliability at these concentrations. Polystyrene MP showed a high negative ζ-potential (− 53.1 ± 11.5).

3.2. Mortality and swimming speed alteration

It was not possible to calculate LC_{50} and EC_{50} for crustaceans exposed to MP, since nauplii and larvae never show any > 50% effect for any end-point and exposure time. Regarding reference toxicants, lethal concentration for barnacle nauplii exposed to cadmium nitrate for 24 and 48 h were 0.98 (confidence limits, C.L. 0.92–1.06) mg L^{-1} and 0.50 (C.L. 0.47–0.54) mg L^{-1}, respectively. Lethal concentration for brine shrimp larvae exposed to potassium dichromate was 34.78 (C.L. 30.36–39.84) mg L^{-1} and 6.01 (C.L. 5.08–7.11) mg L^{-1}.

3.2.1. AmphiBalanus amphitrite

Toxicity tests results with nauplii exposed to different MP concentrations are reported in Fig. 1. No significant effect in mortality was observed in nauplii exposed to all concentrations at both exposure times ($p > 0.05$). Only swimming speed, recorded at 48 h, resulted to be significantly inhibited ($p < 0.05$) at 1 and 10 mg L^{-1}, while no effects were observed after short exposure time (24 h).

3.2.2. Artemia franciscana

Polystyrene MP did not affect brine shrimp larval survival. However, it significantly inhibited swimming speed after 24 h exposure at 10 mg L^{-1} (Fig. 2, $p < 0.05$). Prolonged exposure time to MP significantly accelerated swimming speed at 1 and 10 mg L^{-1} ($p < 0.05$).

Table 1
Physicochemical characterization of 0.1 μm polystyrene particles. Microplastic (MP) size calculated for each concentration (mg L^{-1}) is referred to FSW and distilled water (H_2O) after 0, 24 and 48 h at 25 °C. Due to the high poly-dispersity index (PDI), the values and percentage of peak 1, peak 2 and peak 3 are reported. DLS are the average of a minimum of three separate runs; errors represent the standard deviation over measurements and are intended solely as an indication of the reproducibility of the measurement.

Sample (mg L^{-1})	Solution	Diameter[a] (nm)	PDI[b]	Peak 1	Peak 2	Peak 3
0.01–0 h	FSW	1720	0.488	2579 ± 567.4 (33.9%)	364.1 ± 26.58 (33%)	995.4 ± 184.9 (33%)
0.01–24 h	FSW	3312	0.151	2299 ± 492.7 (51.8%)	4188 ± 822.3 (48.2%)	–
0.01–48 h	FSW	1799	0.396	859.5 ± 106.7 (59.6%)	617.3 ± 72.60 (40.4%)	–
0.1–0 h	FSW	1492	0.885	332.6 ± 36.56 (66.7%)	917 ± 135.7 (33.3%)	–
0.1–24 h	FSW	2471	0.582	2013 ± 476.9 (53.3%)	1214 ± 203.7 (45%)	145.3 ± 15.05 (1.7%)
0.1–48 h	FSW	1719	0.609	849.9 ± 203.2 (100%)	–	–
1.0–0 h	FSW	757.1	0.62	493.7 ± 119.0 (68.6%)	240.4 ± 29.86 (31.4%)	–
1.0–24 h	FSW	1640	0.595	743.2 ± 93.13 (34.9%)	1243 ± 156.3 (32.6%)	354.9 ± 23.02 (32.6%)
1.0–48 h	FSW	2100	0.685	946.0 ± 108.3 (100%)	–	–
10.0–0 h	FSW	1498	0.502	782.1 ± 117.6 (38.2%)	1112 ± 122.4 (31.5%)	371.1 ± 35.54 (30.3%)
10.0–24 h	FSW	4156	0.972	679.6 ± 45.95 (33.3%)	295.3 ± 0.0 (33.3%)	184.4 ± 10.74 (33.3%)
10.0–48 h	FSW	2791	0.806	443.2 ± 27.00 (33.3%)	1380 ± 188.1 (33.3%)	221.7 ± 7.097 (33.3%)
10.0–0 h	H_2O	91.98	0.033	96.8 ± 23.03 (100%)	–	–

[a] z-average hydrodynamic diameter extracted by cumulant analysis of the data.
[b] Polydispersity index from cumulant fitting of the data.

