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Impacts of dietary exposure to different sized polystyrene microplastics alone and with sorbed benzo[*a*]pyrene on biomarkers and whole organism responses in mussels *Mytilus galloprovincialis*

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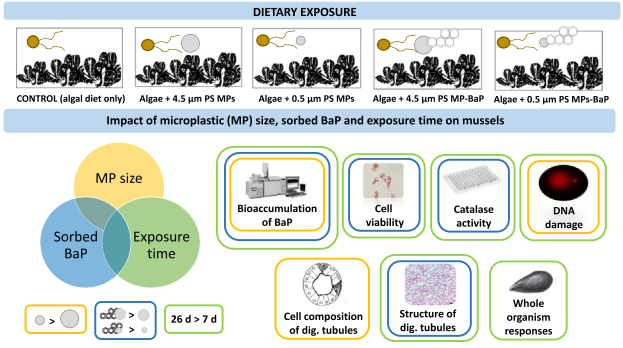
HIGHLIGHTS

- Smaller MPs were able to transfer higher amounts of BaP to mussels than larger MPs
- Bioaccumulation of BaP and most biomarkers showed a time dependent response pattern
- Large MPs were found mostly in stomach contents and also within epithelial cells of digestive system
- Increased effects of MPsBaP respect to MPs alone in hemocyte viability and catalase activity and in digestive gland structure
- Effects dependent on MP size were observed on DNA damage and cell type composition of digestive tubules

GRAPHICAL ABSTRACT



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ABSTRACT

Due to their hydrophobicity and relatively large surface area, microplastics (MPs) can act as carriers of hydrophobic pollutants in the ocean and may facilitate their transfer to organisms. This study examined effects of dietary exposure to polystyrene MPs of 0.5 and 4.5 μm alone and with sorbed benzo[*a*]pyrene (BaP) on mussels *Mytilus galloprovincialis* in order to elucidate the effects of MP size and the presence of sorbed BaP on the organism. MPs were provided daily, mixed with algae, during 26 days at equivalent mass (0.058 mg/L), corresponding to 1000 particles/mL for 4.5 μm MPs and to 7.44×10^5 particles/mL for 0.5 μm MPs. Effects were determined on early cellular biomarkers in hemocytes, structure and cell type composition of digestive tubules (DTs), histopathology and whole organism responses (condition index (CI), clearance rate (CR), food absorption efficiency (AE), respiration rate (RR) and scope for growth (SFG)). BaP concentrations in mussels increased with time, in particular when sorbed to smaller MPs. Large MPs were abundant in the lumen of stomach and DTs, but were also occasionally found within epithelial cells. Effects in all treatments increased with exposure time. MPs with sorbed BaP were more toxic than MPs alone according to hemocyte viability and catalase activity and to the quantitative structure of DT epithelium. Higher toxicity of small MPs compared to larger ones was recorded for DNA damage and cell composition of DTs. At tissue level a slight increase in prevalence of inflammatory responses occurred in

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all exposed groups. At whole organism level a compensatory effect was observed on absorption efficiency across MP treatments at day 26, resulting in increased SFG in mussels exposed to small MPs with sorbed BaP. This could be related to an increased energy need to deal with stress observed in biomarkers. Further work is required to understand the Trojan horse effect of a variety of plastic type, size, shape combinations together with a wide variety of pollutants.

1. Introduction

Plastic debris is now internationally recognized as a ubiquitous pollutant with potentially serious consequences in the marine environment (UNEP, 2011). Whilst the impacts of large plastic debris are well studied, it was only in recent years that interest developed in understanding and quantifying the abundance, distribution and effects of microplastics (MPs), defined as plastic particles <5 mm in their largest dimension (Andrady, 2011; Cole et al., 2011). MPs are largely derived from the in situ mechanical, chemical and biological breakdown of larger plastics. MPs may also enter the marine environment directly. Plastic microbeads used in exfoliating scrubs and synthetic fibres from clothes, such as polyester, are washed into the sea via effluent. Furthermore, industrial sandblasting now utilizes plastic microbeads rather than sand, and accidental industrial spills of pre-manufacture polymer powders and resin beads may occur (Cole et al., 2011). The scientific literature on MPs has grown exponentially and there are now a wealth of papers demonstrating that like large plastics, MPs are a ubiquitous if unevenly distributed pollutant in the oceans (Browne et al., 2011; Cole et al., 2011). Particle concentrations in the water column range from 2×10^{-7} particles/m^3 in the Bearing Sea (Day et al., 1990) to 102,000 particles/m^3 in coastal waters of Sweden (Norén and Naustvoll, 2010) or 9180 particles/m^3 in the North East Pacific Ocean (Desforges et al., 2014), depending on distance from coast and depth (Desforges et al., 2014; Baini et al., 2018). In sediments particle concentrations may vary from 0.21 to >77,000 particles/m^3 (Hidalgo-Ruz et al., 2012). In general, concentrations are highest in coastal sediments particularly around heavily industrialized areas and in oceanic gyres but reported figures may be much higher due to sampling difficulties for smaller MPs (Lusher, 2015). Variations in sampling and quantification methods make it hard to compare MP concentrations in different works (Vandermeersch et al., 2015), highlighting the necessity for standardized methods (ICES, 2015; Li et al., 2019).

Marine organisms have been reported to ingest MPs in nature and the potential for negative effects on organisms after ingestion is a prominent environmental concern (Lusher et al., 2017). Affected organisms include molluscs, echinoderms, cnidarians, polychaete annelids, seabirds, fish and crustaceans (Hoss and Settle, 1990; Jumars, 1993; Thompson et al., 2004; Ward and Shumway, 2004; van Franeker et al., 2011; Hall et al., 2015). The investigation of the effects of MP ingestion in marine organisms is therefore a high priority for environmental science.

Filter feeders and deposit feeders are particularly vulnerable to MP ingestion in the wild as they are able to feed directly on MPs and may even selectively ingest them (Graham and Thompson, 2009). Mussels *Mytilus* spp. have been extensively used as model organisms to study the distribution and impact of marine pollutants (Cajaraville et al., 2000; Viarengo et al., 2007), and in recent years have been identified as a useful model organism for the study of MPs and their co-contaminants. The uptake of MPs (<80 μm) by mussels has been demonstrated both through the gill surface and via transfer of the MPs along gill channels to the mouth and into the digestive gland where translocation into cells could occur (Von Moos et al., 2012). MPs have been shown to appear in the intestinal lumen and epithelium, in digestive tubules (Avio et al., 2015; Paul-Pont et al., 2016) and within the lysosomal compartment (Von Moos et al., 2012). Polystyrene (PS) microspheres of 3 and 9.6 μm have been shown to translocate to the hemolymph were they persisted for up to 48 days, and even entered hemocytes (Browne et al., 2008). Browne et al. (2008) also showed that MP size plays a significant role in the transport and fate of MPs in mussels, as they found that abundance of 3 μm particles in the hemolymph increased by 60% as compared to the 9.6 μm particles. Mussels have an open circulatory system, therefore smaller particles can be seen to pose a greater threat than larger particles due to their enhanced ability to interact with cells throughout the organism (Browne et al., 2008). Toxic effects of virgin MPs in mussels at tissue level or below include changes in transcriptional profiles involving a number of indicators of toxicity, neurotoxicity (Avio et al., 2015), oxidative stress and damage (Paul-Pont et al., 2016), decrease in lysosomal membrane stability, alterations in immune parameters (Pittura et al., 2018) and increases in the formation of granulocytomas (Von Moos et al., 2012), and total histopathological lesions (Paul-Pont et al., 2016; Brate et al., 2018). At organism level, mussels chronically exposed to 30 nm PS nanoplastics showed reduced feeding activity and increased production of pseudofaeces which indicates reduced feeding efficiency (Wegner et al., 2012). However, integrated physiological responses to the impacts of MPs as represented by Scope For Growth (SFG) have yet to be characterized in mussels.

In addition to the effects of MPs on organisms that have initially ingested them, the accumulation of MPs (either absorbed into internal tissues or within the gut lumen) can lead to the trophic transfer of these particles within food chains. The biotransfer of MPs from mussels to crabs and puffer fish has been shown (Farrell and Nelson, 2013; Santana et al., 2017) and field studies have found MPs in mussels grown for aquaculture and in consumer products (Van Cauwenberghe and Janssen, 2014; Li et al., 2018) thereby demonstrating the real world significance of this issue. Furthermore, the ingestion of MPs can facilitate the transport of harmful chemicals to organisms (Browne et al., 2013; Rochman et al., 2013a, 2013b; Lusher, 2015; Ma et al., 2016). This may occur via leaching of chemicals added to plastics during manufacture, such as plasticizers, or via the release of persistent organic pollutants (POPs) which accumulate in MPs in seawater (e.g. polycyclic aromatic hydrocarbons, PAHs) (Lusher, 2015).

Sorption capacity of plastics and desorption rates of pollutants have been characterized for a number of prominent environmental contaminants across a number of polymer types and appear to be highly polymer and pollutant specific (Bakir et al., 2016) making it important to investigate the bioavailability and effects of a wide range of plastic-pollutant combinations. The transfer of chemicals to the body via MPs will ultimately depend on the concentration of the sorbed chemicals relative to the body burden of the contaminant in question derived from other sources (e.g. dietary). However, a number of other factors may also influence MP co-contaminant bioavailability (Koelmans, 2015). These include MP size, surface texture and chemistry all of which may be altered continuously due to the weathering process in nature; the formation of biofilms may also alter the MP surface chemistry (Ziccardi et al., 2016). Indeed size has been identified as a key factor influencing both sorption capacity and sorption/desorption rates of chemicals to and from plastics. Smaller plastics not only have a larger surface area to volume ratio but also have a shorter diffusion pathway; hence smaller plastics can sorb higher contaminant concentrations and exchange these more rapidly with the environment (Velzeboer et al., 2014). Once ingested, gut retention time and fate within the body will influence time available for desorption of chemicals into tissues and the sites of toxic action (Bakir et al., 2016). Again smaller MPs have been highlighted as potentially more dangerous due to their enhanced ability to translocate and increased retention times in mussels and

crabs (>48 days and up to 21 days, respectively) (Browne et al., 2008; Farrell and Nelson, 2013; Watts et al., 2014).

The transfer of sorbed chemical pollutants via MPs has been demonstrated in fish, crustaceans, annelids and bivalve molluscs (Besseling et al., 2013; Oliveira et al., 2013; Rochman et al., 2013a; Avio et al., 2015; Batel et al., 2016, 2018; Paul-Pont et al., 2016; Guilhermino et al., 2018; Pittura et al., 2018). However, the extent to which these co-contaminants cause harmful effects beyond those of the MPs alone is less clear. The common goby (*Pomatoschistus microps*) showed a significant decrease in the activity of acetylcholinesterase (AchE), when exposed to polyethylene (PE) both with and without pyrene (Oliveira et al., 2013), indicating no additional effects of the sorbed pyrene. However, in Japanese medaka (*Oryzias latipes*) a higher level of hepatic stress was induced in fish exposed to PS MPs with sorbed mixed environmental POPs compared to fish exposed to PS alone (Rochman et al., 2013a). Meanwhile, pyrene contaminated PS and PE particles (<100 μm) increased the frequency of micronuclei and inhibited the activity of AchE relative to plastics alone in *M. galloprovincialis* (Avio et al., 2015). Paul-Pont et al. (2016) observed mixed effects in *Mytilus* spp. whereby PS microspheres alone had greater effects than PS with sorbed fluoranthene for certain biomarkers, whilst the reverse was true for other biomarkers. Mixed effects on different biomarkers in *M. galloprovincialis* exposed to low density polyethylene (LDPE) and LDPE with sorbed BaP were also reported by Pittura et al. (2018) but, overall, using the integrative weight of evidence model, LDPE with sorbed BaP was classified as exerting greater hazard than LDPE MPs.

Clearly further work is required to elucidate the effects of a variety of contaminant-plastic combinations in a variety of species using a wide range of plastic sizes. Benzo[*a*]pyrene (BaP) is a priority pollutant (US EPA, 2014; UE, 2008) and has been widely used as a model PAH in ecotoxicology (Banni et al., 2017); it is both genotoxic and carcinogenic and can be found throughout the marine environment (Di et al., 2017). BaP has been reported to cause peroxisome proliferation, oxidative stress, endocrine disruption and genotoxic effects in mussels (Venier et al., 1997; Cancio et al., 1998; Gómez-Mendikute et al., 2002; Orbea et al., 2002; Banni et al., 2017). Because of its highly lipophilic nature BaP has been shown to strongly sorb to MPs and to be transferred via MPs to *Artemia* and then transfer up the food chain from *Artemia* to zebrafish (Batel et al., 2016).

This study aimed to examine the effects of long-term (26 day) dietary exposure of two different sized PS microspheres (4.5 μm and 0.5 μm) alone and with sorbed BaP in mussels *M. galloprovincialis* in order to elucidate the influence of MP size and the presence of sorbed BaP on the organism at different levels of biological organization. An increasing number of studies have used a variety of biomarkers to investigate the effects of MPs and their co-contaminants at cell and molecular levels. Biomarkers are generally used to predict changes at higher levels of biological organization and have been defined as "short-term indicators of long-term biological effects" (Cajaraville et al., 2000). However, in the case of emerging pollutants such as MPs it is important to evidence links between effects at cellular or subcellular levels such as oxidative stress and effects at a whole organism level such as effects on growth, reproduction or survival (Tsangaris et al., 2008). Hence this study adopted a battery of biomarkers approach and assessed effects using biomarkers for cell viability, oxidative stress, genotoxicity and structure and cell type composition of digestive tubules, together with histopathological analysis and measurement of effects on overall organism health (SFG and condition index). Bioaccumulation of BaP in mussel tissues and tissue distribution of MPs alone and with sorbed BaP were also investigated.

