Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation

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**A B S T R A C T**

The effects of polystyrene microbeads (micro-PS; mix of 2 and 6 μm; final concentration: 32 μg L⁻¹) alone or in combination with fluoranthene (30 μg L⁻¹) on marine mussels *Mytilus* spp. were investigated after 7 days of exposure and 7 days of depuration under controlled laboratory conditions. Overall, fluoranthene was mostly associated to algae *Chaetoceros muelleri* (partition coefficient Log Kp = 4.8) used as a food source for mussels during the experiment. When micro-PS were added in the system, a fraction of FLU transferred from the algae to the microbeads as suggested by the higher partition coefficient of micro-PS (Log Kp = 6.6), which confirmed a high affinity of fluoranthene for polystyrene microparticles. However, this did not lead to a modification of fluoranthene bioaccumulation in exposed individuals, suggesting that micro-PS had a minor role in transferring fluoranthene to mussels tissues in comparison with waterborne and foodborne exposures. After depuration, a higher fluoranthene concentration was detected in mussels exposed to micro-PS and fluoranthene, as compared to mussels exposed to fluoranthene alone. This may be related to direct effect of micro-PS on detoxification mechanisms, as suggested by a down regulation of a P-glycoprotein involved in pollutant excretion, but other factors such as an impairment of the filtration activity or presence of remaining beads in the gut cannot be excluded. Micro-PS alone led to an increase in hemocyte mortality and triggered substantial modulation of cellular oxidative balance: increase in reactive oxygen species production in hemocytes and enhancement of anti-oxidant and glutathione-related enzymes in mussel tissues. Highest histopathological damages and levels of anti-oxidant markers were observed in mussels exposed to micro-PS together with fluoranthene. Overall these results suggest that under the experimental conditions of our study micro-PS led to direct toxic effects at tissue, cellular and molecular levels, and modulated fluoranthene kinetics and toxicity in marine mussels.

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1. Introduction

Pollution of the oceans by microplastics, defined as plastic particles of size below <5 mm (NOAA, 2008), originate from the accidental release of primary manufactured plastic particles of micrometric size used in many industrial and household activities (blasting, exfoliates, toothpastes, synthetic clothing), as well as from the fragmentation of larger plastics in the environment (Andrady, 2011). Quantitative studies on micro-debris in open oceans and in intertidal zones in the vicinity of industrial cities
have confirmed the ubiquitous nature of microplastics (Eriksen et al., 2014). According to these authors, microplastics represent more than 92% of the total plastic debris (>0.33 mm) floating at sea, estimated at 5.25 trillion particles in worldwide marine environments. Ingestion of microplastic by marine organisms leading to substantial impacts on major physiological functions such as respiration, nutrition, reproduction, growth and survival has been shown in marine vertebrates and invertebrates (for review see Wright et al., 2013). In addition to physical injuries, the ability of microplastics to efficiently adsorb persistent organic pollutants (POP) has led to an increasing concern related to a potential role of microplastics as vector of POP into marine organisms (Cole et al., 2011; Ivar do Sul and Costa, 2014; Koelmans et al., 2014). Desorption of POP from microplastics was demonstrated to be enhanced under in vitro simulated digestive conditions (Bakir et al., 2014). In vivo experiments conducted on fish (Oliveria et al., 2013; Rochman et al., 2013), mussels (Avio et al., 2015) and lugworms (Besseling et al., 2013) revealed the transfer of chemicals after ingestion of contaminated microplastics, as well as combined effects of both contaminants on neurotransmission, energy production and oxidative metabolism. However, recent studies questioned the importance of such transfer in natural conditions given (i) the baseline contamination levels of seawater and marine organisms and (ii) the low proportion of microplastics in comparison with other suspended particles (organic matter, plankton, detritus, etc.) capable of transferring pollutants probably more efficiently due to their higher abundance in marine ecosystems (Herzke et al., 2016; Koelmans et al., 2016). Therefore, laboratory studies aiming to understand the relative sorption of POP to microplastics in comparison to other occurring media in marine ecosystems are needed to clarify their respective role as vector of organic pollutant for marine organisms.

The present study aims to investigate experimentally (i) the affinity of fluoranthene (FLU) for polystyrene microparticles (micro-PS) in comparison to phytoplankton by assessing its partition among seawater, micro-PS, and marine algae Chaetoceros muelleri used as a food source for mussels; (ii) whether the presence of loaded micro-PS alongside with contaminated algae and seawater may affect FLU bioaccumulation and depuration in marine mussels Mytilus spp., a common biological model in natural environments (Ward and Shumway, 2004); (iii) if the toxicity of FLU is modified by the food composition (micro-PS or green fluorescent polystyrene beads (micro-PS) (Polysciences)) and organic compounds from the micro-PS used as a food source for mussels in natural environments (Ward and Shumway, 2004); (iv) whether the stock solution of FLU set at 30 µg L⁻¹ increased toxicity of FLU in the mussel tank; (v) the detection limit of the technique (Sussarellu et al., 2016). The stock solution of FLU (98% purity, Sigma Aldrich) was prepared in acetone at a concentration of 1 g L⁻¹ before being added to the algal culture (acetone final concentration in the algal culture flask <0.04%v/v and in the mussel tank <0.003% v/v). Micro-PS were added to the algal culture with a light non-ionic detergent (Tween 20—final concentration in the algal culture flask of 0.0001% v/v leading to a final concentration in the mussel tank below 0.00001% v/v) in order to minimize micro-PS clumping and sticking to the flask walls. Acetone and Tween 20 were consistently added at the same concentrations to all algal cultures (supplying control, FLU, micro-PS and micro-PS + FLU tanks) in order to prevent confounding effects due to solvents and detergents. Direct impacts of acetone, Tween, FLU and micro-PS were evaluated on algae over a 24 h period prior to the experiment. No significant effects were observed on biochemical composition, concentration and viability of algae (data not shown). The final concentrations of acetone (0.003%) and Tween-20 (0.00001%) in the experimental tanks were much lower than the toxic levels reported for marine invertebrates (Rodrigues et al., 2013; Ostroumourov, 2003; Sussarellu et al., 2016).