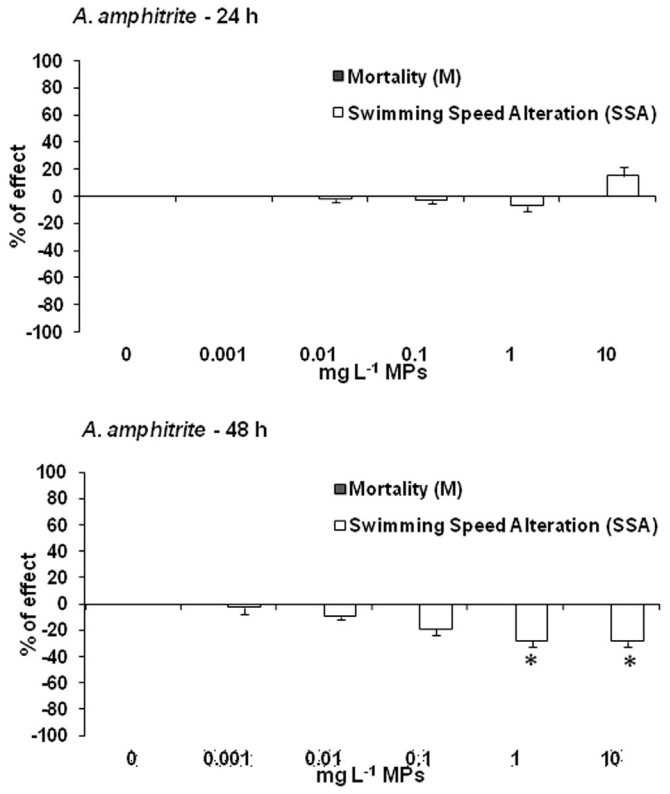

Fig. 1. Percentage of mortality (filled bars) and swimming speed alteration (open bars) of *A. amphitrite* nauplii after 24 and 48 h of exposure to increasing concentrations of polystyrene microplastics (MPs, Mean ± SE, n = 4). Asterisks indicate significant differences between each MP concentration and the control (*p < 0.05).

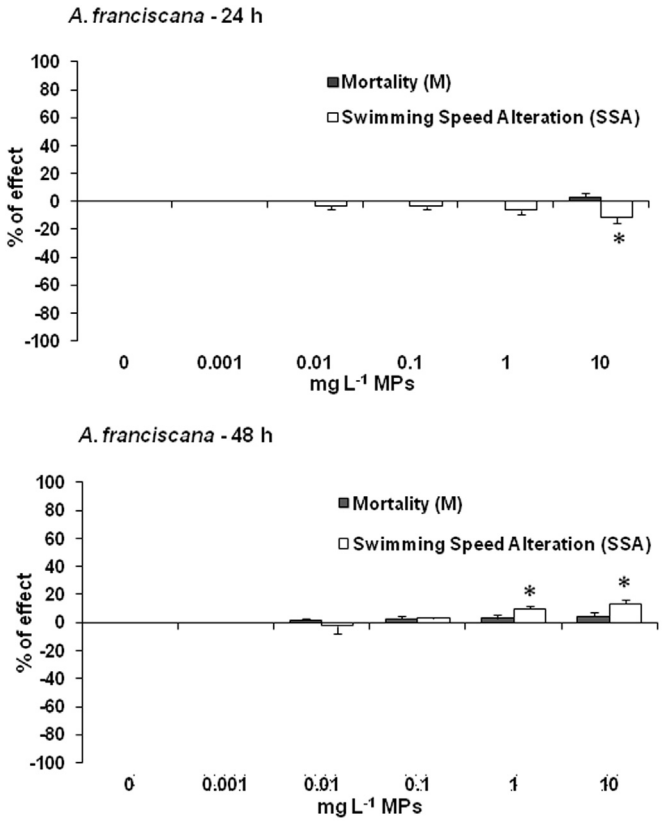

Fig. 2. Percentage of mortality (filled bars) and swimming speed alteration (open bars) of *A. franciscana* larvae after 24 and 48 h of exposure to increasing concentrations of polystyrene microplastics (MPs, Mean ± SE, n = 4). Asterisks indicate significant differences between each MP concentration and the control (*p < 0.05).

3.3. Accumulation

Microscopy observations showed that polystyrene microbeads were ingested and in organisms within 24 and 48 h, and were accumulated in the gut of both crustaceans. In particular, *A. amphitrite* nauplii ingested MP only at very high concentrations (from 1 mg L^{-1}, Fig. 3), whereas brine shrimp larvae tend to accumulate MP at any concentration. Differently from barnacle nauplii that did not excrete MPs, brine shrimps constantly ingested and excreted microbeads. A small percentage (< 20%) of crustacean planktonic stages showed an empty gut. As indicated in Fig. 4, the organisms did not ingest MP in a dose-dependent manner. However, at the highest tested concentration (10 mg L^{-1}), MP build-up was observed in crustacean gut.

3.4. Enzyme activity

Cholinesterase and catalase activity was measurable in all samples belonging to both crustaceans after 48 h exposure (Fig. 4).

3.4.1. Amphibalanus amphitrite

Cholinesterase (AChE) and pseudocholinesterase (PChE) activity in nauplii exposed to MP show a similar trend (Fig. 4), characterized by an