2. Materials and methods

2.1. Sampling and maintenance of mussels

Roughly 600 mussels *Mytilus galloprovincialis* of 3.5–4.5 cm shell length were collected on the 1st of February 2017 from Plentzia, Bay of Biscay (43°24′N, 2°56′W), a relatively clean area (Orbea and Cajaraville, 2006; Bellas et al., 2011, 2014,) and maintained in aquarium facilities at the Plentzia Marine Station (PiE) of the University of the Basque Country (UPV/EHU). According to Ortiz-Zarragoitia et al. (2011) mussels from the sampling area are at advanced gametogenesis in February and spawning starts in March, with a peak in April. Thus, the experimental period was selected to avoid the cold temperature period where mussels remain metabolically less active (Cajaraville et al., 1995; Cancio et al., 1999) whilst at the same time avoiding spawning and post-spawning stress (Bayne et al., 1976).

Seawater from Plentzia was naturally filtered by sand in the uptake wells aided with a pump that sent the water to the Marine Station. Seawater gas balance was controlled in the Station and then passed through a decantation/inertial tank and filtered (particle size ≤3 μm). Mussels were fed with microalgae *Isochrysis galbana* (T-Iso clone) grown in 2 × 40 L plastic culture bags, in a room with controlled temperature at 20 °C under white fluorescent light (GRO-LUX 36 W) with constant aeration. Commercial F2 algae medium (Fritz Aquatics, USA) was supplied to algae cultures according to manufacturer's instructions. Under these conditions, sufficient cells were produced to allow for 10 L of pure culture (cell density ~ 8×10^6 algae cells/mL) to be used each day to feed mussels.

After collection mussels were placed in a single 600 L recirculating seawater system for 6 days without feeding. Then, 110 mussels were distributed in each of 5 static glass aquaria with 40 L seawater and maintained for a further 15 days (light regime 12 L/12D, temperature: 13 °C). Water was changed daily. Feeding conditions during both the acclimation period and subsequent exposure period were set to provide mussels with food around the maintenance ration (1.5% of soft body weight per day in mussels *Mytilus* of the size used in this study; Bayne et al., 1976). Assuming feeding (clearance) rates of 3 L/h per individual mussel, this was achieved by dosing the concentrated stock of the pure phytoplankton culture by means of a multi-channel peristaltic pump (Ismatec, Glattbrugg, Switzerland), at rates set to produce a stable particle concentration of 2500 algal cells/mL in the tanks. Mussels were fed this ration over a period of 22 h/day. Actual tank cell concentration during the acclimation period, recorded with a Coulter Counter Z1 (Beckman, Indianapolis, USA) in water samples taken twice a day (immediately before and 2 h after the water change), ranged from 2500 to 5000 cells/mL, which represents 1 to 2 times the maintenance ration.

During both the acclimation and exposure period all tanks were rinsed with 10 L of seawater during the water change each day in order to prevent the buildup of mussel faeces at the bottom of tanks. Additionally all mussels were checked to ensure that they had closed their valves when exposed to air and post water change all tanks were checked to ensure that mussels had reopened their valves. Water quality parameters were checked daily during the fed portion of the acclimation period in all tanks and in the control tank throughout exposure. Salinity remained stable throughout the experiment and acclimation period at 33 PSU (±0.2) as did pH 8.00 (±0.05), dissolved oxygen >80% and 6.66–8.05 mg/L and NH_4 and NH_3 levels, which were below the threshold for chronic toxicity.

2.2. MPs source and preparation of MPs alone and with sorbed BaP

The plastics chosen for this experiment were unlabeled polystyrene microspheres (Polyscience Inc.; Badener, Germany density 1.05 g/cm^3) of two sizes: 4.5 μm (LMP) and 0.5 μm (SMP). MPs with sorbed BaP (LMPB and SMPB, respectively) were prepared according to Batel et al. (2016) with adaptations described below. 24 h prior to the start of each exposure, 89.2 μL of LMP stock (4.9×10^8 particles/mL) was added to each of two aluminum foil wrapped glass vials containing 10 mL of Milli-Q water in order to create the particle suspensions for the LMP and LMPB treatments. This process was then repeated with the SMP stock (3.64×10^{11} particles/mL) for the SMP and SMPB treatments. 10 μL of 1 mM BaP (Sigma, St. Louis, Missouri) was then added

to each the LMPB and SMPB vials to get a final concentration of 1 μM (252 μg/L) BaP solution containing 0.01% dimethyl sulfoxide (DMSO, purity >99% Sigma, St. Louis, Missouri). All vials were then incubated for 24 h at 300 rpm in an orbital shaker at 18 °C in darkness. These suspensions were then syringe filtered using sterile 0.45 μm and 0.2 μm pore sized filters (Merck Millipore, Darmstadt, Germany) for the LMPB and SMPB treatments, respectively. The plastics were then resuspended in 40 mL of Milli-Q water and added to the algae feed for each respective mussel treatment. This procedure was repeated every day during the 26 day exposure experiment to ensure reproducible dosing of freshly contaminated MPs to mussels. Non-contaminated pristine MPs were incubated, filtered and resuspended in the same way but in Milli-Q water alone. All solutions containing BaP were stored in glass vials wrapped in aluminum foil.

The sorption capacity of the PS microspheres used in this study for BaP has been reported in a separate study (Martínez-Álvarez et al., 2018). According to the Langmuir model, the maximum adsorption capacity (Q_{max}) ranged from 144.99 to 242.89 μg/g and from 30.5 to 67.65 μg/g for 0.5 μm and 4.5 μm MPs, respectively.

2.3. Mussel exposure experiment

After acclimation, mussels were exposed to MPs alone and with sorbed BaP according to the following experimental groups: Control, 4.5 μm MPs alone (LMP), 0.5 μm MPs alone (SMP), 4.5 μm MPs with BaP (LMPB) and 0.5 μm MPs with BaP (SMPB). MPs were provided daily, mixed with algae, at equivalent mass of 0.058 mg/L, corresponding to 1000 particles/mL for 4.5 μm MPs and to 7.44×10^5 particles/mL for 0.5 μm MPs) during 26 days. Selected concentrations of MPs are environmentally relevant according to Eriksen et al. (2013) and Lechner et al. (2014).

During exposure mussels were fed continuously with *I. galbana*, at the same rations administered during the acclimation period. Mussels were exposed to plastics together with algae and a daily water change was performed. The control aquaria received pure algae whereas the exposure aquaria received algae mixed with plastics (with/without BaP). Aeration was used to keep both plastics and algae in suspension. During the experiment, mortality recorded in each exposure group was 3 mussels in the LMP group, 4 in the SMPs, 1 in the LMPB and 3 in the SMPB. No mortality was recorded in control mussels.

Mussel sampling and dissection took place after 7 and 26 days exposure. 15 individuals were taken per experimental group and frozen whole at −40 °C for analysis of BaP burden in tissues by GC–MS. Hemolymph was withdrawn from the posterior adductor muscle of 8 mussels per experimental group and used to measure neutral red (NR) uptake, catalase activity (CAT) and DNA damage (Comet assay). Another 10 mussels per experimental group were dissected and half of their digestive gland, gills and gonad preserved in formalin and processed for paraffin histology. The other halves of digestive glands were frozen in liquid nitrogen for cryostat histology. Finally, 7 individuals were taken per experimental group for the analysis of SFG. Following sampling on day 7 dosing of algae was recalculated in order for maintenance rations to remain the same for the reduced number of mussels present in the tanks.

2.4. Bioaccumulation of BaP in mussel soft tissues

Whole mussels for chemical analysis (15 per experimental group) were stored at −40 °C and analyzed at the General Services SGiker of UPV/EHU for BaP determination using GC–MS (SIM mode). The lyophilized tissues were homogenized and then extracted using a MARX microwave (CEM, Mathews, NC, USA) and cleaned through solid phase extraction (SPE Vacuum Manifold System, Millipore). The extracts obtained were concentrated through evaporation with nitrogen flow, filtered and encapsulated in chromatographic vials. Concentration of BaP was measured in an Agilent (6890) Gas Chromatograph with Mass Detector (Agilent 5975C) (Navarro et al., 2008) and given in ng/g dry weight. Calibration standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and regression coefficients obtained for the calibration curves were always above 0.995. Limit of quantification for BaP was 1 ppb.

2.5. Tissue distribution of MPs in mussels

Digestive gland, gills and mantle tissue of 10 mussels per experimental group were dissected out. Half of the digestive gland, half of the gills and whole mantle were fixed in 4% formalin in individual cassettes and processed using an alternative tissue dehydration protocol which utilizes N butyl alcohol (Stiles, 1934) in order to prevent the degradation of the PS microspheres by the organic solvents used in the standard histology tissue processing procedure. Then, samples were embedded in paraffin and sections of 5 μm thickness were cut in a Leitz 1512 microtome (Leica Instruments, Wetzlar, Germany). For staining, slides were placed in a heater at 130 °C for 10 min and then immersed in N butyl alcohol at 60 °C for another 10 min to remove the paraffin. Then, slides were re-hydrated in a series of decreasing alcohol concentrations and stained with hematoxylin/eosin (Gamble and Wilson, 2002). Sections were observed under a BX51 light microscope (Olympus, Tokyo, Japan) and the prevalences of mussels showing MPs in the digestive gland, gills and gonad were calculated as percentages. For the quantification of the number of large MPs in the digestive gland, one section containing the digestive tract was selected per mussel.

2.6. Cellular biomarkers in mussel hemocytes

On each sampling day, the hemolymph of 8 mussels per treatment was withdrawn from the posterior adductor muscle and immediately cell viability, catalase activity and DNA damage were measured in hemocytes of individual mussels.

Neutral red (NR) uptake was assessed in hemocytes according to Borenfreund and Puerner (1985) with modifications as explained below. The assay is based on the incorporation of a cationic neutral red dye into the lysosomes of live cells. Hemolymph of 8 animals per group was collected and seeded into 4 replicates in a 96-well flat bottom microplate. Cells were kept at 18 °C in a FRIOCELL incubator (MMM group, Planegg, Germany) for 30 min in order to allow cell attachment. Afterwards, cells were incubated with NR solution (0.04% in PBS, pH 7.3–7.4) for 1 h in the same incubator, and washed several times with PBS to eliminate non-incorporated dye. Then, NR was extracted from intact cells using an acetic acid (0.5%) ethanol (50%) solution and absorbance was measured at 550 nm in a Biotek EON microplate reader (Winooski, USA). Cell concentration was determined in the hemolymph of each animal using a Bright-Line™ Hemacytometer (Sigma Aldrich, St. Louis, USA) and used to normalize absorbance data.

Catalase activity (CAT) was assessed according to Aebi (1984) with modifications as explained below. The method is based on the conversion of H_2O_2 into H_2O and O_2 by catalase. Hemolymph of 8 animals per group was centrifuged at 300*g* for 10 min at 4 °C, and pellets were resuspended and homogenized in TVBE buffer (1 mM sodium bicarbonate, 1 mM EDTA, 0.1% ethanol and 0.01% Triton X-100, pH 7.4) and stored at −80 °C for further analysis. Then, samples of each animal were added into 4 replicates in a UV/VIS 96-well microplate and consumption of H_2O_2 (20 mM H_2O_2 in 50 mM phosphate buffer pH 7) was measured every 22 s during 4 min at 240 nm in a Bio-tek EL 312 microplate reader (Winooski, USA). Protein concentration was measured in each sample following Lowry et al. (1951) and used to normalize absorbance data. CAT activity was expressed as $\Delta Abs \cdot mg\ protein^{-1}$.

The Comet assay was performed on mussel hemocytes following Raisuddin and Jha (2004) with modifications, as described in Katsumiti et al. (2015). Briefly, hemolymph of 8 animals per group was centrifuged at 300*g* for 10 min at 4 °C and pellets were resuspended in 0.5% low melting point agarose and placed on slides coated with 1%

normal agarose. Slides were kept at 4 °C for 10 min in order to allow agarose solidification. Slides were then immersed in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N lauroyl sarcosine, 1% Triton X-100, 10% DMSO; pH 10) for 1 h at 4 °C in the dark to remove cellular proteins. Afterwards, slides were washed with distilled water and transferred to a tank containing a chilled electrophoresis solution (1 N NaOH and 200 mM EDTA; pH 13) and kept for 20 min to allow alkaline DNA unwinding. Electrophoresis was carried out at 300 mA, 25 V for 30 min in the same electrophoresis solution. Then, slides were incubated with neutralization buffer (0.4 M Tris-HCl; pH 7.5) and fixed with chilled methanol for 3 min. For analysis, slides (2 per animal) were stained with ethidium bromide (10 μg/L) and 50 randomly selected cells per slide were analyzed under an Olympus BX61 fluorescence microscope (Hamburg, Germany) and scored using the Komet 5.5 imaging software (Andor Bioimaging, Liverpool, UK). DNA strand breaks were determined as percentage of DNA in the comet tail.