Once micro-PS and FLU were added to the algal cultures, the

2. Material and methods

2.1. Mussel collection and acclimatization

Mussels (58.6 ± 9.6 mm, mean ± SD) were collected at the Pointe d’Armorique in the Bay of Brest (48°19’20.29”N, Brittany, France), a site known to exhibit low PAH concentrations (Lacroix et al., 2015). The sampling site is located within a zone of overlap between Mytilus edulis and Mytilus galloprovincialis (Bierne et al., 2003), the mussel population is thus considered as a “species complex” (Lacroix et al., 2014a), and is referred to as Mytilus spp. Mussels were acclimatized in a flow-through aerated 100 L-tank supplied with natural filtered seawater (20, 10, 5 and 1 µm mesh size; active carbon filter) for 6 weeks. Mussels were fed daily with diatoms (Chaetoceros muelleri) using peristaltic pumps at a ratio of 3% w/w organic matter per gram of mussel tissue (dry weight, dw) during the acclimation phase in order to maintain bivalves in healthy conditions. The average dry weight was 0.65 g per individual.

2.2. Mussel exposure

After the acclimation, mussels were transferred to 30 L tanks (24 mussels per tank) filled with filtered seawater maintained at 17.2 ± 1.3 °C (mean ± SD). Four experimental conditions were set up in triplicates: control, FLU (fluoranthene), micro-PS (polystyrene microbeads) and micro-PS + FLU (fluoranthene and polystyrene microbeads). Control mussels were not exposed to any stressor (micro-PS or fluoranthene) and were fed daily with fresh C. muelleri culture. Exposed mussels were subjected to daily doses of fluoranthene (FLU) set at 30 µg L⁻¹ day⁻¹ and/or monodisperse yellow-green fluorescent polystyrene beads (micro-PS) (Polysciences) supplied together with the daily prepared algal culture (C. muelleri) for a period of 7 days. Micro-PS of different sizes were used in order to reflect the spectra of food particles ingested by mussels in natural environments (Ward and Shumway, 2004): 2 µm (1800 microbeads ml⁻¹ day⁻¹) and 6 µm (200 microbeads ml⁻¹ day⁻¹) beads, obtaining a final concentration of 2000 microbeads ml⁻¹ day⁻¹. This corresponded to a mass concentration of 32 µg PS L⁻¹ day⁻¹. The leaching of chemicals (styrene, additive and fluorochrome) and organic compounds from the micro-PS used in this study was tested; plastics did not release compounds at significant levels above 0.1 ng L⁻¹, the detection limit of the technique (Sussarellu et al., 2016).

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daily prepared mixtures were gently stirred for 45 min before being supplied to the tanks containing the mussels using peristaltic pumps (flow rate: 6.5 mL min⁻¹; feeding duration: 5 h). The food ratio for control and exposed mussels was 1.5% organic matter per gram of mussel tissue per day, corresponding to 2 10⁵ cells mL⁻¹ per tank per day. As the average mass per cell is 45.8 pg cell⁻¹ for C. muelleri (Robert et al., 2004), the quantity of algae added per tank per day was 9.16 mg L⁻¹. The relative proportions of PS/FLU/algae was around 1/1/289 given the concentration of each component (micro-PS = 32 μg/L; FLU = 30 μg/L; Algae = 9160 μg L⁻¹). In order to ascertain that the mussels efficiently ingested algae and were not overfed, water samples were daily collected at the end of the feeding period to assess the concentration of algae remaining in the mussels tanks by flow cytometry. No algae were detected in water or at the bottom of the tanks, suggesting adequate food consumption. Preliminary experiments were performed to ensure that sorption of fluoranthene inside the peristaltic tubes remained negligible and that no extra polymer particles were produced by the tubing wear.

During the 7 days of exposure, mortality monitoring and water renewal were performed daily prior to the addition of food (with and without contaminants). At the end of the exposure period, mussels were anaesthetized (i.e. the shells were carefully brushed and rinsed to avoid any transfer of micro-PS or FLU) and transferred to clean 30 L tanks for 7 days of depuration with similar seawater and food conditions as those used during the exposure phase.  