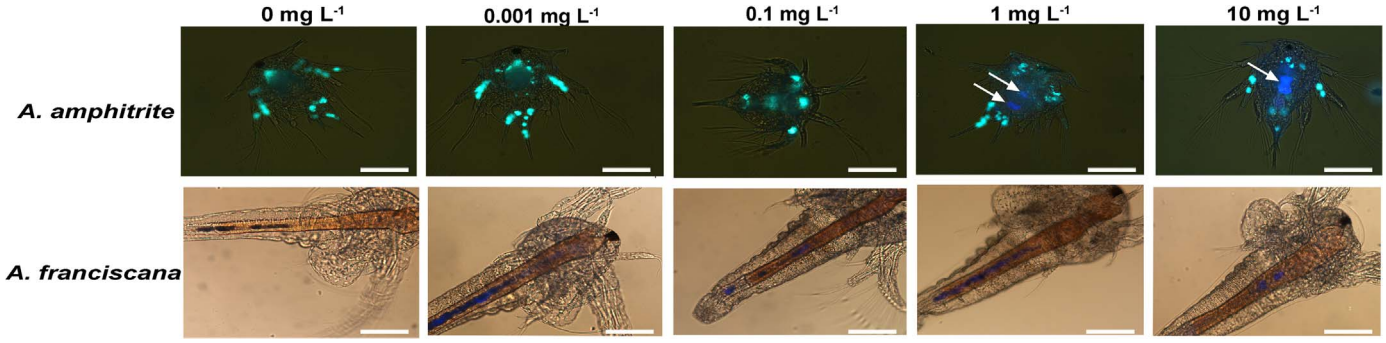

Fig. 3. Representative microscopy images of *A. amphitrite* nauplii and *A. franciscana* larvae revealing polystyrene microplastics (MPs) inside the invertebrates after 48 h of the control and with exposure to 0.001, 0.1, 1 and 10 mg L^{-1} (as indicated). Note that nauplii did not accumulate MPs up to 1 mg L^{-1} (arrows). Bar = 100 µm.

increase at low concentrations followed by a decrease at 1 mg L^{-1}. A significant increase was observed in both AChE and PChE activity, from 0.001 to 0.1 mg L^{-1} as compared to controls ($p < 0.05$), while the highest MP concentration significantly affected only PChE activity. Catalase was inhibited at 0.001 mg L^{-1} and it significantly increased from 0.1 mg L^{-1} upwards ($p < 0.05$).

3.4.2. *Artemia franciscana*

AChE and PChE were affected by polystyrene MP in *A. franciscana.* AChE was impaired only by 0.001 and 0.01 mg L^{-1} concentration of MP, where a significant difference was observed between exposed larvae and controls ($p < 0.05$). No significant difference was found at the other concentrations. PChE significantly increased at 0.01 and 0.1 mg L^{-1} ($p < 0.05$), while no differences were observed between controls and larvae exposed to other concentrations. At all tested MP concentrations, a significant catalase activity increase was induced compared to controls ($p < 0.05$), which however was not consistent with concentrations.

4. Discussion

We reported the effects of polystyrene beads (Ø 0.1 µm) on MP accumulation, mortality, swimming behavior, and enzymatic activity in barnacle nauplii and brine shrimp larvae at environmental and high concentrations. Both planktonic larvae were able to ingest MP: this accumulation did not affect survival, but caused sub-lethal effects. Marine crustaceans have been found to ingest different MP particles (Batel et al., 2016; Cole et al., 2013; Powell et al., 1990; Setälä et al., 2014). The uptake of different size, less than 1 µm, polystyrene microbeads has been documented in the gut of copepod nauplii and adults (Lee et al., 2013) and brine shrimp larvae (Bergami et al., 2016). Our results confirm these findings, since brine shrimp larvae ingested 0.1 µm polystyrene beads after 24 h and 48 h, which were localized in the gut.

Microbeads were constantly ingested and excreted in brine shrimp larvae exposed to all concentrations. However, this phenomenon could not be observed in barnacle nauplii, which would only accumulate polystyrene particles from 1 mg L^{-1} MP upwards. Nauplii have a