2.7. Lysosomal enzyme histochemistry in mussel digestive gland

Samples of digestive gland for lysosomal enzyme histochemistry were frozen in liquid nitrogen and stored at −80 °C. The 10 digestive gland samples per experimental group were frozen together in two plastic chucks, each containing 5 half digestive glands attached to the chuck with cryoMbed. Eight 10 μm sections were cut using a Leica CM 3050 S cryostat (Leica Instruments, Wetzlar, Germany), frozen and stored at −80 °C. The obtained sections were then stained for lysosomal β hexosaminidase activity according to UNEP-RAMOGE (1999). Additional serial sections from control mussels at day 7 were stained in the same way but without the substrate β *N* acetylhexosaminide and also stained for lipofuscins using the Schmorl's reaction according to Pearse (1972).

2.8. Cell composition, quantitative structure and histopathology of mussel digestive gland

For these analyses, digestive gland paraffin sections stained with hematoxylin/eosin described in Section 2.5 were used. Cell composition of digestive tubules was measured in terms of volume density of basophilic cells (VvBAS, given as $\mu m^3/\mu m^3$) and the structure of digestive tubules was quantified as mean epithelial thickness to mean diverticular radius (MET/MDR, μm/μm) and mean luminal radius to MET (MLR/MET, μm/μm) by using a stereological method (Bignell et al., 2012; De los Ríos et al., 2013). Hits on basophilic cells, digestive cells and lumen of digestive tubules were recorded using a modified Weibel graticule (multipurpose test system M-168) with a drawing-tube attached to a Leitz Laborlux S microscope (Stuttgart, Germany) using a 40× magnification in 3 fields per section in 10 mussels per experimental group. Then, the different parameters were calculated as described in Bignell et al. (2012).

Prevalences of general histopathological alterations were recorded for the same 10 mussels per experimental group and given as percentages. The following alterations were recorded: occurrence of parasites, fibrosis in the connective tissue, necrosis of digestive tubule epithelium, focal and diffuse hemocytic infiltrations, presence of brown cells in connective tissue and brown aggregates in epithelium of the digestive tract (Garmendia et al., 2011; Bignell et al., 2012; Ruiz et al., 2014).

2.9. Gamete development, gonad index and histopathology of mussel gonad

For this, mantle paraffin sections stained with hematoxylin/eosin described in Section 2.5 were used. Six gamete developmental stages were distinguished according to Seed (1969) and a gonad index value was assigned to each mussel following Kim et al. (2006) in one section per mussel and 10 mussels per experimental group. The gonad index ranged from 0 (resting gonad) to 5 (mature gonad), showing increasing GI values (0 to 5) during gametogenesis phases and decreasing GI values (5 to 1) at spawning and post-spawning phases.

Prevalences of general histopathological alterations were recorded for the same 10 mussels per experimental group and given as percentages. The following alterations were recorded: occurrence of parasites, fibrosis in the connective tissue, focal and diffuse hemocytic infiltrations and oocyte atresia (Ortiz-Zarragoitia and Cajaraville, 2006, 2010; Ruiz et al., 2014). For oocyte atresia, the following semiquantitative scale was used: 0 normal gonad, 1 less than half the follicles are affected, 2 about half the follicles are affected, 3 more than half the follicles are affected and 4 all follicles affected (Kim et al., 2006).

2.10. Whole organism responses

On each sampling day, 7 mussels from each treatment were collected to measure the physiological parameters and condition index. To this end, mussels from each treatment were placed in separate 20 L aerated static tanks filled with clean seawater. To allow for individual collection of biodeposits, each mussel was placed inside a 250 mL chamber, completely submerged. Feeding conditions prior (12 h) and during physiological measurements (2 days) were set to allow for rates of fecal production to be compatible with measurements of absorption efficiency (AE) based on gravimetric determinations. On the other hand, ashed silt particles (<30 μm) were added to *I. galbana* cultures to fulfill the requirements for an inorganic tracer in measurements of AE with the Conover method. This mixed diet (~70% organic content) was dosed to the feeding tanks by means of a multi-channel peristaltic pump (Ismatec, Glattbrugg, Switzerland), at a rate set to provide for particle concentrations of 15,000 particles/mL. Feeding conditions were maintained stable by frequent checking of particle concentration using a Coulter Counter Coulter Z1 (mean of 3 replicates) and adjustment of pumping rates. Actual concentrations across all experiments ranged between 10,000 and 30,000 particles/mL, above the threshold to produce sufficient faeces and below the threshold for pseudofaeces production (Navarro et al., 1996).

Clearance rate (CR: L/h) was measured as volume of water cleared of particles per animal per hour according to Coughlan's (1969) method, based on recording the exponential decrease of particle concentration over the time in a static system. For each treatment, 8 beakers filled with 2.5 L seawater provided with gentle aeration were used: i.e. 7 replicates each containing 1 mussel and 1 control for sedimentation. Each replicate was sampled for particle concentration (Coulter Counter Z1) at least 6 times at 10–20 min intervals. Respiration rate (RR: J/h) was used as a proxy for metabolic rate and was estimated from rates of O_2 consumption according to Gnaiger (1983). Individual mussels ($n = 7$) were placed in sealed 250 mL chambers, fitted with O_2xyDot®Sensor and filled with seawater. O_2 concentration was recorded using an Oxygen Analizer (OxySense 5250i, New Castle, UK), every 10 min for at least 7 times and the mean average of two O_2 measurements from each sampling point was used. One control (chamber without animal) was used with each treatment for checking O_2 concentration stability. Absorption efficiency (AE: fraction) was measured according to Conover's (1966) method based on the ratio of organic contents in food and faeces. To characterize food suspensions, duplicate water samples (0.8 to 2.5 L) taken from the feeding tanks were filtered onto ashed, pre-weighted Whatman® GF/C glass-fibre filters (Sigma-Aldrich, St. Louis, Missouri, USA), which were subsequently processed to determine concentrations of total particulate matter (TPM: mg/L) and inorganic and organic particulate matter (PIM and POM: mg/L). Salts retained in the filters were rinsed out with a solution of isotonic ammonium formate, then filters were dried at 110 °C, weighed, ashed at 450 °C and weighed again. TPM and PIM were estimated, respectively, as the dry and ash weight increment of the filters and POM as the weight loss of this filtered material on ashing. Organic content (OC) was estimated as POM/TPM. For faeces collection mussels were allowed to acclimate to the stable particle concentrations in each treatment for at least 12 h before individual

chambers were cleared of any solid material. Mussels were then left to feed and faeces produced collected by pipette. Faeces were then filtered onto ashed, pre-weighted GF/C glass-fibre filters and processed for organic content determination as indicated for food suspensions. Absorption rate (AR J/h) was computed as the product of CR (L/h), POM (mg/L), AE (fraction) and the energy content of food (=18.75 J/mg POM for *I. galbana* cells; Whyte, 1987). Scope for growth (SFG: J/h) was then calculated as AR – RR (Navarro et al., 1991).

After physiological determinations soft tissues of each animal were excised from the shells, dried at 80 °C for 24 h and weighted. Physiological rates were then standardized to the mean dry weight of mussel's tissue (0.2646 g) using size-scaling b values of 0.67 and 0.75 for CR and RR, respectively (Navarro et al., 1991). Mussel shell lengths were also recorded with vernier caliper and a condition index calculated as: tissue dry weight (g) / [shell length (cm)]^3.

2.11. Data analysis

All data were tested for normality and homogeneity of variance using the Ryan-Joiner and Levene's tests, respectively. Normally distributed data which met the assumptions of homogeneity of variances were assessed via two-way ANOVAs with treatment and sampling day as factors and treatment x day as an interaction. Where differences were found at a $p < 0.05$ significance level, Tukey's HSD post hoc test was used to find the source of any differences observed. Data which did not met the above assumptions were analyzed by separate one-way Kruskal-Wallis tests to look for differences between treatments within days, and by Mann-Whitney *U* tests to look for differences between days within treatments. Where differences were found at a $p < 0.05$ significance level in the Kruskal-Wallis test, Dunn's post hoc tests were used to determine the source of the observed differences. Parametric tests were analyzed in Minitab 17 (Minitab Inc.) and non-parametric tests were analyzed in SPSS 24 (IBM Analytics, Armonk, NY).

3. Results

3.1. Bioaccumulation of BaP in mussel soft tissues

BaP concentrations were very low approaching detection limits in all control and plastic alone treatments (all <2.2 ng/g dry weight) across both sampling days (Table 1). At day 7 BaP concentrations increased in mussels exposed to plastics with sorbed BaP, values ranging from 17.3 to 66.7 ng/g dry weight (Table 1) thereby demonstrating the transfer of BaP from MPs to mussel tissues. BaP tissue concentrations were notably higher in both MP with sorbed BaP treated groups at day 26 compared to day 7 (96.1–306 ng/g dry weight) indicating the bioaccumulation of BaP in mussel tissues (Table 1). These differences amounted to an approximate 2.5 to 10 fold increase across days in BaP tissue burden in LMPB samples and a roughly 4 to 6 fold increase in SMPB samples. In addition, mussels in the SMPB treatment had considerably higher BaP tissue burdens compared to LMPB treated mussels across both days. This difference remained roughly the same at day 26 compared to day 7, with SMPB treated mussels having roughly 2–4 fold greater BaP tissue burden compared to LMPB mussels at day 7 and a 2–3 fold greater BaP tissue burden at day 26.

Table 1
Bioaccumulation of benzo[*a*]pyrene (BaP) in mussel soft tissues (ng/g dry weight) in control mussels and in mussels dietarily exposed for 7 and 26 days to 4.5 μm MPs (LMP), 0.5 μm MPs (SMP), LMP with sorbed BaP (LMPB) and SMP with sorbed BaP (SMPB). Data are given for 15 mussels per experimental group in 2 replicates.

	Control		LMP		SMP		LMPB		SMPB	
	1	2	1	2	1	2	1	2	1	2
7 d	<1	1.3	2.2	1.1	1	<1	37	17.3	54	66.7
26 d	2	<1	<1	2.1	1.3	1.4	96.1	163.7	306.7	277.4

3.2. Tissue distribution of MPs in mussels

Large 4.5 μm MPs were localized and quantified in different tissues of mussels exposed to LMP and LMPB (Table 2). At least 80% of treated mussels presented large MPs in the digestive gland in both treated groups. Only 10 to 40% of treated mussels showed large MPs in gill tissues and these were only occasionally observed in gonad tissues (Table 2).

In sections of the digestive gland, large MPs were very abundant in the lumen of the stomach mixed with stomach contents (Fig. 1A, Table 3). Large MPs were also found in the lumen of digestive tubules, associated to cell debris (Fig. 1B) but at lower amounts than in the lumen of the stomach (Table 3). Occasionally individual large MPs were observed within epithelial cells of the digestive tract (Fig. 1C), ducts (Fig. 1D) and digestive tubules (Table 3). Few large MPs were also found isolated in the connective tissue (Fig. 1D). In the gills, large MPs were found interspersed between gill filaments (Fig. 1E) but never within gill epithelial cells. In mantle sections, large MPs were found in the connective tissue surrounding gonad follicles but not within the follicles. Overall, there was a high variability in numbers of MPs among different individuals in each exposure group (Table S1 of Supplementary material). The highest amount of MPs (120.60 ± 53.28) was found in mussels exposed to LMP for 7 days and the number of MPs decreased at 26 days in this group to 17 ± 10.57 particles (Table 3). Conversely, mussels exposed to LMPB treatment showed lower amounts of MPs at day 7 (11.70 ± 3.60) compared to the LMP group but numbers increased with exposure time up to 69 ± 41.91 particles (Table 3).

Small 0.5 μm MPs were hardly seen in the histological sections of mussels exposed to SMP and SMPB. Only in few individuals large numbers of particle aggregates were observed in the lumen of the stomach, mixed with stomach contents.

3.3. Cellular biomarkers in mussel hemocytes

There was no significant effect of any treatment on NR uptake at day 7. At day 26 hemocytes from mussels from both LMPB and SMPB groups showed a decrease in NR compared to controls, thereby indicating a decrease in cell viability in hemocytes from these treatments (Fig. 2A). LMPB and SMPB treatments also showed a significant decrease in NR compared to their respective day 7 values. The reduced uptake of NR in the LMPB and SMPB groups can also clearly be observed in the micrographs in Fig. 2B–F. Further, hemocytes from mussels in LMPB and SMPB groups showed altered morphology, cells becoming rounded and detaching from the substrate (Fig. 2E–F).

At day 7 there was a significant increase in CAT in hemocytes of both LMPB and SMPB mussels compared to the control (Fig. 2G). However at day 26 this pattern was reversed, with hemocytes of mussels exposed to LMPB and SMPB showing a significant decrease in CAT compared to the control. LMPB and SMPB mussel hemocytes also showed significantly reduced CAT compared to their respective day 7 values (Fig. 2G).

Background levels of DNA damage were observed in the control groups at mean values of 27.67% ± SE 0.78 and 25.28% ± SE 0.76% tail

Table 2
Prevalence of mussels showing MPs in different tissues of control mussels and of mussels dietarily exposed to 4.5 μm MPs (LMP) and to LMP with sorbed BaP (LMPB) along 7 and 26 days. Data are shown as percentages of 8–10 mussels per experimental group (one section per mussel). Not observed, n.o.

	Localization	Control	LMP	LMPB
7 d	Digestive gland	n.o.	80	90
	Gills	n.o.	40	20
	Gonad	n.o.	10	20
26 d	Digestive gland	n.o.	90	100
	Gills	n.o.	20	10
	Gonad	n.o.	12.5	n.o.