2.3. Assessment of fluoranthene partition in algal cultures

The partition of fluoranthene among seawater, marine algae and micro-PS was assessed after 45 min of contact (time for which the algae cultures started to be supplied to the tanks containing the mussels) and also after 5 h of contact (time for which the food supply stopped) in the “FLU” and “micro-PS + FLU” algal cultures. For each time point, 10 mL of each algal culture were sampled and centrifuged at 2000 rpm for 10 min at 4 °C to pellet the algae. The 6 μm beads exhibited similar size and density than C. muelleri cells; therefore it was impossible to discriminate them from the algal cells using classical centrifugation or filtration methods. As a consequence, >90% of the 6 μm beads were pelleted with the algae. Due to their lower size and density, the 2 μm beads remained in suspension. Microscopical examinations were backed up by flow cytometry analyses to confirm that >95% of the 2 μm micro-PS remained in the supernatant, and >98% of the algae alongside with >90% of the 6 μm beads were pelleted after centrifugation. The supernatant was filtered on a fiberglass filter (Whatman, 0.7 μm mesh) to retain the 2 μm micro-PS. The filter and the filtrate were separately kept to assess the quantity of fluoranthene associated with the 2 μm microbeads (F2) and dissolved in seawater (Fd), respectively. The pellet (containing algae and the 6 μm micro-PS) was re-suspended in 4 mL ethanol absolute (molecular grade) and this was used to measure the quantity of fluoranthene associated with the pellet (Fp). The fractions of FLU dissolved in water (Fd) and associated with the 2 μm micro-PS (F2) and with the pellet (Fd) were directly measured using a Stir Bar Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry (SBSE-TD-GC-MS) method described in Lacroix et al. (2014b). FLU was quantified relatively to [2H10]-FLU using a calibration curve ranging from 1 ng to 10 μg per bar. The limit of quantification (LOQ) was 0.2 μg g⁻¹ wet weight (WW). Results of fluoranthene content in gills and digestive gland were expressed as μg FLU g⁻¹ wet tissue weight (WW).

2.5. Fluoranthene quantification

FLU was quantified in digestive gland using a Stir Bar Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry (SBSE-TD-GC-MS) method described in Lacroix et al. (2014b). FLU was quantified relatively to [2H10]-FLU using a calibration curve ranging from 1 ng to 10 μg per bar. The limit of quantification (LOQ) was 0.2 μg g⁻¹ wet weight (WW). Results of fluoranthene content in gills and digestive gland were expressed as μg FLU g⁻¹ wet tissue weight (WW).

2.6. Flow cytometric analyses

Morphological and functional analyses of collected hemocytes were performed on a BD FACsVerse flow cytometer (BD Biosciences, France). Hemocyte mortality was assessed according to Haberkorn et al. (2010) and expressed as the percentage of dead cells present in each sample. The concentration of circulating hemocytes (all hemocytes, granulocytes and hyalinocytes) was also determined. Phagocytosis activity was calculated as the percentage of hemocytes that ingested three fluorescent beads or more (=active hemocytes), while phagocytosis capacity was estimated as the average number of beads engulfed by active hemocytes (Hégarèt et al., 2003). ROS production was measured using a DCFH-DA assay as described in Lambert et al. (2003) and was expressed as the mean geometric fluorescence (in arbitrary units, A.U.).

2.7. Antioxidant enzyme activities

An aliquot of 50 mg of ground digestive gland was homogenized (1:4, w/v) in K-phosphate buffer 100 mM, pH 7.6 containing 0.15 M KCl, 1 mM DTT and 1 mM EDTA in a sonicator UP 200S (0.5 cycle and 60% of amplitude with two rounds of 5 pulses). Samples were then centrifuged at 10,000 × g for 20 min (4 °C). Supernatants were used for all enzymatic assays as well as protein quantification according to Lowry et al. (1951) by using bovine serum albumin as standard. Superoxide-dismutase (SOD) was measured using SOD-Assay kit-WST and was expressed in U min⁻¹ mg protein⁻¹. Catalase (CAT) was measured according to Claiborne (1985) and associated to algae or micro-PS (2 and 6 μm) by the aqueous phase concentration (μg/L).

2.4. Mussel sampling

Mussels were sampled at the end of each phase (exposure = T7; depuration = T14). A total of 21 mussels were collected per condition: 9 mussels (3 mussels per replicate tank) were collected for histology and histopathology analyses; 12 mussels (4 mussels per replicate tank) were collected for hemolymph sampling, FLU quantification, gene expression and enzyme activity. Hemolymph was withdrawn from the adductor muscle using a 1-ml hypodermic syringe (25 G needle) before being filtered on 80 μm nylon mesh and kept on ice until flow cytometric analyses. The digestive gland and gills of the same animals were dissected in RNase-free conditions, snap-frozen in liquid nitrogen, crushed to a fine powder at −196 °C with a mixer mill MM400 (Retsch) and stored at −80 °C until RNA extraction, enzymatic assays and FLU quantification. For histological observations, a cross section of mussel tissues (including digestive gland, gills, mantle and gonad) was fixed in modified Davidson’s solution (Latendresse & al., 2002) for 48 h and further processed as described in Fabioux et al. (2005). Microscopic observations were performed in order to localize the micro-PS in tissues. A five-level semi-quantitative scale was established to assess the intensity of histopathological conditions.

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expressed as μmol of H₂O₂ consumed min⁻¹ mg⁻¹ protein. Glutathione reductase (GR) activity was measured according to Ramos-Martinez et al. (1983) and expressed as nmol of NADPH oxidized min⁻¹ mg⁻¹ protein. Glutathione S-transferase (GST) was measured according to Habig et al. (1974) and was expressed as nmol min⁻¹ mg protein⁻¹. Lipid peroxidation (LPO) was quantified following Buege and Aust (1978) and expressed as nmol MDA mg protein⁻¹.

3. Gene expression analyses

3.1. Total RNA extraction and cDNA synthesis

An aliquot of 50 mg of grounded tissue was homogenized in 0.5 ml of Tri Reagent (Ambion) using a Precellys® 24 grinder coupled to a Cryolys® cooling system (Bertin technologies) for total RNA extraction. An aliquot of 40 μg RNA was then treated with the RTS DNase™ Kit (1U/3 μg total RNA, MoBio). RNA purity and concentration were measured using a Nanodrop spectrophotometer (Thermo Scientific) and RNA integrity was assessed using RNA nanochips and Agilent RNA 6000 nanoreagents (Agilent Technologies). RNA Integrity Numbers (RIN) were 8.3 ± 1.0 and 6.6 ± 0.6 (mean ± standard deviation) for gills and digestive gland samples, respectively. 2.5 μg RNA were reverse-transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas).