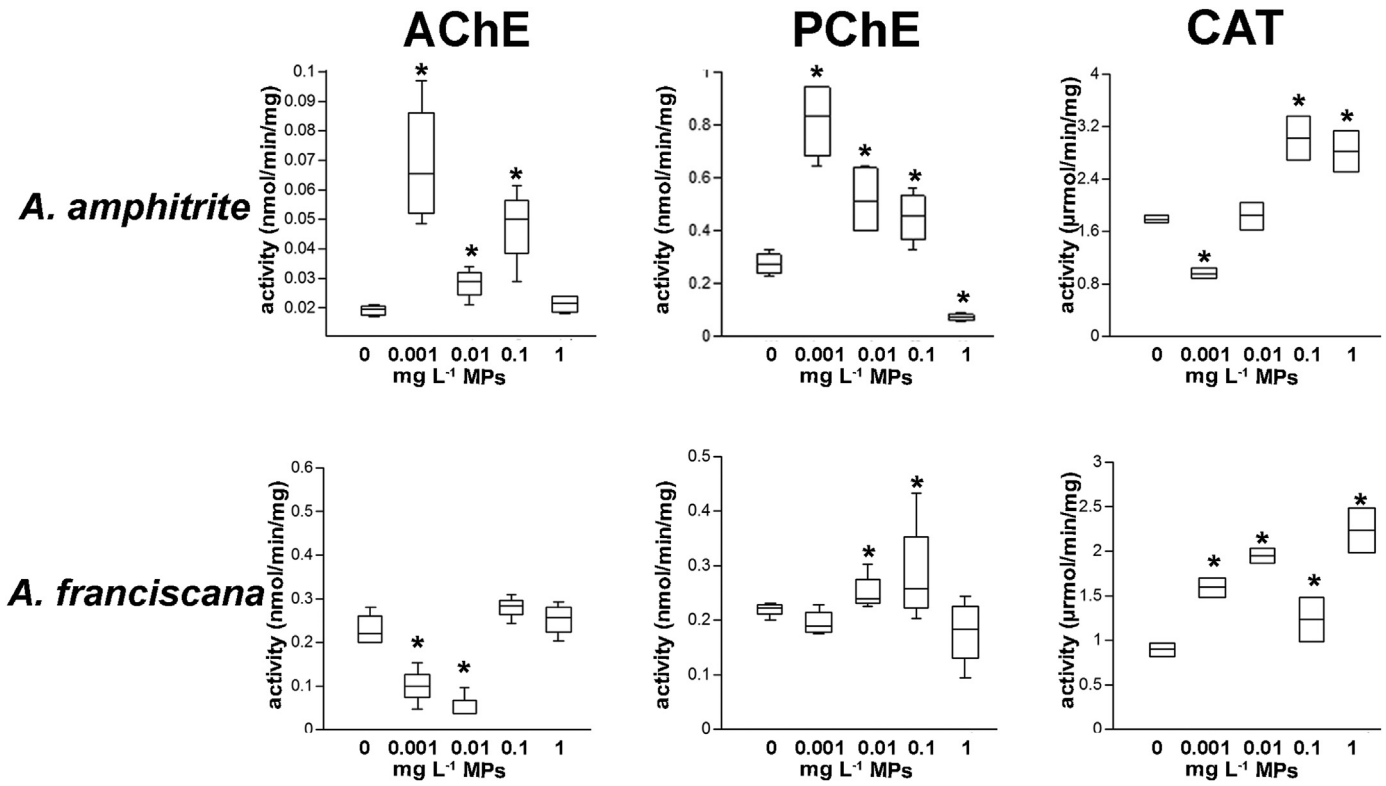

Fig. 4. Effect of 48 h exposure to microplastics (MPs) on ChE activities (acetylcholinesterase, AChE, propionylcholinesterase, PChE; catalase, CAT) of *A. amphitrite* nauplii and *A. franciscana* larvae (Mean ± SE, n = 4). Asterisks indicate measurements that were significantly different from controls (*$p < 0.05$).

peritrophic membrane (Rainbow and Walker, 1977) just like brine shrimp larvae (Abatzopoulos et al., 2002). Therefore, MP excretion – although not directly observed under the microscope – is likely to occur also in these organisms. Barnacle ingestion was only observed at high MP concentrations, while sub-lethal effects (AchE, PChE and catalase activity impairment) occurred at all concentrations (Figs. 3 and 4). However, nauplii might have ingested MP even at lower concentrations (0.001 mg L^{-1}), since they usually feed on microalgae of the same order of magnitude (i.e. *Tetraselmis suecica*; Piazza et al., 2014).

Survival of these crustaceans was not affected by polystyrene MP at any concentration, in accordance with previous studies on other crustaceans, including brine shrimps exposed to other > 1 μm plastic beads (Bergami et al., 2016; Lee et al., 2013). Recent research indicates that zooplankton survival may be significantly affected when exposed to polystyrene MP for long exposure periods (Cole et al., 2015). In this case, mortality increased in a time-dependent manner in copepods fed daily on MP for up to 4 days. Despite high MP concentrations used in this study (> 1 mg L^{-1}), no mortality was observed in barnacles and brine shrimps after 2 days. These findings suggest that experimental set-up and exposure time are two parameters that should be further taken into account for MP toxicity assessment. In this regard, chronic tests could be performed in order to verify whether survival can be affected by these MPs.

When suspended in seawater, microbeads formed agglomerates (resulting in high hydrodynamic values) ranging from ≈ 700 nm up to several μm in diameter. It is worth noting that aggregation took place as soon as microbeads came in contact with seawater, with peaks ranging from 757 up to 1720 nm at 0 h. MP may end up in significant aggregations in seawater due to the counterbalance of several parameters, such as the presence of salts, natural organic matter and colloids, with size and surface chemistry (Bergami et al., 2016; Corsi et al., 2014). Indeed, when two different phases are in contact, if either phase is a charged particle and the other one is an electrolyte solution, the ions dissolved in the medium with charge opposite to the particle surface tend to surround the particle, in order to keep the solution neutral. The arrangement of charges located in one phase and those fixed in the particle surface form what is called 'electric double layer' (Ortega-Vinuesa and Bastos-Gonzalez, 2001). The stability of materials stabilized with ionic surfactants is disrupted by high ionic strength media. This is the case, for example, of seawater. At high salt concentrations, the accumulation of counterions near the interface screens particle charge, decreasing nanodispersion stability, because of decreased repulsion between particles and due to agglomerate forming particles (Lopez-Leon et al., 2005). The presence of salts has indeed been reported to screen particle surface charges, leading to the observed aggregation (Wegner et al., 2012). According to previous studies on the same polymer particles of different size (Della Torre et al., 2014; Lee et al., 2013), polystyrene MP showed high negative ζ-potential. The negative charge and seawater aggregation is likely to protect marine organisms from MP toxicity, as previously reported for other nanomaterials (Canesi et al., 2012). Therefore, 0.1 μm polystyrene bead size and surface chemistry in FWS could imply a low interaction with planktonic crustaceans, as demonstrated from the absence of mortality in both crustaceans.