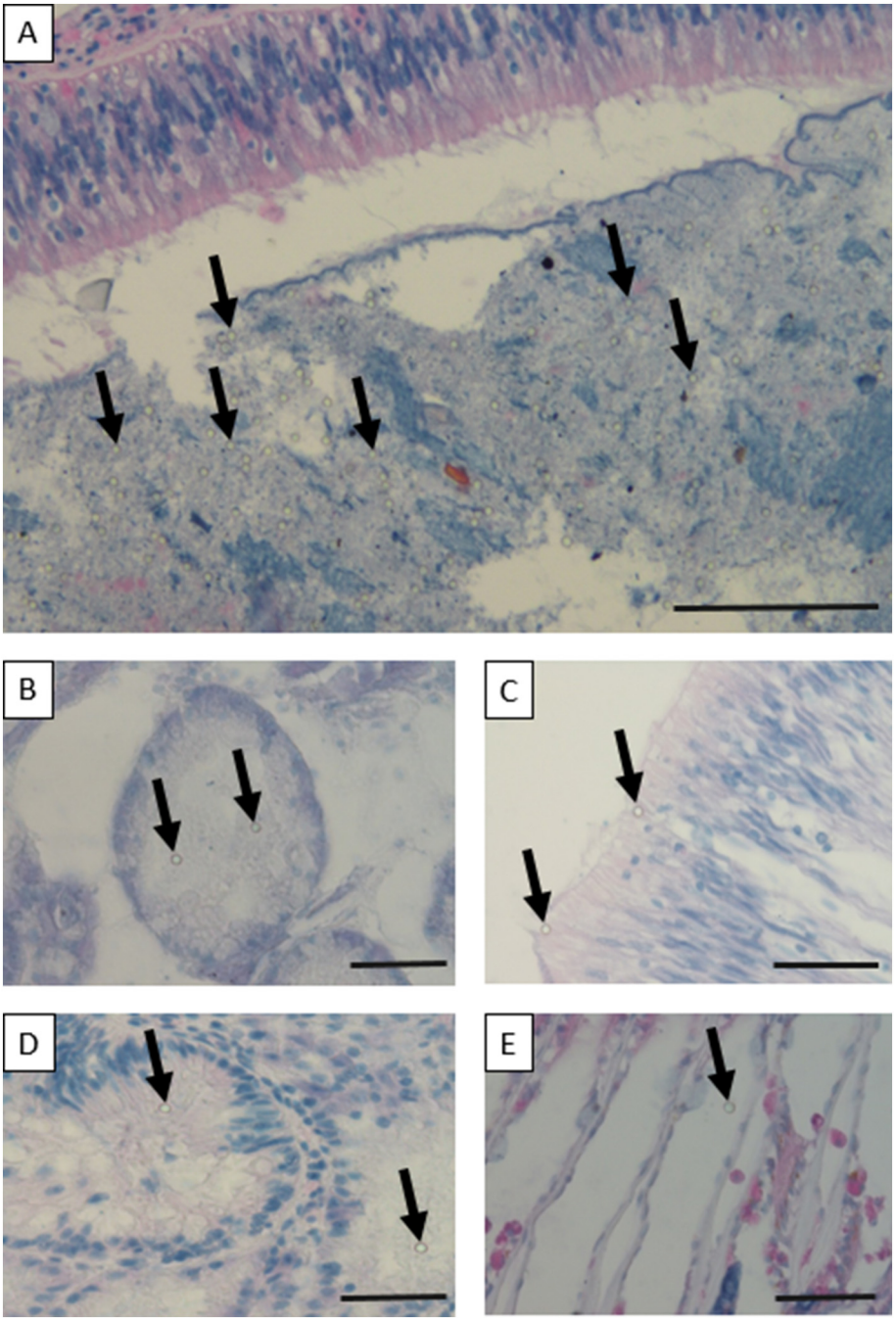

Fig. 1. Bright field micrographs of mussel paraffin sections showing the presence of 4.5 μm MPs (black arrows) in: A) stomach lumen of a mussel exposed to LMP for 7 days; B) lumen of digestive tubule of a mussel exposed to LMPB for 7 days; C) stomach epithelium of a mussel exposed to LMP for 7 days; D) digestive duct and connective tissue of a mussel exposed to LMPB for 7 days; E) gills of a mussel exposed to LMP for 7 days. Scale bars: A: 100 μm; B–E: 50 μm.

DNA for days 7 and 26, respectively, and showed no significant change between days (Fig. 3A). At day 7 the LMPB treatment displayed a significantly higher level of DNA damage as compared to the control and LMP treatment. At day 26 all treatments showed significantly higher levels of DNA strand breaks compared to the control except for LMPB which had higher than but not significantly different levels of DNA damage from the control. Highest levels of DNA damage were observed in the SMP treatment (mean 40.12% ± SE 0.72% tail DNA) which were also significantly higher than in the LMP treatment (mean 35.67% ± SE 0.71% tail DNA). There was no significant difference in DNA damage levels between SMP and SMPB mussels whereas levels in LMPB were significantly lower than in LMP mussels. DNA strand breaks increased significantly in LMP, SMP and SMPB treatments between day 7 and day 26. However in LMPB mussels DNA strand breaks decreased significantly from day 7 to day 26 (Fig. 3A). Results shown in Fig. 3A are illustrated in the fluorescence micrographs in Fig. 3B–F.

3.4. Lysosomal enzyme histochemistry in mussel digestive gland

Although the staining procedure for the lysosomal membrane stability test worked and lysosomes containing hexosaminidase activity could clearly be seen (Fig. 4A,B), a very strong yellow-brown background staining was present in digestive tubule cells in all treatments. The level of this staining was too high to be able to clearly distinguish staining level of lysosomes and hence determine lysosomal membrane labilization period. Background staining was also high in serial cryostat

Table 3
Tissue distribution of MPs in the digestive gland of mussels dietarily exposed to 4.5 μm MPs (LMP) and to LMP with sorbed BaP (LMPB) along 7 and 26 days. Data are shown as means and standard errors of the number of MPs in sections of the digestive gland of 10 mussels per experimental group (one section per mussel). Not observed, n.o.

	Localization		LMP	LMPB
7 d	Stomach	Lumen food	126.78 ± 55.08	3.80 ± 1.78
		Lumen mucus	0.56 ± 0.36	0.20 ± 0.20
		Epithelium	1.67 ± 0.69	1.10 ± 0.53
	Ducts	Lumen	0.20 ± 0.13	0.40 ± 0.27
		Epithelium	0.10 ± 0.10	0.50 ± 0.31
	Digestive tubules	Lumen	0.80 ± 0.61	3.90 ± 3.11
		Epithelium	0.30 ± 0.21	0.10 ± 0.10
	Connective tissue		3.10 ± 1.16	1.70 ± 0.58
	Total MPs		120.60 ± 53.28	11.70 ± 3.60
	Total MPs within epithelial cells		1.90 ± 0.84	1.70 ± 0.58
26 d	Stomach	Lumen food	12.78 ± 11.06	56.90 ± 35.20
		Lumen mucus	0.78 ± 0.49	2.30 ± 1.99
		Epithelium	0.89 ± 0.42	2.30 ± 0.75
	Ducts	Lumen	2.40 ± 1.67	4.50 ± 4.50
		Epithelium	n.o.	0.30 ± 0.30
	Digestive tubules	Lumen	n.o.	0.80 ± 0.14
		Epithelium	n.o.	n.o.
	Connective tissue		1.16 ± 0.96	2.30 ± 1.11
	Total MPs		17 ± 10.57	69 ± 41.91
	Total MPs within epithelial cells		0.80 ± 0.42	2.60 ± 0.91

sections of 5 control digestive glands from day 7 stained for hexosaminidase activity but without the substrate β *N* acetylhexosaminide (Fig. 4C,D). This, along with the observation of unstained sections within the same series (Fig. 4E,F), confirmed that the staining observed was not an artefact and appeared to correspond to lipofuscin-like pigments, which can accumulate in lysosomes as the indigestible end product of intracellular digestion and have a yellow-brown coloration. This was confirmed by the Schmorl's reaction and lipofuscin like pigments were clearly seen as the indigo stained inclusions within the epithelial cells of the digestive tubules (Fig. 4G,H). Lipofuscin levels in the digestive gland of control mussels were too high to clearly distinguish differences in lipofuscin accumulation between treatments.

3.5. Cell composition, quantitative structure and histopathology of mussel digestive gland

In general, in both control and treatment groups, the appearance of digestive tubules indicated a high digestive activity. In many tubules, cells in digestive epithelia were very high and contained a great number of large vesicles that obliterated the lumen (Fig. 5A,B,C). In other tubules, the digestive epithelium was thin and the large lumen appeared full of cell debris (including entire digestive cells). Tubules with totally disorganized necrotic areas were also observed (Fig. 5B) in a single mussel each in treatments LMP at 7 days and LMP and SMP at 26 days (Table 4). Low prevalences of inflammatory responses such as fibrosis (Fig. 5C), hemocytic infiltration (Fig. 5D) and accumulation of brown cells in the connective tissue (Fig. 5E) and in the epithelium of the digestive tract (Fig. 5F) were observed in mussels exposed to both sizes of MPs alone or with sorbed BaP in both sampling times (Table 4). The parasite *Mytilicola intestinalis* was observed in the digestive tract of a relevant number of individuals but there were no clear trends of parasite prevalence with treatments or along the exposure time (Table 4).

At day 7 of exposure, the volume density of basophilic cells (VvBAS) was significantly higher in mussels exposed to SMP and SMPB compared to the controls and to mussels exposed to LMP (Fig. 6A). No significant differences were observed at day 26 of exposure with respect to controls, but a significantly higher VvBAS was recorded in mussels exposed to SMP in comparison to mussels exposed to LMP. No significant differences in VvBAS were recorded along the exposure within each treatment (Fig. 6A).

Regarding the two parameters indicative of the structural integrity of the digestive tubules, mean epithelial thickness to mean diverticular radius (MET/MDR) and mean luminal radius to mean epithelial thickness (MLR/MET), no differences among treatments were observed at day 7 of exposure (Fig. 6B,C). At day 26 of exposure, mussels exposed to SMPB displayed significantly lower MET/MDR and higher MLR/MET than mussels treated with SMP. Values of MET/MDR decreased significantly between days 7 and 26 in LMP and SMPB mussels whereas MLR/MET increased significantly with time in SMPB (Fig. 6B,C).

3.6. Gamete development, gonad index and histopathology of mussel gonad

Percentages of gametogenic stages were similar in all the different experimental groups, both controls and treated (Fig. S1 of Supplementary Material). Mature gonad was the predominant stage in all mussels at day 7. Meanwhile at day 26 of exposure, the number of individuals with spawning gonad increased. The mean value for gonad index was around 4 for all experimental groups in both sampling days (Fig. S1 of Supplementary Material).

In general, no significant histopathological alterations were observed in mussel gonads (Fig. 5G). Fibrosis was recorded at low prevalences in mussels exposed to LMP, LMPB and SMPB at 7 days of exposure and in mussels exposed to LMPB at 26 days of exposure. Hemocytic infiltration (Fig. 5H) and oocyte atresia (Fig. 5I) were observed at low prevalences in both controls and in treated mussels at both sampling times (Table S2 of Supplementary Material). Oocyte atresia was found mostly at stage 1, with less than half the follicles being affected. A digenean trematode parasite was found in one mussel of the LMP treatment at day 26.

3.7. Whole organism responses

Mussels appeared in good health throughout the experimental period and were observed to close their valves tightly during the emersion of the daily water change and reopen their valves almost immediately following reimmersion. All physiological data met the assumptions to be analyzed by parametric tests. Two way ANOVAs revealed significant effects of treatment for all parameters tested except CR (Table 5). Effect of exposure time was significant for CR and RR, whereas significant effects of the interaction between treatment and time were found for all parameters except AE (Table 5).

For CI, post-hoc testing revealed no significant differences between mean values at day 7 (Fig. S2 of Supplementary Material). Whilst by day 26 mussels exposed to SMP increased significantly their condition index compared to mussels from day 7 and presented higher values than the rest of treatments except SMPB (Fig. S2 of Supplementary Material).

For CR, the results displayed an overall high degree of variability among treatments throughout the study which reduced from day 7 to day 26 (Fig. S3 of Supplementary Material). No significant effect of treatments on CR was noticed (Table 5). except for SMP at day 7 that was significantly higher than LMP and SMPB. Maximum value achieved with SMP treatment in the short-term response (3.77 L/h) declined significantly after 26 days (1.82 L/h) (Fig. S3 of Supplementary Material).

Similar to CR, there were no significant differences in RR compared to control values for any treatment in either sampling day, and no significant differences in control respiration rates between days (Fig. S4 of Supplementary Material). Post hoc analysis revealed that, at day 7, SMPB mussels had a significantly lower RR than the rest of groups except the control, and the RR of LMP mussels decreased significantly from day 7 to day 26 (Fig. S4 of Supplementary Material).

There were no significant differences in AE among treatments at day 7 and no significant difference between days by treatment. In contrast, at day 26, all treatments showed an increased AE as compared to the control, this increase being significant for SMP, LMPB and SMPB (Fig. 7A).