Gene expression analyses were carried out using random hexamers. A cDNA sample was reverse-transcribed with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). A positive control (reference cDNA sample) and negative (MilliQ water) controls were included in each PCR run. Primers were designed for each target gene, and melting temperature (Tm) are listed in Table 1. Assays were performed in triplicate according to the protocol described by Lacroix et al. (2014a) and expressed as relative quantification on the C₀ basis. The geometric mean of these three genes was therefore used as an index to normalize target gene expression. The normalization index to 1 (E) was calculated according to Pfaffl (2001): E₀ = 2⁻ΔC₀Target (reference – sample)

R = E₀Target / E₀Index

4. Statistical analyses

All quantitative variables were analyzed using a two-way ANOVA in order to determine possible interactive effects between the two independent variables called factors (microplastics and fluoranthene) on each parameter that constitutes the dependent variable (Sokal and Rohlf, 1981). Normality was assumed and homogeneity of variance was verified with Cochran’s test (data were log10 transformed when homogeneity of variance was not achieved). Percentages of phagocytic and of dead hemocytes were arcsin transformed to meet homogeneity requirements. Intensities of histopathological conditions (semi-quantitative data) were compared statistically using the Mann–Whitney U-test to assess differences attributable to the conditions (micro-PS, FLU or the combination of both) after exposure and depuration periods. All tests were performed using the STATISTICA 10 software for Windows.

5. Results

5.1. Fluoranthene partition in algal cultures

No difference was observed between 45 min and 5 h in the quantity of FLU measured in each fraction (F2, Fd and Fp) suggesting that sorption equilibrium occurred. In the FLU condition (i.e. no micro-PS added in the algal culture), fluoranthene was mainly associated with algae (89%) in comparison with the fraction of FLU dissolved in water (11%) (Table 2). Algae exhibited a Log KpA of 4.84. In micro-PS + FLU condition, the fraction of FLU dissolved in water was similar (12%) but the fraction of FLU associated to algae was reduced (67%) and a significant fraction of FLU appeared associated to the micro-PS (21%). This is reflected by a higher Log KpMPS (6.58) in comparison with Log KpA (4.77) (Table 2).

5.2. Fluoranthene quantification in mussel tissues

At T7, mussels exposed to FLU alone or micro-PS + FLU showed similar concentrations of FLU in gills (12.1 ± 0.8 μg g⁻¹ and 13.5 ± 1.1 μg g⁻¹, mean ± SE, respectively) and digestive gland (117.1 ± 10.7 μg g⁻¹ and 89.2 ± 8.4 μg g⁻¹, mean ± SE, respectively) (p > 0.05; Fig. 1). Negligible amounts of FLU (<2 μg g⁻¹) were detected in tissues of control and micro-PS exposed mussels. At T14, FLU concentration in gills was similar in mussels exposed to micro-PS alone (36.9 ± 6.7 μg g⁻¹, mean ± SE) than in mussels exposed to micro-PS + FLU (61.3 ± 4.8 μg g⁻¹, mean ± SE) (p < 0.001; Fig. 1). No FLU was detected in tissues of control and micro-PS exposed mussels.

5.3. Histology and histopathology

At T7, micro-PS were detected exclusively inside the digestive tract and the intestine of all mussels exposed to micro-PS, regardless of the exposure to FLU (Fig. 2A). At T14, some microbeads were still observed in the intestine of mussels exposed to micro-PS and micro-PS + FLU (<1–5 beads/histological section/animal). A few micro-PS were also observed stuck in the mucus on the outer side of the gills epithelium (Fig. 2B). There was no observation of micro-PS in any other tissue.

At T7, a significant increase in total histopathological lesions/abnormalities was demonstrated in mussels exposed to FLU, micro-PS and micro-PS + FLU, in comparison with controls (Fig. 3). Significantly higher hemocyte infiltration and ceroids (stress induced lipofuscin-pigments) were observed in the stomach and digestive gland of all mussels exposed to FLU in comparison to mussels exposed to micro-PS alone (Fig. 2 and 3). At T14, significantly higher histopathological lesions/abnormalities were
observed in mussels exposed to micro-PS + FLU, in comparison with all other treatments (Fig. 3). This difference was mainly driven by higher hemocyte infiltration and ceroids detected in gills, gonads, digestive glands and intestine of micro-PS + FLU exposed mussels.

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<th>Transcript name</th>
<th>Abbreviation</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Efficiency (G/DG)</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Accession number (species)</th>
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<td>GCAAAAGGCCAAACTCCTCAT</td>
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<td>249</td>
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Catalase * cat | CACAGG7GTCCTCCTCTGT | CCTCCGAGATGCCGATGTAT | 1.86/1.85       | 235              | 81.5       | AY580271 (me)/AY743716 (mg) | Lacroix et al., 2014a      |
| Cu/Zn-Superoxide Dismutase * sod         | CATTCCCAGATCACCACA | GGAACAGTCCTTTGACTCA | 1.93/1.90       | 214              | 82.2       | AFS118746 (me)/PM177867 (mg) | Lacroix et al., 2014a      |
| Se-dependant-Glutathione peroxidase * gpx | AGCGTAAAGACGCTCATC | TCTTGTCACAGGTTCCCATATG | 2.00/2.06       | 119              | 79.7       | HQ891311 (mg) | Lacroix et al., 2014a      |