Interestingly, the only significant effects were constituted by sub-lethal responses, thus observed for swimming behavior and enzymatic activity in barnacle nauplii and brine shrimp larvae. Behavioral responses, such as swimming behavior, have been proved to be more sensitive than mortality in different marine invertebrates, including crustaceans (Gambardella et al., 2014, 2015a; Huang et al., 2016; Morgana et al., 2016; Oliveira et al., 2012). According to these findings, we observed a significant effect on crustacean swimming activity at high MP concentrations (1 and 10 mg L^{-1}), while no lethal effects were observed. High concentrations of nanomaterials dispersed in seawater affect the swimming of crustacean larvae (Mesarič et al., 2015). Likewise, the high amount of MP aggregates observed at the highest concentrations may alter swimming activity of larval stages, resulting in mechanical disturbance.

To our knowledge, this is the first report on swimming behavior alteration in planktonic marine invertebrates and crustaceans exposed to MP. Recently, Sussarellu et al. (2016) have reported some sperm velocity inhibition (− 23%) in adult oysters exposed to polystyrene microspheres for two months. Swimming speed is an ecologically relevant parameter to assess the effect of contaminants even at concentrations that do not cause mortality (Faimali et al., 2016). On this basis, swimming behavior may be a suitable sub-lethal end-point to assess the effect of high concentration MP. Since about 20–30% of significant alteration in naupliar and larval swimming was observed at concentrations that did not induce mortality, we propose that swimming activity should be further considered in the assessment of high MP pollution. Interestingly, the significant behavioral sub-lethal effects found at organism level corresponded to enzymatic activity impairment. However, those changes resulted to be significant even when swimming activity was not affected (Fig. 1, Fig. 2 and Fig. 4), thus suggesting that molecular impairments are more sensitive than behavioral ones. Indeed, biochemical responses, in terms of either reduced or increased activity, occur at any concentration. In this study, we analyzed cholinesterases and catalase, since they are considered to be damage and defence biomarkers. Cholinesterases are indicative for neurotoxicity, while catalase is an oxidative stress indicator in marine invertebrates, involved in hydrogen peroxide detoxification (Jemec et al., 2008). All these enzymes have been found in barnacles and brine shrimps and were proved to be reliable biomarkers (Baek et al., 2015; Desai and Prakash, 2009; Faimali et al., 2003; Falugi, 1988; Gambardella et al., 2014; Varò et al., 2002; Venkteswara Rao et al., 2007). When stress occurs, it is generally followed by cholinesterase increase (Gambardella et al., 2013; Massoulié et al., 1993). In particular, inflamed cells and tissues have been related to a greater amount of Ach compared to healthy ones (de Oliveira et al., 2012). AChE and PChE expression increase corresponding to a hormetic effect in barnacle nauplii could be a response to stress caused by polystyrene MP, which seem to primarily affect organisms at the lowest tested concentration (0.001 mg L^{-1}), at which the highest cholinesterase activity was obtained. This assumption is also supported by significantly inhibited catalase activity: due to hydroxyl radical production leading to hydrogen peroxide production, in turn responsible for catalase inactivation, decreased catalase activity is a defense response (Halliwell and Gutteridge, 1999). Therefore, observed inhibition may be correlated to hydroxyl radical production, although further investigations are required to support this assumption.

AChE inhibition observed in brine shrimp larvae exposed to low MP concentrations (0.001–0.01 mg L^{-1}) may be responsible for a high number of uncleaved Ach, resulting in a toxic effect, as demonstrated for marine invertebrates exposed to nanomaterials (Gambardella et al., 2015b). Similar results have been recently reported for the marine fish *Pomatoschistus microps* exposed to polyethylene MP, where AChE activity was inhibited causing oxidative damage (Oliveira et al., 2013; Ferreira et al., 2016). In our study, significant inhibition (by an average of 44% and 23% for 0.001 and 0.01 mg L^{-1} of MPs, respectively) was reported, considered high enough to affect nervous functions (Ludke et al., 1975). Noteworthy, this toxic effect does not persist at concentrations from 0.1 mg L^{-1} upwards, with only a significant inflammation process in PChE expression, suggesting some still ongoing stress. Furthermore, in brine shrimps, catalase activity was significantly enhanced following MP exposure compared to controls, indicating that MP stimulate oxidative burst, as previously reported for mussels exposed to nanomaterials (Canesi et al., 2010).

5. Conclusions

We demonstrated that polystyrene microbeads which accumulate in barnacle and brine shrimp planktonic stages, only affect sub-lethal

responses at environmental and high MP concentrations. We propose that, in the future, swimming activity should be considered as a suitable tool for detecting high MP contamination level, while cholinesterases and catalase may be good biomarkers for assessing polystyrene microbead pollution in marine crustaceans.