Differences in SFG among treatments reflected mainly the behavior of clearance rates: At day 7 SMP mussels exhibited significantly higher SFG values than the rest of groups, except LMPB (Fig. 7B). Whilst at day 26 all treatments had a higher SFG compared to the control, an

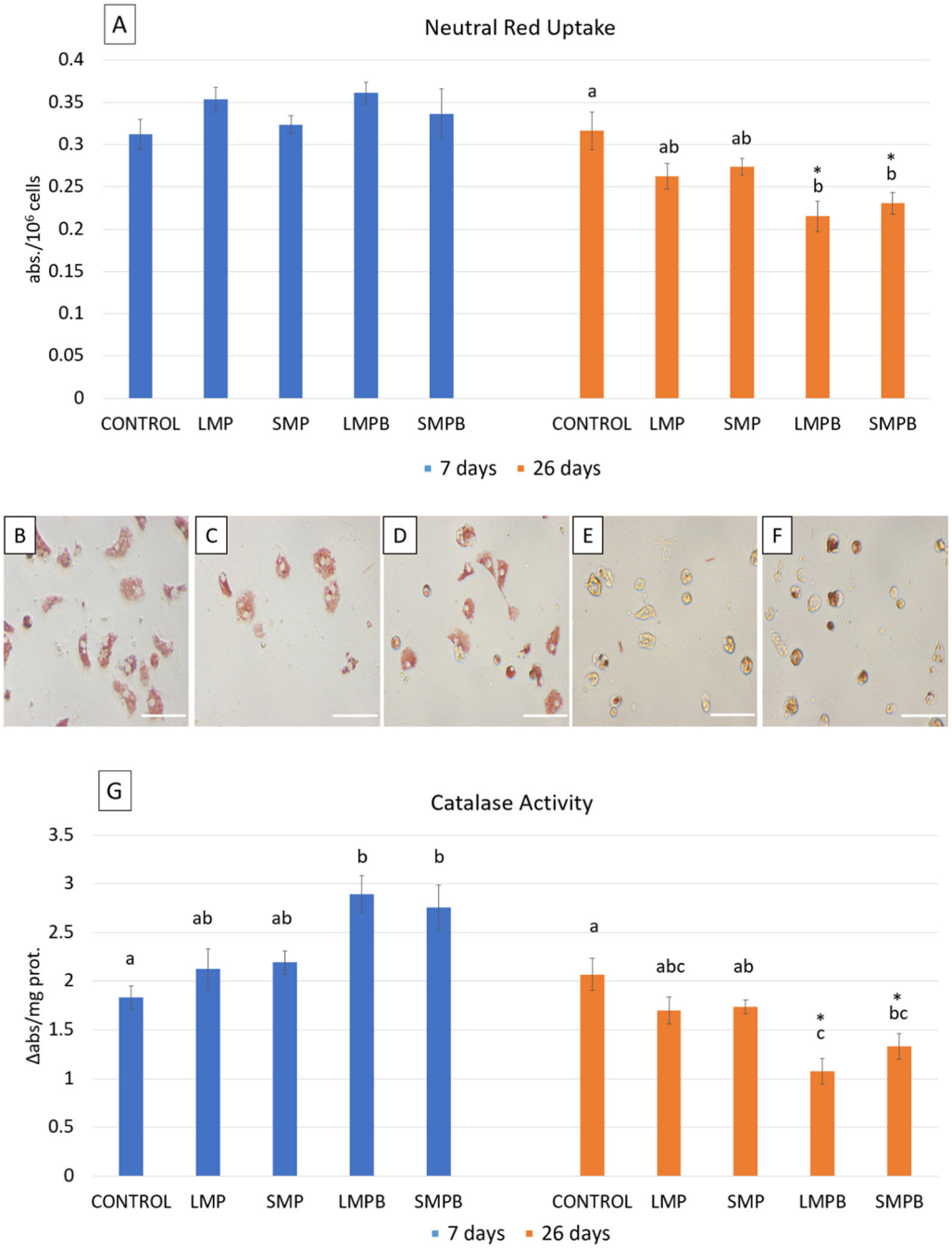

Fig. 2. A: Neutral red uptake (given as absorbance/10^6 cells) and G: catalase activity (given as absorbance difference/mg protein) in hemocytes of control mussels and in mussels dietarily exposed for 7 and 26 days to 4.5 μm MPs (LMP), 0.5 μm MPs (SMP), LMP with sorbed BaP (LMPB) and SMP with sorbed BaP (SMPB). Data are given as mean values and standard errors (8 mussels per experimental group, 4 replicates per mussel). Letters indicate significant differences among treatments within the same day (Kruskal-Wallis H test followed by Dunn's post hoc test, $p < 0.05$). Asterisks indicate a significant difference between days within the same treatment (Mann-Whitney *U* test, $p < 0.05$). B–F: Bright field micrographs of mussel hemocytes during the NR assay for hemocytes from control (B), LMP (C), SMP (D), LMPB (E), and SMPB (F) treatments at day 26. Scale bars: 50 μm.

effect which was only significant for the difference between the control and SMPB. In addition, SMP mussels displayed significantly lower SFG at day 26 compared to day 7 (Fig. 7B).

4. Discussion

4.1. Bioaccumulation of BaP in mussel soft tissues

The present study investigated the effects of long-term (26 days) dietary exposure of two different sized PS microspheres (4.5 μm and 0.5 μm) alone and with sorbed BaP in mussels in order to elucidate the influence of MP size and the presence of sorbed BaP on the organism at different levels of biological organization. Results on BaP accumulation in mussel whole tissues clearly indicate that BaP was not present in significant amounts as a background contaminant; that BaP was transferred from the plastics to the mussels, and that BaP bioaccumulated in the mussel tissue of the BaP treated groups, an effect which increased with exposure time. Furthermore, as hypothesized, notably larger amounts of BaP were transferred to the mussel tissue in the SMPB treatment as compared to the larger plastics likely due to their larger surface to volume ratio which facilitates both the adsorption to and the desorption of PAHs from the plastic surface (Koelmans, 2015). In addition, concentrations

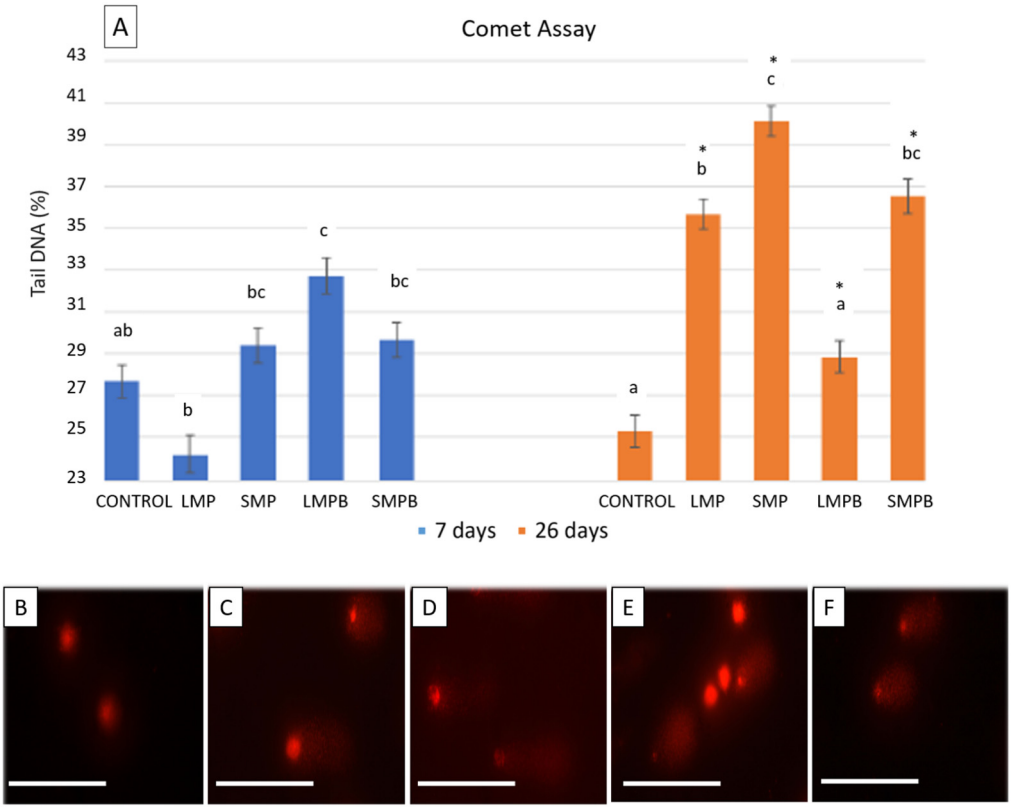

Fig. 3. A: DNA strand breaks (given as % tail DNA) in hemocytes of control mussels and in mussels dietarily exposed for 7 and 26 days to 4.5 μm MPs (LMP), 0.5 μm MPs (SMP), LMP with sorbed BaP (LMPB) and SMP with sorbed BaP (SMPB). Data are given as mean values and standard errors (8 mussels per experimental group, 50 cells in 2 slides per mussel). Letters indicate significant differences among treatments within the same day (Kruskal-Wallis H test followed by Dunn's post hoc test, $p < 0.05$). Asterisks indicate a significant difference between days within the same treatment (Mann-Whitney U test, $p < 0.05$). B–F: Comet assay fluorescence micrographs displaying typical levels of DNA strand breaks for hemocytes from control (B), LMP (C), SMP (D), LMPB (E), and SMPB (F) treatments at day 26. Scale bars: 100 μm.

of plastic particles administered were equivalent by weight, but not by number of particles, therefore the SMPB treated mussels were exposed to much higher plastic particle numbers compared to the LMPB treated mussels. Again this indicates that the SMPB treatment represented a much higher surface area for the adsorption and desorption of BaP. The fact that the differences in BaP tissue burden between LMPB and SMPB mussels remained the same between days suggests that, although small MPs transfer larger amounts of BaP to mussels than larger MPs, degree of bioaccumulation over time was not increased with decreasing plastic size.

Avio et al. (2015) investigated the transfer of the PAH pyrene via MPs to *M. galloprovincialis*. After 7 days exposure there was a 13 fold increase in tissue concentrations in exposed mussels compared to the controls. Accumulation in the present study was markedly greater with up to a 37 fold increase in BaP tissue burdens in mussels exposed to LMPB and a 66 fold increase in mussels exposed to SMPB relative to

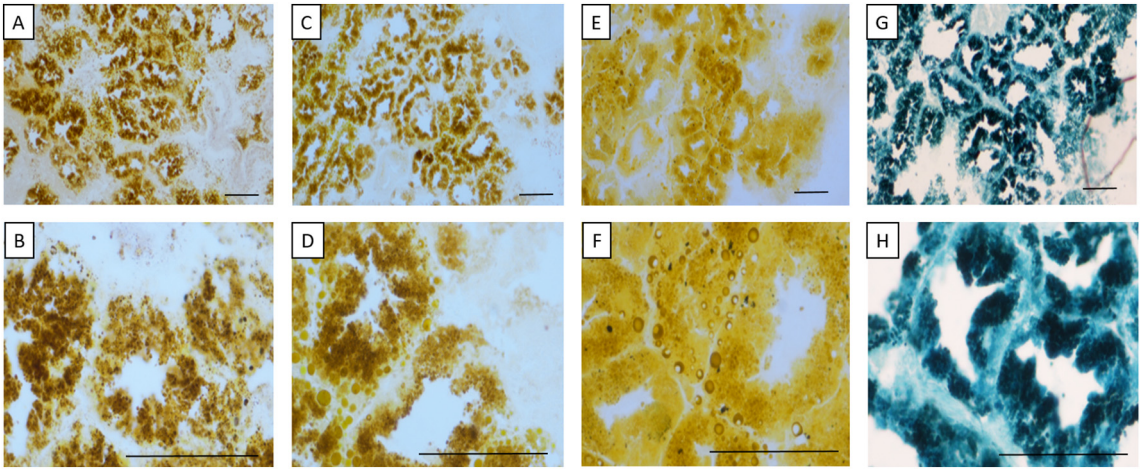

Fig. 4. Bright field micrographs of serial cryostat sections of the digestive gland of control mussels from day 7 with different stains to illustrate high degree of background staining and presence of lipofuscins: A,B) histochemical detection of lysosomal β-hexosaminidase activity; C,D) same as A, B but omitting incubation with the substrate; E,F) unstained; and G,H) Schmorl's reaction for lipofuscins. Scale bars: 100 μm.