Cytochrome P450-1-like-1 * cyp11 | TGGTGGCAATGTTATGGCCTGGA | GGGCAAGAACAAACTCCTCGTA | ND/1.95         | 150              | 77.5       | JX859178 (me) | Lacroix et al., 2014a      |
| Cytochrome P450-3-like-2 * cyp32 | CAGACCGCGCAAATGATA | GCTCCAGGCGAAAAGAAGG | 1.87/1.85       | 194              | 80.1       | B479539 (me) | Lacroix et al., 2014a      |

α-Glutathione-S-transferase * αgst | CGACTCTATAGCCTGAGATAT | AGAAGCGGAAACTACAAGAGG | 1.92/2.04       | 152              | 77.5       | Locus 38757 (a) | Lacroix et al., 2014a      |
| β-Glutathione-S-transferase * βgst | CACTCTTGAGGAATCCTGAGAC | CACTCTTGAGGACATCCTGGA | 2.05/1.95       | 104              | 78.4       | Locus 42054 (a) | Lacroix et al., 2014a      |
| σ-Glutathione-S-transferase * σgst | CCTGGTGCCGAGACGCTACTA | TTGGGCATCTGTCCTGTGTAT | 1.92/NA         | 131              | 78.0       | FL494070 (mg) | Lacroix et al., 2014a      |

Growth arrest and DNA damage inducible aadd45α | CACATTCCCTCAACTCTCTC | GCCGGAACAGACGTAAACAGT | 1.96/1.89       | 140              | 78.7       | AJ623737 (mg) | Lacroix et al., 2014a      |
| α-Amylase * amylase | CTTGGGGTAGCTGTTTTTA | TCCAAATGTCGGGTGCTTTT | ND/1.91         | 232              | 79.2       | EU386958 (me) | Lacroix et al., 2014a      |
| Pyruvate kinase * pk | GAGTCCTGAGGTCCTCAC | GTAGTACACAGGAGGTCCTAC | 1.83/1.88       | 228              | 81.6       | Locus 282832 (me) | Lacroix et al., 2014a      |
| Isocitrate dehydrogenase [NADP] cytoplasmic * idp | GGAGGTACTGTATTTCGTGAGGC | TGATCTCCATAAGCATTGCT | 1.93/1.97       | 104              | 76.9       | Locus 282835 (me) | Lacroix et al., 2014a      |
| Glyceraldehyde 3-phosphate dehydrogenase * gapdh | GTCGTTGTAGTGGAGAGTCC | GGCCTCCTCATTCTGATGT | 1.84/1.84       | 220              | 78.7       | FL496349 (mg) | Lacroix et al., 2014a      |
| Hexokinase * hk | CCAATATGACAATTGCCGTTGA | GGAGACCCACAGAAGCTACCATCA | 1.91/1.91       | 148              | 78.2       | JN595865 (mg) | Lacroix et al., 2014a      |
| P-53 tumor suppressor-like * p53 | CAAACATTGCCCAATCCGA | GCCGCGTGCTGATATGATCT | 1.85/1.89       | 228              | 80.1       | AFS157942 (me)/DIQ158079 (mg) | Lacroix et al., 2014a      |
| ABCB/P-glycoprotein-like protein * pgp | CACTGTTGGAGCGGCTTGA | GTGTCTCGGCTGTTGCTCT | 1.92/1.86       | 116              | 82.7       | AF397107 (me)/EF057747 (mg) | Lacroix et al., 2014a      |
| Lysozyme * lys | AGGTTTGGTCATCTTCTTG | TGACTGTCGGGACACAAAGA | 1.94/1.92       | 173              | 81.6       | AF334662 (me)/AF334665 (mg) | Lacroix et al., 2014a      |
| Caspase 3/7-3 * casp37-3 | CAATGTGTAATAAGGAGAACCTTGTG | GTTATGATGACTGGCTCTC | 1.84/1.93       | 146              | 76.5       | HQ424453 (mg) | Lacroix et al., 2014a      |
5.4. Hemocyte parameters

After seven days of exposure, the percentage of dead hemocytes increased significantly in mussels exposed to micro-PS and FLU, alone or in combination, in comparison with controls (Fig. 4A, Table 3). ROS production was significantly higher in mussels exposed to micro-PS and FLU alone, in comparison with control individuals and mussels exposed to micro-PS + FLU (Fig. 4B, Table 3). Significant interactions between both stressors were demonstrated on percentage of dead hemocytes, phagocytosis capacity and ROS production (Fig. 4, Table 3). No effect of micro-PS and FLU alone or in combination was observed on phagocytosis activity and hemocyte concentration at that time.

At the end of the depuration, the percentage of dead hemocytes remained significantly higher in mussels exposed to micro-PS and FLU, in single or in combination, in comparison with controls (Fig. 4A, Table 3). All micro-PS exposed mussels exhibited significantly lower granulocyte concentration (1.2 ± 0.2 10^5 cells mL^-1 and 1.1 ± 0.2 10^5 cells mL^-1 for mussels exposed to micro-PS and micro-PS + FLU, respectively) in comparison with controls (3.4 ± 0.5 10^5 cells mL^-1) and mussels exposed to FLU (2.7 ± 0.5 10^5 cells mL^-1) (Table 3). This decrease was also reflected in the total hemocyte count with mean cells concentrations of 5.4 ± 1.1 10^6 cells mL^-1, 4.8 ± 0.8 10^6 cells mL^-1, 2.8 ± 0.5 10^6 cells mL^-1 and 2.3 ± 0.4 10^5 cells mL^-1 in controls and mussels exposed to FLU, micro-PS and micro-PS + FLU, respectively (Table 3). All micro-PS exposed mussels demonstrated significantly higher phagocytosis capacity as compared to control individuals (Fig. 4B, Table 3). No effect of micro-PS and FLU alone or in combination was observed on phagocytosis activity, ROS production and hyalinocyte concentration at that time.