Acknowledgments

This work has been supported by RITMARE (Ricerca Italiana per il MARE) Flagship Project, a National Research Program funded by the Italian Ministry of University and Research (MIUR) and by EPHEMARE (Ecotoxicological effects of microplastics in marine ecosystems) Project, a JPI Oceans.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at. http://dx.doi.org/10.1016/j.ecoenv.2017.07.036

References

Abatzopoulos, T.J., Beardmore, J., Clegg, J.S., Sorgeloos, P., 2002. *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers, Dordrecht.

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

APAT IRSA CNR 8070, 2003. Metodo di valutazione della tossicità acuta con *Artemia* sp. APAT (Agenzia per la Protezione dell'Ambiente e per i servizi Tecnici) IRSA (Istituto di Ricerca Sulle Acque) CNR. pp. 1043–1049.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. 198, 211–222.

Baek, I., Choi, H.-J., Rhee, J.-S., 2015. Inhibitory effects of biocides on hatching and acetylcholinesterase activity in the brine shrimp *Artemia salina*. Toxicol. Environ. Health Sci. 7, 303–308.

Batel, A., Linti, F., Scherer, M., Erdinger, L., Braunbeck, T., 2016. The transfer of benzo[a] pyrene from microplastics to artemia nauplii and further to zebrafish via a trophic food web experiment – CYP1a induction and visual tracking of persistent organic pollutants. Environ. Toxicol. Chem. 35, 1656–1666.

Bergami, E., Bocci, E., Vannuccini, M.L., Monopoli, M., Salvati, A., Dawson, K.A., Corsi, I., 2016. Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp *Artemia franciscana* larvae. Ecotoxicol. Environ. Safe 126, 18–25.

Bhattacharya, P., Lin, S., Turner, J.P., Ke, P.C., 2010. Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. J. Phys. Chem. C 114, 16556–16561.

Braun, G., Mulloney, B., 1994. Acetylcholinesterase activity in neurons of crayfish abdominal ganglia. J. Comp. Neurol. 350, 272–280.

Brillant, M.G.S., MacDonald, B.A., 2000. Postingestive selection in the sea scallop, *Placopecten magellanicus* (Gmelin): the role of particle size and density. J. Exp. Mar. Biol. Ecol. 253, 211–227.

Canesi, L., Fabbri, R., Gallo, G., Vallotto, D., Marcomini, A., Pojana, G., 2010. Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (Nano carbon black, C60 fullerene, Nano-TiO_2, Nano-SiO_2). Aquat. Toxicol. 100, 168–177.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., Gallo, G., 2012. Bivalve mollusks as a unique target group for nanoparticle toxicity. Mar. Environ. Res. 76, 16–21.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646–6655.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod *Calanus helgolandicus*. Environ. Sci. Technol. 49, 1130–1137.

Corsi, I., Cherr, G.N., Lenihan, H.S., Labille, J., Hassellov, M., Canesi, L., Dondero, F., Frenzilli, G., Hristozov, D., Puntes, V., Della Torre, C., Pinsino, A., Libralato, G., Marcomini, A., Sabbioni, E., Matranga, V., 2014. Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. NanoFocus 8, 9694–9709.

Costa, E., Piazza, V., Gambardella, C., Moresco, R., Prato, E., Biandolino, F., Cassin, D., Botteri, M., Maurizio, D., D'Adamo, R., Fabbrocini, A., Faimali, M., Garaventa, F., 2016. Ecotoxicological effects of sediments from Mar piccolo, South Italy: toxicity testing with organisms from different trophic levels. Environ. Sci. Pollut. Res. 23, 12755–12769.

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Dawson, K.A., Corsi, I., 2014. Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of the sea urchin embryos *Paracentrotus lividus*. Environ. Sci. Technol. 48, 12302–12311.

de Oliveira, P., Gomes, A.Q., Pacheco, T.R., de Almeida, V.V., Saldanha, C., Calado, A., 2012. Cell-specific regulation of acetylcholinesterase expression under inflammatory conditions. Clin. Hemorheol. Microcirc. 51, 129–137.

Desai, D.V., Prakash, S., 2009. Physiological responses to hypoxia and anoxia in *Balanus amphitrite* (Cirripedia: Thoracica). Mar. Ecol. Prog. Ser. 390, 157–166.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of cholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Faimali, M., Falugi, C., Gallus, L., Piazza, V., Tagliafierro, G., 2003. Involvement of acetylcholine in settlement of *Balanus amphitrite*. Biofouling 19, 213–220.

Faimali, M., Garaventa, F., Piazza, V., Magillo, F., Greco, G., Corrà, C., Giacco, E., Gallus, L., Falugi, C., 2006. Swimming speed alteration of larvae of *Balanus amphitrite* as behavioural end-point for laboratory toxicological bioassays. Mar. Biol. 149, 87–96.

Faimali, M., Gambardella, C., Costa, E., Piazza, V., Morgana, S., Estévez-Calvar, N., Garaventa, F., 2017. Old model organisms and new behavioral end-points: swimming alteration as an ecotoxicological response. Mar. Environ. Res. 128, 36–45. http://dx.doi.org/10.1016/j.marenvres.2016.05.006.

Falugi, C., 1988. Localization and possible functions of cholinesterase activities in *Balanus amphitrite* embryos and larvae. Acta Embryol. Morphol. Exp. New Ser. 9, 133–156.