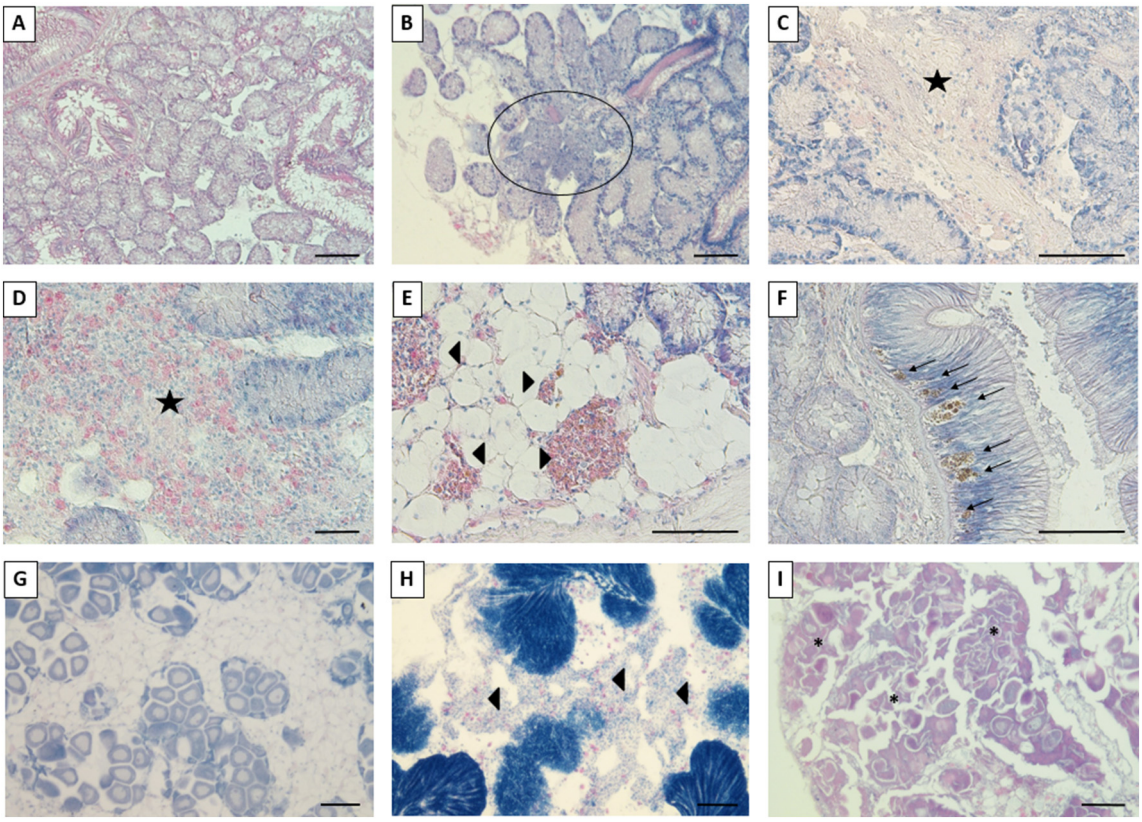

Fig. 5. Bright field micrographs of mussel paraffin sections showing histopathological alterations in mussels digestive glands and gonads: A) control mussel at day 7; B) mussel after 7 days of exposure to LMP showing a necrotic area (encircled) in digestive tubules; C) mussel after 7 days of exposure to LMP showing fibrosis in the connective tissue (star); D) mussel after 7 days of exposure to SMP showing hemocytic infiltration (star); E) mussel after 7 days of exposure to LMPB showing accumulation of brown cells in connective tissue (arrow heads); F) mussel after 26 days of exposure to SMPB showing brown cell aggregations in the epithelium of digestive tract; G) control female at day 7; H) male mussel after 7 days of exposure to SMP showing hemocytic infiltration (arrow heads); I) female mussel after 7 days of exposure to SMPB showing oocyte atresia (asterisks). Scale bars: 100 μm.

the control at day 7. Whilst at day 26 the difference in BaP tissue burdens between controls and mussels exposed to plastics with sorbed BaP were even greater with up to a 150 fold increase in SMPB vs control and a roughly 80 fold increase in LMPB vs the control. Avio et al. (2015) used heterogeneously sized (1 to 100 μm) PS or PE MPs. As such, it is likely that the majority of the particles used in their study were significantly larger than the two particle sizes used in this study which may explain the differences seen. In addition, different MP polymers and concentrations were used in the two studies. Similarly, a lower bioaccumulation than in the present work was observed by Pittura et al. (2018) after exposure of *M. galloprovincialis* to 20–25 μm LDPE MPs with sorbed BaP, equivalent to a waterborne exposure of 150 ng/L BaP for 28 days. Further, in the present study, in contrast to the majority of MP studies, mussels were fed continuously with microalgae. Okay et al. (2006) found that *Mytilus* spp. accumulated higher amounts of pyrene with increased algal feed concentration, hence this could be another reason why BaP bioaccumulation in the present study was higher than that documented previously (Avio et al., 2015; Pittura et al., 2018). Various studies have noted the increased sorption capacities and sorption/desorption rates with decreased MP size (Koelmans, 2015), however to the authors' knowledge this is the first study to demonstrate an increased transfer of an organic pollutant to tissues of a marine organism as a consequence of decreased MP size, in line with findings in freshwater organisms *Daphnia magna* (Ma et al., 2016) and *Danio rerio* (Chen et al., 2017).

Table 4
Prevalence of histopathological alterations in digestive gland of control mussels and of mussels dietarily exposed for 7 and 26 days to 4.5 μm MPs (LMP), 0.5 μm MPs (SMP), LMP with sorbed BaP (LMPB) and SMP with sorbed BaP (SMPB). Data are shown in percentages of 10 mussels per experimental group (one section per mussel). Not observed, n.o.

			Control	LMP	LMPB	SMP	SMPB
7 d	Fibrosis		n.o.	10	10	n.o.	30
	Hemocytic infiltration	Local	n.o.	n.o.	10	20	10
		Diffuse	n.o.	n.o.	n.o.	10	10
	Brown cells in connective tissue		n.o.	n.o.	10	10	n.o.
	Brown aggregates in epithelium of digestive tract		n.o.	n.o.	n.o.	10	10
	Necrosis		n.o.	10	n.o.	n.o.	n.o.
	Parasites		20	50	n.o.	20	20
26 d	Fibrosis		n.o.	n.o.	10	n.o.	n.o.
	Hemocytic infiltration	Local	n.o	n.o	10	10	10
		Diffuse	n.o.	10	n.o.	n.o.	n.o.
	Brown cells in connective tissue		n.o.	10	n.o.	n.o.	10
	Brown aggregates in epithelium of digestive tract		n.o.	n.o.	n.o.	n.o.	20
	Necrosis		n.o.	10	n.o.	10	n.o.
	Parasites		20	40	20	20	10

The fact that plastics were found in mussel tissues, especially in the lumen of the gastrointestinal tract, suggests that BaP transferred from MPs to mussels via MP ingestion. This agrees with the higher accumulation of PAHs found in the digestive gland compared to gills in mussels exposed to MPs with PAHs (Paul-Pont et al., 2016; Pittura et al., 2018). But it may be that the plastics used were not the only transfer path. Many studies have shown that when MPs with sorbed PAHs are added to clean seawater the PAHs immediately begin to desorb in order to reach equilibrium concentrations with the surrounding seawater (Bakir et al., 2016; Ziccardi et al., 2016). Desorption dynamics depend on MP size, surface chemistry and texture, degree of weathering and fouling and also seawater temperature, pH and background PAH concentrations (Koelmans, 2015). Therefore, it is likely that the

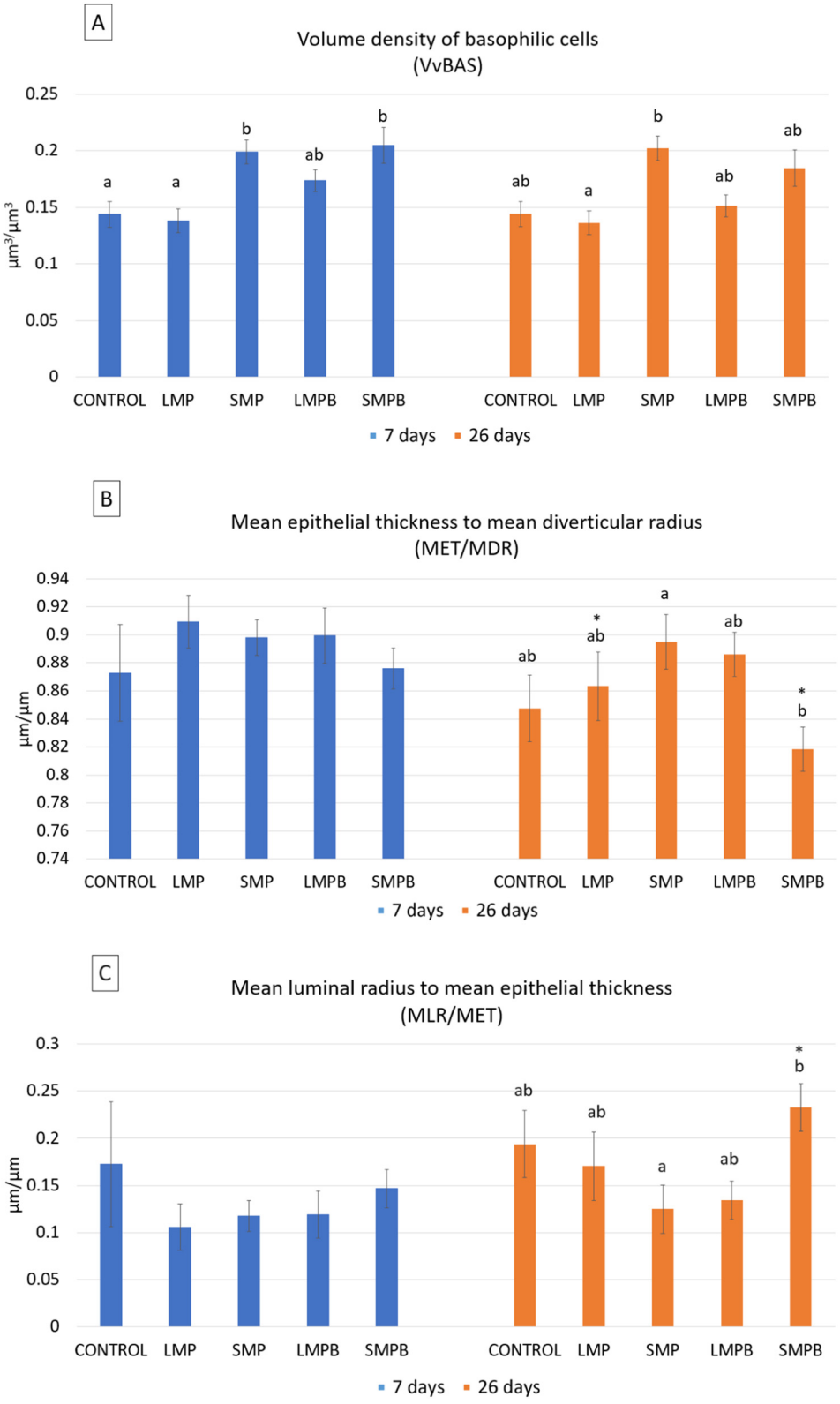

Fig. 6. A: Volume density of basophilic cells ($\mu m^3/\mu m^3$), B: Mean epithelial thickness to mean diverticular radius (MET/MDR, µm/µm) and C: mean luminal radius to mean epithelial thickness (MLR/MET, µm/µm) in control mussels and in mussels dietarily exposed for 7 and 26 days to 4.5 µm MPs (LMP), 0.5 µm MPs (SMP), LMP with sorbed BaP (LMPB) and SMP with sorbed BaP (SMPB). Data are given as mean values and standard errors of 10 mussels per experimental group. Letters indicate significant differences among treatments within the same day (Kruskal-Wallis H test followed by Dunn's post hoc test, $p < 0.05$). Asterisks indicate a significant difference between days within the same treatment (Mann-Whitney U test, $p < 0.05$).

seawater represented an additional path for the transfer of BaP to the mussels in this study. In addition, the plastics with the sorbed BaP were added to a mixture of clean algae and seawater which was gradually and continuously administered to the mussel tanks over a period of 22 h daily. Therefore, there was a long period for potential desorption of BaP from the MPs and potential sorption onto algae.

Table 5
Summary of two-way ANOVAs to analyze the effects of treatment, sampling time and their interaction on the studied parameters: Condition Index (CI), Clearance Rate (CR), Respiration Rate (RR), Absorption Efficiency (AE) and Scope for Growth (SFG). Characters in bold indicate significant differences ($p < 0.05$). df: degrees of freedom; F: Fisher's F-ratio; p: probability of F.

Parameter	Factor	df	F	Significance
CI	Treatment	4	3.06	$\mathbf{p = 0.023}$
	Day	1	3.09	$p = 0.084$
	Treatment × day	4	4.31	$\mathbf{p = 0.004}$
CR	Treatment	4	1.96	$p = 0.113$
	Day	1	9.67	$\mathbf{p = 0.003}$
	Treatment × day	4	11.67	$\mathbf{p < 0.001}$
RR	Treatment	4	4.24	$\mathbf{p = 0.004}$
	Day	1	6.81	$\mathbf{p = 0.012}$
	Treatment × day	4	4.64	$\mathbf{p = 0.003}$
AE	Treatment	4	4.90	$\mathbf{p = 0.002}$
	Day	1	0.18	$p = 0.675$
	Treatment × day	4	2.17	$p = 0.085$
SFG	Treatment	4	5.20	$\mathbf{p = 0.001}$
	Day	1	3.67	$p = 0.061$
	Treatment × day	4	7.14	$\mathbf{p < 0.001}$

I. galbana has a high lipid content (24%, Creswell, 2010) and would therefore likely have a higher affinity for BaP than seawater. This idea is supported by the results of Paul-Pont et al. (2016) who investigated the partitioning of the PAH fluoranthene in mixtures of algae and seawater, and of algae, PS microspheres (2 μm and 6 μm) and seawater. They found that when fluoranthene was added to algae and seawater without MPs, the PAH was largely associated with the algae (89%) with a fraction of only 11% remaining in water. However, when MPs were added, the fraction of dissolved fluoranthene remained similar (12%) whilst the fraction associated with the algae decreased to 67%. The remaining fraction (21%) was associated to the MPs (Paul-Pont et al., 2016). PS microspheres of 2 μm have also been observed to attach to microalgae aggregates in laboratory studies (Long et al., 2015, 2017). This factor may have therefore affected the partitioning of BaP between MPs, algae and seawater by providing a direct pathway between MPs and algae.