5.5. Anti-oxidant enzyme activities and lipid peroxidation

At T7 a significant effect of micro-PS exposure was demonstrated on CAT activity with a reduced activity in mussels exposed to micro-PS and micro-PS + FLU, respectively (52.6 ± 3.9 and 66.6 ± 8.7 μmol min^-1 mg^-1), in comparison with controls and mussels exposed to FLU alone (70.4 ± 7.1 μmol min^-1 mg^-1 and 82.9 ± 4.3 μmol min^-1 mg^-1, respectively) (Fig. 5, Table 4). Lipid peroxidation (LPO) was also significantly reduced in mussels exposed to micro-PS alone or in combination with FLU (0.9 ± 0.2 and 1.1 ± 0.2 TBARS mg^-1, respectively) compared to controls (1.4 ± 0.2 TBARS mg^-1) and mussels exposed to FLU alone (1.8 ± 0.1 TBARS mg^-1) (Fig. 5, Table 4). Activities of GR and SOD were significantly higher in mussels exposed to FLU (17.1 ± 1.7 nmol min^-1 mg^-1 and 61.1 ± 4.8 U min^-1 mg^-1, mean ± SE, respectively) compared to control mussels (9.6 ± 0.4 nmol min^-1 mg^-1 and 47.0 ± 2.3 U min^-1 mg^-1, respectively) (Fig. 5, Table 4). At T14, significant effects of micro-PS and FLU exposures were observed on GST and SOD, with an increase in enzyme activities in all exposed mussels. Highest GST and SOD activities (16.9 ± 1.4 nmol min^-1 mg^-1 and 50.7 ± 3.5 U min^-1 mg^-1, mean ± SE, respectively) were observed in the micro-PS + FLU condition in comparison with all other treatments (Fig. 5, Table 4). Significant effects of micro-PS were observed on LPO levels with the lowest concentration being measured in micro-PS + FLU exposed mussels (Fig. 5, Table 4). A significant interaction between micro-PS and FLU was observed on the activity of the GR: FLU exposure led to a decrease in GR activity in the absence of micro-PS (12.0 ± 0.5 nmol min^-1 mg^-1 vs. 10.7 ± 0.9 nmol min^-1 mg^-1, in control and FLU exposed mussels, respectively), while a significantly higher activity was observed in micro-PS + FLU exposed mussels (12.8 ± 0.8 nmol min^-1 mg^-1) in comparison with micro-PS exposed animals (9.7 ± 0.4 nmol min^-1 mg^-1) (Fig. 5, Table 4).

5.6. Gene expression

At T7, significant effects of micro-PS were observed in mRNA levels only in gills. Compared to controls, mRNA level of luy increased 2.2 and 1.2 folds, respectively in micro-PS and micro-PS + FLU exposed mussels; the mRNA levels of cat were 0.7 and 0.8 times lower, respectively in micro-PS and micro-PS + FLU exposed mussels (Table 5). At T14 in gills, exposure to micro-PS led to a significant increase in mRNA level for pk by 1.1 and 1.4 times in mussels exposed to micro-PS and micro-PS + FLU, respectively, compared to controls (Table 6). The mRNA level of sod increased...
significantly by 1.1, 1.2 and 1.5 times in mussels exposed to FLU, micro-PS and micro-PS + FLU, respectively, compared to controls. The mRNA levels of *gpx* and *idp* genes were significantly higher in gills of mussels exposed to FLU alone (by 2.3 and 2.4 times,

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**Fig. 2.** Histological observations in mussels after 7 days of exposure to fluoranthene and micro-PS. A: Fluorescent 6 μm and 2 μm micro-PS (arrows) in the intestine (INT), Ep: epithelium; B: Micro-PS (arrows) in gills (G); C: Cerooids (CER) in intestine (INT), Ep: epithelium; D: normal gills; E: Vacuolization in gills (G VAC); F: Alteration in intestine epithelium (AEp), normal intestine epithelium (NEp); G: Hemocyte infiltration (HI) in conjunctive tissue of digestive gland (full arrows). Hemocyte in diapedesis in intestine epithelium (empty arrows); H: Normal digestive tubules (DT); I: Hemocytes infiltration in conjunctive tissues of digestive gland.
compared to controls, with highest inductions observed in mussels exposed to micro-PS + FLU (3.2, 1.5 and 1.7 times, respectively) (Tables 5 and 6). Exposure to FLU induced a diminution of \( \sigma \) \( \text{gst} \) mRNA level by 0.7 times in comparison with controls. Overall the experiment, no effects of micro-PS or FLU or their combination were observed on cyc11, cyc32, \( \omega \) \( \text{gst} \), \( \text{gst} \), \( gadd45a \), gapdh, hk, p53, casp37-3 mRNA levels.