Ferreira, P., Fonte, E., Soares, M.E., Carvalho, F., Guilhermino, L., 2016. Effects of multi-stressors on juveniles of the marine fish *Pomatoschistus microps*: gold nanoparticles, microplastics and temperature. Aquat. Toxicol. 170, 89–103.

Finney, D.J., 1978. Statistical Method In Biological Assay, 3rd ed. Charles Griffin & Co. Ltd, London, England.

Gambardella, C., Aluigi, M.G., Ferrando, S., Gallus, L., Ramoino, P., Gatti, A.M., Rottigni, M., Falugi, C., 2013. Developmental abnormalities and changes in cholinesterase activity in sea urchin embryos and larvae from sperm exposed to engineered nanoparticles. Aquat. Toxicol. 130–131, 77–85.

Gambardella, C., Mesarič, T., Milivojevič, T., Sepčič, K., Gallus, L., Carbone, S., Ferrando, S., Faimali, M., 2014. Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: evaluation of mortality and behavioural and biochemicalresponses. Environ. Monit. Assess. 186, 4249–4259.

Gambardella, C., Costa, E., Piazza, V., Fabbrocini, A., Magi, E., Faimali, M., Garaventa, F., 2015a. Effect of silver nanoparticles on marine organisms belonging to different trophic levels. Mar. Environ. Res. 111, 41–49.

Gambardella, C., Morgana, S., Di Bari, G., Ramoino, P., Bramini, M., Diaspro, A., Falugi, C., Faimali, M., 2015b. Multidisciplinary screening of toxicity induced by silica nanoparticles during sea urchin development. Chemosphere 139, 486–495.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology 19, 512–519.

GESAMP, 2015. Sources, fate and effects of microplastics in the marine environment:a global assessment. In: Kershaw, P.J (ed.) (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). GESAMP Reports & Studies no. 90 – Microplastics in the Ocean. p. 96.

Gewert, B., Plassmann, M.M., MacLeod, M., 2015. Pathways for degradation of plastic polymers floating in the marine environment. Environ. Sci. Proc. Imp. 17, 1513–1521.

Hall, N.M., Berry, K.L.E., Rintoul, L., Hoogenboom, M.O., 2015. Microplastic ingestion by scleractinian corals. Mar. Biol. 162, 725–732.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press, New York.

Huang, Y., Persoone, G., Nugegoda, D., Wlodkowic, D., 2016. Enabling sub-lethal behavioral ecotoxicity biotests using microfluidic lab-on-a-chip technology. Sens. Act. B Chem. 226, 289–298.

Jemec, A., Tisler, T., Drobne, D., Sepčić, K., Jamnik, P., Roš, M., 2008. Biochemical biomarkers in chronically metal stressed daphnids. Comp. Biochem. Physiol. C 147, 61–68.

Jeong, C.-B., Kang, H.-M., Lee, M.-C., Hwanf, D.-S., Hwang, U.-K., Zhou, B., Souissi, S., Lee, S.-J., Lee, J.-S., 2016. Microplastic size-dependent toxicity, oxidative stress induction, and p-jnk and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). Environ. Sci. Technol. 50, 8849–8857.

Ivar do Sul, J.A., Costa, M.F., 2014. The present and future of microplastic pollution in the marine environment. Environ. Pollut. 185, 352–364.

Kaposi, K., Mos, B., Kelaher, B.P., Dworjanyn, S.A., 2013. Ingestion of microplastic has limited impact on a marine larva. Environ. Sci. Technol. 48, 1638–1645.

Koelmans, A.A., Besseling, E., Shim, W.J., 2015. Nanoplastics in the aquatic environment. Critical review. In: Bergmann, M., Gutow, L., Klages, M. (Eds.), Marine Anthropogene Litter. Springer, Berlin, pp. 325–340.

Lee, K.W., Shim, W.J., Kwon, O.Y., Kang, J.-H., 2013. Size-dependent effects of micro polystyrene particles in marine copepod *Tigriopus japonicus*. Environ. Sci. Technol. 47, 11278–11283.

Li, H.-X., Getzinger, G.J., Ferguson, P.L., Orihuela, B., Zhu, M., Rittschof, D., 2016. Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle *Amphibalanus amphitrite*. Environ. Sci. Technol. 50, 924–931.

Libralato, G., 2014. The case of *Artemia* spp. in nanoecotoxicology. Mar. Environ. Res. 101, 38–43.

Lithner, D., Larsson, A., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci. Total Environ. 409, 3309–3324.

Lopez-Leon, T., Carvalho, E.L.S., Seijo, B., Ortega-Vinuesa, J.L., Bastos-Gonzalez, D., 2005. Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior. J. Colloid Interface Sci. 283, 344–351.

Ludke, J.L., Hill, E.F., Dieter, M.P., 1975. Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. Arch. Environ. Contam. Toxicol. 3, 1–21.