4.2. Tissue distribution of MPs in mussels

Tissue distribution of large 4.5 μm MPs in mussels was in agreement with previous works (Browne et al., 2008; Von Moos et al., 2012; Avio et al., 2015; Paul-Pont et al., 2016; Magni et al., 2018; Pittura et al.,

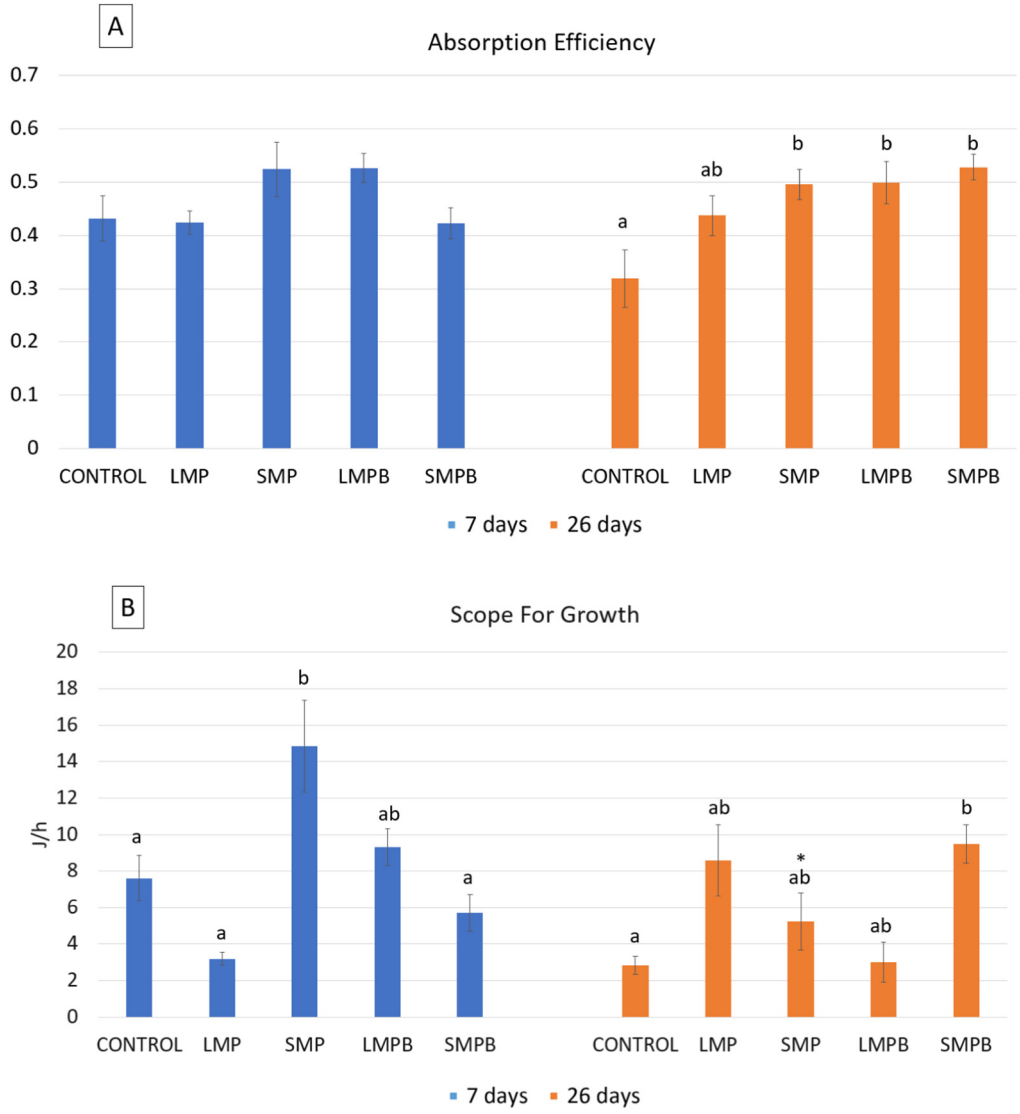

Fig. 7. A: Absorption efficiency (fraction) and B: Scope for Growth (J/h) in control mussels and in mussels dietarily exposed for 7 and 26 days to 4.5 μm MPs (LMP), 0.5 μm MPs (SMP), LMP with sorbed BaP (LMPB) and SMP with sorbed BaP (SMPB). Data are given as mean values and standard errors of 7 mussels per experimental group. Letters indicate significant differences among treatments within the same day and asterisks indicate a significant difference between days within the same treatment (2-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

2018). Thus, plastics in mussels dietarily exposed to LMP and LMPB were abundant in the lumen of stomach, mixed with stomach contents, and in the lumen of digestive tubules, associated to cell debris, although there was a marked interindividual variability that could be associated to heterogeneous distribution of MPs along the digestive tract. Occasionally large MPs were found within epithelial cells of the stomach, ducts and digestive tubules and in the connective tissue. Large MPs were also found between gill filaments, but not within epithelial cells of the gills. Overall, results reinforce the idea that MPs get in contact with mussel gill surfaces and are then transported into the mouth via ciliae movement and later to the digestive system through ingestion (Browne et al., 2008; Von Moos et al., 2012; Brate et al., 2018; Kolandhasamy et al.; 2018). Many works have already underlined the importance of MP size for MP ingestion and assimilation in mussels (Browne et al., 2008; Van Cauwenberghe et al., 2015; Kolandhasamay et al., 2018; Qu et al., 2018; Li et al., 2019) although it is not yet clear which particle size has the greatest accumulation potential in mussels and if accumulation can be polymer-type dependent (Li et al., 2019). The 4.5 μm LMPs were similar in size to microalgae provided as food and they were retained and internalized in mussel tissues. On the other hand, 0.5 μm SMPs would possibly not be retained as retention efficiency of particles in gills is close <15% for particles below 1 μm in size (Ward and Shumway, 2004). However, 0.5 μm particles may enter cells by endocytosis, as shown in mussel hemocytes (Cajaraville and Pal, 1995). Even though 0.5 μm SMPs can be hardly distinguished under the light microscope, these were occasionally observed as large aggregates in the lumen of stomach. Further transmission electron microscopy analyses are necessary to decipher mechanisms of internalization into cells and elimination from cells of mussels.

4.3. Early cellular biomarkers in mussel hemocytes

In the present work, catalase activity (CAT), neutral red (NR) uptake and DNA strand breaks were measured in hemocytes as these cells are pivotal components of the immune and xenobiotic detoxification system of mussels (Gómez-Mendikute et al., 2002) and are known to uptake MPs in a wide range of sizes (Browne et al., 2008; Pittura et al., 2018). The results for CAT in hemocytes of mussels exposed to LMPB and SMPB followed the classic bell-shaped curve (Viarengo et al., 2007), wherein CAT increased significantly compared to the control at day 7 but was significantly lower than the control at day 26. These results therefore suggest the onset of oxidative stress at day 7, followed by a decrease in catalase activity at day 26 as the antioxidant system was overwhelmed. These results were accompanied by a significant reduction in NR uptake in both treatments of MPs with sorbed BaP at day 26. A decrease in NR uptake is indicative of a decrease in cell viability (Borenfreund and Puerner, 1985; Repetto et al., 2008; Katsumiti et al., 2015). Hence, the results from the NR assay clearly indicate decreased hemocyte viability in mussels exposed to LMPB and SMPB at day 26, which can be related to the increased accumulation of BaP in mussel tissues from day 7 to day 26. The fact that effects on CAT and NR were observed in LMPB and SMPB treatments but not in either of the MP alone groups demonstrates a toxic effect of BaP transferred by the MPs on the oxidative balance and cell viability of mussel hemocytes in these treatments. BaP is known to induce oxidative stress in mussels (Akcha et al., 2000; Banni et al., 2017), and also to cause dose-dependent increases in ROS production and decreases in NR uptake in mussel hemocytes (Gómez-Mendikute et al., 2002; Gómez-Mendikute and Cajaraville, 2003). In agreement with our results, lysosomal membrane stability (LMS) was reduced in hemocytes of mussels exposed for 7 days to LDPE MPs with sorbed BaP compared to LDPE MPs alone, although not after 14 and 28 days (Pittura et al., 2018). Phagocytosis increased in hemocytes of mussels exposed for 7 days to LDPE MPs alone compared to controls but not to LDPE with sorbed BaP whereas at longer exposure times (14 and 28 days) phagocytosis decreased in hemocytes of both mussels exposed to LDPE alone and with sorbed BaP (Pittura et al., 2018). Avio et al. (2015) reported decreased LMS in mussels exposed to PS (<100 μm) alone and with sorbed pyrene. However, antioxidant enzyme activities, including CAT in digestive gland, did not change after MP exposure alone or with adsorbed pyrene (Avio et al., 2015) or BaP (Pittura et al., 2018). Meanwhile, Paul-Pont et al. (2016) found that 7 days of exposure to micro PS alone and with fluoranthene caused reduced CAT and other indications of oxidative stress in mussels. Significant effects on LMS and oxidative stress were also reported in mussels exposed to a virgin high density polyethylene (HDPE) fluff (<80 μm) (Von Moos et al., 2012). Détrée and Gallardo-Escárate (2017) also observed oxidative stress and immunological effects in mussels *M. galloprovincialis* after short exposure (24 h) to a high concentration (1.5×10^7 particle/L) of PE microbeads. Finally, there was no effect of MP size on CAT activity or NR uptake. This contrasts a report of increased antioxidant enzyme activities and intracellular ROS levels with decreasing MP size in rotifers (*Brachionus koreanus*) exposed to PS microspheres (0.05, 0.5, and 6 μm) at 20 μg/mL over 24 h (Jeong et al., 2016). These effects were accompanied by subsequent reductions in fecundity and survival time.

Background levels of DNA damage observed in the control groups were at similar levels to those found in hemocytes of mussels collected in the same region in previous studies (Katsumiti et al., 2015) and did not change between days indicating no effect of time spent in aquaria on DNA damage. Levels of DNA damage displayed a wide degree of variability at day 7 with only mussels exposed to LMPB showing higher DNA damage than control mussels. However, at day 26 differences were more apparent with all treatments except for LMPB, showing significantly higher levels of DNA strand breaks compared to the day 26 control and to their day 7 counterparts. These results therefore indicate an effect of increased genotoxicity with exposure time in SMP, LMP and SMPB treatments. In contrast to this, levels of DNA damage in mussels exposed to LMPB decreased significantly between day 7 and day 26, although DNA damage in the LMPB treatment at day 26 was still higher than in the day 26 control but not significantly. It is unclear why levels of DNA damage would reduce in the LMPB treatment with time. One possible explanation could be that mussels in this treatment were able to more effectively repair the DNA damage observed than mussels in other treatments. Indeed, BaP not only causes DNA strand breaks but also initiates DNA strand break repair pathways in mice (Tung et al., 2014), as also suggested in mussels (Bihari et al., 1990). Banni et al. (2017) found increased transcription levels of genes involved in DNA repair in mussels exposed to BaP.

At day 26 mussels exposed to LMPB and SMPB both had lower percentages of tail DNA than mussels exposed to LMP and SMP, respectively (significant for the difference between LMP and LMPB), indicating that, despite increased BaP tissue burdens with time in these groups, there was no additional toxic effect of BaP on mussel DNA as compared to MPs alone. These results could be related to the above mentioned initiation of DNA repair pathways caused by exposure to BaP (Bihari et al., 1990) leading to increased strand break repair in treatments of MPs with sorbed BaP. Avio et al. (2015) also found increased effects of MPs alone as compared to MPs with sorbed pyrene in the comet assay, however none of their results were significantly different from the control. Similarly, Pittura et al. (2018) did not find significant effects on DNA damage in mussels exposed to LDPE and LDPE with sorbed BaP for 28 days, as neither did Santana et al. (2018) after exposing *Perna perna* mussels to polyvinyl chloride (PVC) for 90 days. In contrast, Ribeiro et al. (2017) and Brandts et al. (2018) found DNA damage after exposing clams *Scrobicularia plana* for 7 days to 1 mg/L 20 μm PS MPs and mussels *M. galloprovincialis* for 96 h to 0.05, 0.5, 5 and 50 mg/L PS nanoparticles, respectively. The effects of MPs alone on DNA strand breaks could be related to their observed pro-oxidant effects in marine organisms (Von Moos et al., 2012; Browne et al., 2013; Paul-Pont et al., 2016; Détrée and Gallardo-Escárate, 2017). The genotoxicity of BaP is due to its metabolism to reactive metabolites such as BaP diol epoxide, which is known to bind covalently to DNA,

and BaP quinones which induce oxidative stress (Canova et al., 1998; Tung et al., 2014). Banni et al. (2017) found that DNA strand breaks caused by BaP exposure as measured by the comet assay exhibited a dose response pattern in *Mytilus* spp. Hence, it may be that the BaP tissue burdens in this study were not high enough to cause an increased effect on DNA strand breaks compared to the effects of the plastics alone. The comet assay results also showed that mussels exposed to both SMP and SMPB displayed significantly higher levels of DNA damage compared to mussels exposed to LMP and LMPB, respectively, indicating an effect of MP size on genotoxicity with smaller plastics causing increased levels of DNA damage. To the authors knowledge this effect of size of MPs on DNA damage has not been demonstrated previously and is probably related to the higher particle numbers in the treatments with 0.5 μm MPs and to the increased ability of smaller particles to interact with mussel tissues, translocate and even enter into mussel cells compared to larger particles (Browne et al., 2008), as discussed above.