6. Discussion

6.1. Micro-PS exhibited high sorption capacity for fluoranthene

The present study evidenced that micro-PS exhibited a higher sorption capacity for fluoranthene than marine algae \( \text{C. muelleri} \) as indicated by the partition coefficient log \( K_p \) values, and this confirmed a strong affinity of fluoranthene for polystyrene, especially when considering the relative mass proportion of algae and micro-PS fed to the mussels (289:1) in the context of our study. Polyethylene (PE) and polyvinylchloride (PVC) also demonstrated high sorption capacity, as expressed with Log \( K_p \) values, for phenanthrene and dichlorodiphenyltrichloroethane (DDT) (Bakir et al., 2012). Similarly, polystyrene (PS) and PE microparticles exhibited high sorption capacity for pyrene (Avio et al., 2015), and polypropylene (PP) pellets immersed in Tokyo Bay also showed high adsorption coefficients for polychlorobiphenyls (PCB) and dichlorodiphenyltrichloroethane (DDE) (Mato et al., 2001). However, as some sediment and suspended particles may exhibit similar or even higher adsorption coefficient than microplastics (Mato et al., 2001; Velzeboer et al., 2014), it may be reasonable to question the respective role of each component in the contamination of marine organisms.

6.2. Micro-PS had negligible effect on fluoranthene bioaccumulation but altered its depuration in marine mussels

The similar fluoranthene bioaccumulation in all exposed mussels at T7 may be explained by the fact that all FLU fractions (on algae, on micro-PS and dissolved in water) were available for mussels. This actually shows that the Trojan horse effect of micro-PS (i.e. facilitating the uptake of organic contaminants by marine organisms) was negligible in the context of our study as compared to water and food exposures, especially given the low proportion of micro-PS relatively to microalgae. This is in agreement with a recent study that critically reviewed all available data regarding this hypothesis (field, laboratory and modelling studies) and concluded that given the low abundance of plastic particles relative to other media present in the oceans (marine phytoplankton in our case), exposure to POP via plastic is likely to be negligible compared to natural pathways (Koelmans et al., 2016).

At the end of the depuration phase, the highest FLU concentrations measured in the digestive glands of micro-PS + FLU exposed mussels may be related to (i) some loaded micro-PS remaining in mussels tissues; (ii) a time lag in the kinetics of FLU desorption/assimilation from micro-PS that were not assimilated as the micro-algae were; (iii) an indirect effect of micro-PS exposure on the general metabolism of mussels resulting in a reduction in FLU depuration. Indeed, low metabolism and activity are associated with low PAH clearance rates (Lotufo, 1998; Al-Subiai et al., 2012); and (iv) a possible direct impact of micro-PS on PAH detoxification processes, as suggested by a decrease in \( \text{P-glycoprotein} \) mRNA levels in all mussels exposed to micro-PS. Indeed, \( \text{P-glycoproteins} \) are transmembrane proteins primarily involved in the efflux of a wide range of compounds including unmodified xenobiotics and PAH (Smital et al., 2003). Impacts of polyethylene microbeads on detoxification mechanisms were previously demonstrated in...
common goby *Pomatoschistus microps* juveniles and seabass *Dicentrarchus labrax* larvae (Mazurais et al., 2015; Oliveira et al., 2013). The high fluoranthene concentration remaining in tissues of mussels exposed to micro-PS and FLU at the end of the depuration may explain the highest levels of ceroids, hemocyte infiltration and tissue lesions, known to be associated with PAH (Kim et al., 2008; Al-Subiai et al., 2012), observed in this condition.

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6.3. Micro-PS exposure affected mussels physiology

6.3.1. Modulation of digestion and energy metabolism

The induction of glycolysis and digestive activity upon micro-PS exposure may sign increased energy requirements in response to the implementation of anti-oxidant and detoxification processes (Palais et al., 2012). This mechanism would allow the animal to cope with experimental stress and maintain homeostasis, as suggested in a study conducted by Van Cauwenbergh et al. (2015) who demonstrated a 25% increase in energy consumption in mussels exposed to micro-PS for 14 days in comparison with controls. Alternatively, the increase in digestive activity could be explained by a compensatory effect on food intake and enhancement of mechanical digestion upon particles exposure, as also hypothesized in oysters (Sussarellu et al., 2016). An increase in absorption efficiency was for instance demonstrated in mussels exposed to moderate quantities of silt in relation to an improvement of the mechanical disruption in the stomach due to the presence of particles (Bayne et al., 1987). In the present study, a control condition using non-plastic inorganic particles of same size (silt, clay, silica) is lacking to discriminate whether the overall observed effects of micro-PS were due to the plastic nature of the particles or to the particles as such. This point should be addressed in further experiments.

6.3.2. Modulation of anti-oxidant defences and oxidative damages

Micro-PS exposure alone significantly modulated the cell oxidative system in our study. Such perturbations were also observed in mussels exposed to polystyrene (PS) and polyethylene (PE) alone or in combination with pyrene (Avio et al., 2015). Reactive oxygen species (ROS) production in hemocytes is naturally occurring (Galloway and Depledge, 2001) but overproduction of ROS may lead to oxidative damages (Lesser, 2006). In our study, the significant rise of ROS in hemocytes upon 7 days of micro-PS exposure seemed to have been well controlled as no anti-oxidant markers were activated and no sign of lipid peroxidation (LPO) was observed at that time. A biphasic response of the catalase involved in the neutralization of the hydrogen peroxide ($H_2O_2$) is hypothesized with a possible activation within the first days of exposure and consequently followed by a decrease in gene expression and protein activity afterwards (T7). Such compensatory effect was previously observed in eels and mussels (Regoli et al., 2011; Romeo et al., 2003). Also, implication of other enzymes involved in $H_2O_2$ neutralization such as glutathione peroxidase (GPX) cannot be yet clarified. After depuration, the SOD activity in micro-PS exposed mussels reflected the need for a greater capacity to rapidly convert $O_2$ into the less damaging hydrogen peroxide ($H_2O_2$) thus contributing to prevent host cellular oxidative damage (Jo et al., 2008). In the micro-PS + FLU exposed mussels, an efficient neutralization of the ROS is suggested by the activation of anti-oxidant and glutathione related markers and low LPO levels. Low LPO levels suggesting a suitable neutralization of ROS was previously hypothesized in the crustacean Carcinus maenas (Rodrigues et al., 2013), and the molluscs Perna viridis (Cheung et al., 2001) and Chlamys farreri (Xiu et al., 2014) exposed to FLU.