Manfra, L., Savorelli, F., Di Lorenzo, B., Libralato, G., Comin, S., Floris, B., Francese, M., Gallo, M.L., Gartner, I., Guida, M., Leoni, T., Marino, G., Martelli, F., Palazzi, D., Prato, E., Righini, P., Rossi, E., Volpi Ghirardini, A., Migliore, L., 2015. Intercalibration of ecotoxicity testing protocols with *Artemia franciscana*. Ecol. Indic. 57, 41–47.

Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., Vallette, F.M., 1993. Molecular and cellular biology of cholinesterases. Prog. Neurobiol. 41, 31–91.

Mesarič, T., Gambardella, C., Milivojević, T., Faimali, M., Drobne, D., Falugi, C., Makoveč, M., Jemec, A., Sepčić, K., 2015. High surface adsorption properties of carbon-based nanomaterials are responsible for mortality, swimming inhibition, and biochemical responses in *Artemia salina* larvae. Aquat. Toxicol. 163, 121–129.

Morgana, S., Gambardella, C., Falugi, C., Pronzato, R., Garaventa, F., Faimali, M., 2016. Swimming speed alteration in the early developmental stages of *Paracentrotus lividus* sea urchin as ecotoxicological endpoint. Mar. Environ. Res. 115, 11–19.

Nasser, F., Lynch, I., 2015. Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on *Daphnia magna*. J. Proteom. 137, 45–51.

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereora, C.D.S., Turra, A., 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). Mar. Pollut. Bull. 92, 99–104.

Oliveira, C., Almeida, J.R., Guilhermino, L., Soares, A.M.V.M., Gravato, C., 2012. Acute effects of deltamethrin on swimming velocity and biomarkers of the common prawn *Palaemon serratus*. Aquat. Toxicol. 124, 209–216.

Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). Ecol. Ind. 34, 641–647.

Ortega-Vinuesa, J.L., Bastos-Gonzalez, D., 2001. A review of factors affecting the performances of latex agglutination tests. J. Biomater. Sci. Polym. 12, 379–408.

Piazza, V., Dragic, I., Sepcic, K., Faimali, M., Garaventa, F., Turk, T., Berne, S., 2014. Antifouling activity of synthetic alkylpyridium polymers using the barnacle model. Mar. Drugs 12, 1959–1976.

Piazza, V., Gambardella, C., Canepa, S., Costa, E., Faimali, M., Garaventa, F., 2016. Temperature and salinity effects on cadmium toxicity on lethal and sublethal responses of *Amphibalanus amphitrite* nauplii. Ecotoxicol. Environ. Safe 123, 8–17.

Powell, M.D., Berry, A.J., 1990. Ingestion and regurgitation of living and inert materials by the estuarine copepod *Eurytemora affinis* (Poppe) and the influence of salinity. Estuar. Coast. Shelf Sci. 31, 763–773.

PlasticsEurope Plastics – The Facts 2014/2015. 〈http://www.plasticseurope.org/Document/plastics-the-facts-20142015.aspx?FolID=2〉.

Rainbow, P.S., Walker, G., 1977. The functional morphology and development of the alimentary tract of larval and juvenile barnacles (Cirripedia: Thoracica). Mar. Biol. 42, 337–349.

Setälä, O., Fleming-Lehtinen, V., Lehtiniemi, M., 2014. Ingestion and transfer of microplastics in the planktonic food web. Environ. Pollut. 185, 77–83.

Sjollema, S.B., Redondo-Hasselerharm, P., Leslie, H.A., Kraaethaak, A.D., 2016. Do plastic particles affect microalgal photosynthesis and growth? Aquat. Toxicol. 170, 259–261.

Sun, X., Li, Q., Zhu, M., Liang, J., Zheng, S., Xhao, Y., 2017. Ingestion of microplastics by natural zoo plankton groups in the northern South China Sea. Mar. Pollut. Bull. 115, 217–224.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Eve, M., Pernet, J., La Goic, N., Quillien, V., Mingant, C., Epelboin, Y., Corporeau, C., Guyomarch, J., Robbens, J., Paul-Pont, I., Soudant, P., Huvet, A., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. Proc. Natl. Acad. Sci. USA 113, 2430–2435.

Van Cauwenberghe, L., Claessens, M., Vandegehuchte, M.B., Janssen, C.R., 2015. Microplastics are taken up by mussels (*Mytilus edulis*) and lungworms (*Arenicola marina*) living in natural habitats. Environ. Pollut. 199, 10–17.

Varò, I., Navarro, J.C., Amat, F., Guilhermino, L., 2002. Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. Chemosphere 48, 563–569.

Venkateswara Rao, J., Kavitha, P., Jakka, N.M., Sridhar, V., Usman, P.K., 2007. Toxicity of organophosphates on morphology and locomotor behavior in brine shrimp, *Artemia salina*. Arch. Environ. Contam. Toxicol. 53, 227–232.

Wegner, A., Besseling, E., Foekema, E.M., Kamermans, P., Koelmans, A.A., 2012. Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L). Environ. Toxicol. Chem. 31, 2490–2497.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492.

Yarsan, E., Yipel, M., 2013. The important terms of marine pollution “Biomarkers and Biomonitoring, Bioaccumulation, Bioconcentration, Biomagnification”. J. Mol. Biomark. Diagn. S1, 003. http://dx.doi.org/10.4172/2155-9929.S1-003.