4.4. Cell and tissue biomarkers in mussel digestive gland and gonad

Background staining in mussel digestive gland cryosections stained for LMS was too high to determine the lysosomal membrane labilization period. The staining seen corresponded largely to partially digested algae but also to the presence of lipofuscins, as evidenced by Schmorl's reaction. In addition, the possibility of using lipofuscin accumulation as a biomarker for oxidative stress was dismissed as lipofuscin levels in the digestive gland of control mussels were too high to clearly distinguish differences in lipofuscin accumulation between treatments. The presence of the high background staining was due to the fact that the mussels were continually fed in this study whereas usually mussels are starved or fed intermittently in studies investigating LMS in the digestive gland. The same result was found by Izagirre (2007) who compared the effects of different dietary regimes on ability to measure LMS and Blanco-Rayón et al. (2019) reported increased levels of lipofuscins in continuously fed mussels compared to starved ones. The general appearance of the digestive gland in paraffin histological sections of both control and treated mussels also indicated a high digestive activity. In many digestive tubules epithelial cells were relatively high and the lumen was obliterated. In other tubules, the digestive epithelium was thin and the large lumen appeared full of cell debris (including entire digestive cells). Changes in the cell composition of the digestive epithelium and in the structure of digestive tubules can be measured in terms of volume density of basophilic cells (VvBAS) and mean epithelial thickness to mean diverticular radius (MET/MDR) and mean luminal radius to MET (MLR/MET), respectively (Bignell et al., 2012; De los Ríos et al., 2013). These parameters are commonly used biomarkers in ecosystem health assessment, as they significantly reflect changes in the general health status of mussels (Marigómez et al., 2006; Zorita et al., 2006; Garmendia et al., 2011; De los Ríos et al., 2013). The digestive tubules are lined by a single and dynamic epithelium, comprised of two cell types: digestive and basophilic cells. Under normal physiological conditions, basophilic cells are less abundant than digestive cells in digestive tubules, but this is reversed under stress conditions by basophilic cell hypertrophy and digestive cell loss (Zaldibar et al., 2007). In the present work, at day 7 of exposure values of VvBAS in mussels exposed to SMP and SMPB (0.19 ± 0.01 $\mu m^3/\mu m^3$ and 0.2 ± 0.01 $\mu m^3/\mu m^3$, respectively) were significantly higher than in control mussels and in mussels exposed to LMP. These values indicate a high stress level according to the thresholds reported by Bignell et al. (2012) and have been observed in mussels after the Prestige oil spill (Cajaraville et al., 2006; Marigómez et al., 2006; Garmendia et al., 2011). At day 26 of exposure there were less differences among exposure groups but mussels exposed to SMP still showed higher VvBAS values than those exposed to LMP, clearly demonstrating a significant influence of MP size on the cell composition of digestive tubules, as observed for DNA damage. Additional effects of BaP compared to the plastics alone or effects of exposure time were not observed in VvBAS, also in line with results of DNA damage. These results can be explained taking into account that alterations in the cell composition of digestive tubules is a fast inducible and reversible response (Soto et al., 2002; Zaldibar et al., 2007).

In contrast, the parameters indicative of the structure of digestive tubules evidenced additional effects of BaP compared to the plastics alone and effects of exposure time, but no effect of plastic size. Thus, differences in MET/MDR and MLR/MET among treatments were recorded only at day 26 of exposure, mussels exposed to SMPB showing significantly lower MET/MDR and higher MLR/MET compared to mussels exposed to SMP. These data indicate that mussels treated with small MPs with sorbed BaP showed signs of thinning of digestive epithelium and atrophy of digestive tubules, even though values of MLR/MET were below the threshold (0.7 μm/μm) suggested as hallmark of this histopathological condition (Bignell et al., 2012). In general, values of MLR/MET were low for all mussels in the present work, possibly due to the high digestion activity seen in cryostat and paraffin sections, related to the continuous feeding regime during the experiment. Digestive tubule epithelial thinning has been widely associated to exposure of mussels to hydrocarbons (Cajaraville et al., 1992; Garmendia et al., 2011) and may lead to a reduced ability to digest food material (Zorita et al., 2006). Digestive tubules with necrotic areas were observed in few mussels exposed to small and large MPs alone (SMP, LMP) but prevalences were too low to reach a consistent conclusion about the influence of MPs on this histopathological alteration. Similarly, inflammatory responses such as fibrosis, hemocytic infiltration and accumulation of brown cells in the connective tissue and in the epithelium of the digestive tract were observed at low prevalences in mussels exposed to both sizes of MPs alone or with sorbed BaP. Due to the pivotal role of hemocytes in internal defense, hemocytic infiltration has been interpreted as a repair process following tissue damage (Des Voigne and Sparks, 1968; Gosling, 2015). In agreement with our results, a general increased presence of hemocytes has been reported in different organs of mussels exposed to MPs (Brate et al., 2018). Formation of granulocytomas has also been observed after exposure of mussels to MPs (Von Moos et al., 2012). Paul-Pont et al. (2016) found increased hemocytic infiltration in stomach and digestive gland of mussels co-exposed to PS MPs and fluoranthene in comparison to mussels exposed to PS alone. In contrast, Pittura et al. (2018) did not observe any histological alterations in mussels exposed to LDPE MPs alone or with sorbed BaP. Clearly further work is necessary to confirm the slight increase in prevalence of inflammatory reactions observed in the present work in mussels exposed to PS MPs alone and with sorbed BaP. On the other hand, in the present work no alterations were observed in gametogenic development or in gonad histopathology of mussels exposed to MPs of both sizes alone or with sorbed BaP in comparison to control mussels. In oysters after 2 months of exposure to MPs, a reduction in oocyte numbers, oocyte diameter and sperm mobility was observed (Sussarellu et al., 2016). Moreover, parental exposure had detrimental effects on offspring with lower rates of D-larval yield and larval development (Sussarellu et al., 2016), indicating again the need for further work on ecologically relevant endpoints such as reproduction and development of mussels and other marine organisms.

4.5. Whole organism responses

In the present work, effects on condition index (CI) as well as on feeding (CR), food absorption efficiency (AE), metabolism (RR) and their combination into an energy budget in the form of scope for growth (SFG) were measured as ecologically relevant whole organism responses. The results for CI suggest that there was no impact of time spent in aquaria on mussel condition. The difference seen between days for mussels exposed to SMP are likely due to differing CIs among mussels at the beginning of the experiment rather than a change caused due to the effects of differing feeding conditions or the effects of the plastics themselves. This explanation is supported by the results from

the physiological variables. In agreement with our results, Santana et al. (2018) and Ribeiro et al. (2017) did not observe changes in the CI of mussels *Perna perna* after long (90 days) exposure to 0.1–1 μm PVC particles and clams *Scrobicularia plana* exposed for 14 days to 1 mg/L 20 μm PS MPs, respectively. In contrast, long term (8 months) dietary exposure to environmentally relevant concentrations of polypropylene rope caused a reduction in condition and feeding activity in the Norwegian lobster *Nephrops norvegicus* (Welden and Cowie, 2016). Differences in polymer and type of material tested and exposure time as well as species differences in feeding strategy may account for the different results.

There was no difference in CR or RR for the control mussels between days and CR and RR did not differ significantly from the control for any treatment across both sampling days indicating that there was no effect of any treatment or of exposure time on CR or RR. It is possible that MP and BaP concentrations despite having an effect on cell and tissue level responses were not high enough to cause an effect on mussel feeding and metabolism. Van Cauwenberghe et al. (2015) observed a 25% increase in RR in *M. edulis* following a 14 day exposure to 10, 30 and 90 μm PS microspheres at 110 particles/mL. This increase in RR was interpreted as a stress response as animals have to utilize a greater amount of energy in order to maintain homeostasis (Van Cauwenberghe et al., 2015). Alternatively other studies have shown depressed metabolic activity in response to MP exposure, that could occur in order to preserve energy due to reductions in available nutrients caused by alterations in digestive efficiency resulting from gut MP accumulation (Welden and Cowie, 2016). Concerning the lack of response of CR to MP exposure, it should be noted that feeding behavior of mussels is strongly affected by relatively minor changes in the inorganic (not assimilable) fraction of the seston (Navarro et al., 1996). This could account for the contrasting effects that have been attributed to suspended MPs as regards to feeding rates in bivalves, ranging from stimulatory (Sussarellu et al., 2016) to inhibitory effects (Wegner et al., 2012; Rist et al., 2016; Xu et al., 2017) depending on factors such as concentration and size of particles. Responses can also vary depending on the bivalve species studied. Thus, oysters (*O. edulis*) and mussels (*M. edulis*) exposed to 25 μg/L polylactic acid or HDPE MPs presented just the opposite response in relation to filtration rate; whilst mussels tend to reduce the filtration rate oysters response was to increase it (Green et al., 2017). On the other hand, in mussels *P. perna* exposed for 90 days to PVC MPs no differences in CR were observed between exposed and control mussels. Further, Rist et al. (2016) found no additional effect of exposure to sorbed fluoranthene as compared to PVC MPs alone on CR or RR in *P. viridis* after 44 days exposure.

In contrast to the results for CR and RR significant results were found in AE. Values of this parameter were not significantly different among treatments in mussels sampled at day 7 but increased significantly with respect to the control for most treatments (namely, SMP, LMPB and SMPB) in those sampled at day 26. The pattern shown is in accordance with the hypothesized order of impact of the treatments i.e. smaller MPs causing more effects than larger MPs and MPs with sorbed BaP causing more effects than MPs alone. Sussarellu et al. (2016) also found an increase in microalgal consumption and in AE in oysters (*Crassostrea gigas*) exposed to virgin 2 and 6 μm PS microspheres over 2 months and Paul-Pont et al. (2016) observed the induction of glycolysis and digestive activity in *Mytilus* spp. following a 7 day exposure to the same PS microspheres. These results were interpreted as a possible compensatory mechanism to cope with the increased energy requirements of stressed conditions. In line with this interpretation, the increased AE observed in this work in mussels exposed for 26 days to small MPs and to both sizes of MPs with sorbed BaP may constitute a mechanism to increase energy intake in response to damage observed at cell level (e.g., oxidative stress, cytotoxicity and DNA damage in hemocytes) and tissue level (structure of digestive tubules).

The integrative parameter SFG largely followed the pattern of results for CR. At day 7, mussels exposed to SMP showed significantly higher SFG than the rest of experimental groups except LMPB whereas at day 26 the only significant difference observed was that mussels exposed to SMPB showed significantly higher SFG than the day 26 control mussels. These results again support the idea of a general compensatory effect of MPs, regardless of size of MPs or presence of BaP, in line with results of Sussarellu et al. (2016). In contrast, Xu et al. (2017) found that a significant reduction in CR but no change in AE or respiration led to a reduced energy budget in clams *Atactodea striata* exposed to PS (63–250 μm) at the same concentration used in this study for the large MPs (1000 particles/mL). The differences compared to the results found in this study could be related to the greater size of MPs used. In crustaceans, reductions in feeding activity leading to reductions in SFG and body mass have been found in *Carcinus maenas* and *N. norvegicus* respectively following chronic dietary exposure to MP fibres (Watts et al., 2015; Welden and Cowie, 2016). Similar reductions in energy budget have also been seen in lugworms following chronic exposure to MPs with reduced feeding and weight loss correlated with increased MP concentration (Besseling et al., 2013; Wright et al., 2013). However, no increased effects of sorbed contaminants have yet been observed on energy budget in comparison to MPs alone (Besseling et al., 2013; Browne et al., 2013; Rist et al., 2016). Further studies are therefore required to better elucidate the effects of MP co-contaminants in relation to MPs alone on integrative indices of physiological condition in an array of marine organisms with different feeding and digestion strategies.

5. Conclusions

This study demonstrated that BaP transferred from MPs to mussels and bioaccumulated in mussel tissues with increased exposure time, and that smaller MPs (0.5 μm) posed an increased hazard in terms of the transfer of BaP to tissues than larger MPs (4.5 μm). Effects of MPs and MPs with sorbed BaP were observed at environmentally relevant concentrations on a range of cellular and tissue level biomarkers and whole organism responses. Effects increased with exposure time or only developed following longer term exposure in the majority of responses studied, highlighting the importance of long term chronic exposure studies in the investigation of the effects of MPs and their co-contaminants. Increased effects of sorbed BaP compared to MPs alone were demonstrated on cell viability and catalase activity of hemocytes and on the structure of digestive tubules in the digestive gland but there was no additional effect of sorbed BaP on DNA damage in hemocytes, despite its genotoxic potential. Increased effects of smaller MPs were observed on DNA damage and on cell composition of digestive tubules, indicating the need of testing a variety of MP sizes in studies investigating MP potential toxicity. At a whole organism level, a hormetic effect was demonstrated on SFG. This appeared to represent a compensatory response, whereby exposed mussels increased their food absorption efficiency in order to increase energy intake to make up for energy expended dealing with stress observed in cell and tissue biomarkers. Thus, the present work evidenced a link between MP effects at different levels of biological organization. Further work is still required under realistic scenarios on the effects of a variety of plastic type, size, shape combinations together with a wide variety of pollutants in order to understand the hazards posed by MPs and their relevance as carriers of other pollutants in the marine environment.

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Declaration of Competing Interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.05.161.

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