6.3.3. Modulation of hemocytes mortality and activities

Micro-PS exposure impaired major hemocyte parameters. Based on the absence of observed translocation, direct toxicity due to contact with micro-PS or leaching of chemicals were excluded to explain the high percentage of dead hemocytes in micro-PS exposed mussels. Instead, this may result from a modification of the circulating hemocyte concentration or balance in hemolymph. For instance, the recruitment of active hemocytes for incursion in tissues could explain the decrease in circulating granulocyte and total hemocyte concentrations observed in micro-PS exposed mussels, which would subsequently modify the balance of live circulating hemocytes in the hemolymph (Hégaret et al., 2007). Modulation of mussel immunity was also demonstrated through the increase in lyso mRNA levels in gills of mussels exposed to micro-PS, related either to (i) a direct effect of micro-PS, as demonstrated for a wide range of pollutants including metals, hydrocarbons, carbon nanoparticles, oestrogenic compounds (Renault, 2015), polyethylene beads (Von Moos et al., 2012) and styrene monomers (Mamaca et al., 2005); or to (ii) an increase in the digestive activity observed in the present study, as most lysozymes are known to play a dual role in bivalve immunity and digestion of microbial food particles (Allam and Raftos, 2015). Finally, the absence of clear effects of micro-PS exposure on phagocytosis is consistent with the study conducted by Browne et al. (2008). However, statistically significant interactions between both stressors suggest a potential modification of the bioavailability or toxicity of FLU by micro-PS. Indeed, intracellular distribution, and toxicity of fluoranthene carried by algae and micro-PS may have been quite different due to the nature and the assimilation or not of the carrier. Hemocytes of bivalves are not restricted to immune processes and are involved in many other physiological functions including nutrient transport and digestion, tissue and shell formation, detoxification and maintenance of homeostasis (Donaghly et al., 2009). Therefore, the impact of micro-PS on hemocyte activities should be considered in a larger context than immune responses, i.e. at the whole animal homeostasis, and longer-term studies are needed to clarify the chronic effect of microplastics exposure in real environmental conditions.

7. Conclusion

Despite a high sorption of fluoranthene on micro-PS, this did not enhance fluoranthene bioaccumulation in the specific conditions of this experiment; i.e. when mussels were also exposed to fluoranthene via water and micro-algae. Micro-PS concentration used in this study (0.032 mg L$^{-1}$) was lower than those used in most studies on marine invertebrates (range 0.8—2500 mg L$^{-1}$) (Avio et al., 2015; Besseling et al., 2013; Wegner et al., 2012; Von Moos et al., 2011; Romo et al., 2011; Wegner et al., 2012; Von Moos et al., 2011).
Fig. 5. Anti-oxidant enzyme activities (CAT, SOD, GST, GR) and lipid peroxidation (LPO) measured in digestive gland of mussels after exposure (T7) and depuration (T14). MP or micro-PS: microplastics; FLU: fluoranthene. Results are expressed as mean percentage of mortality ± standard error (SE) (n = 9).

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Table 4
Results of the two-way ANOVA performed on the enzyme activities measured in mussels exposed to microplastics (micro-PS) and fluoranthene (FLU) alone or in combination after exposure (T7) and depuration (T14). Only parameters exhibiting levels significantly modulated by micro-PS and/or FLU are presented here. P-values < 0.05 are in bold and italic character.

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<th>Experimental conditions</th>
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<th>T14 p-value</th>
<th>T7 p-value</th>
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Table 5
Relative gene expression in gills (G) and digestive glands (DG) of mussels exposed to microplastics (micro-PS) and fluoranthene (FLU) alone or in combination after exposure (T7) and depuration (T14). Only anti-oxidant genes exhibiting mRNA levels significantly modulated by micro-PS and/or FLU are presented here, alongside with the results of the two-way ANOVA (p-value). P-values < 0.05 are in bold and italic character. Arrows represent the way of induction, ▲: up-regulation; ▼: down-regulation; †: interaction.

Table 6
Relative gene expression in gills (G) and digestive glands (DG) of mussels exposed to microplastics (micro-PS) and fluoranthene (FLU) alone or in combination after exposure (T7) and depuration (T14). Only genes exhibiting mRNA levels significantly modulated by micro-PS and/or FLU are presented here, alongside with the results of the two-way ANOVA (p-value). P-values < 0.05 are in bold character highlighted in grey. Arrows represent the way of induction, ▲: up-regulation; ▼: down-regulation; †: interaction.
balance, an increase in histopathological damages, percentage of dead hemocytes and lysozyme mRNA levels, which suggested an impairment of the bivalve metabolism upon micro-PS exposure. As the toxic endpoints highlighted here were observed in specific and restrictive experimental conditions, the energetic costs to the animals (in terms of maintenance costs, immune responses, detoxification and oxidative balance regulation) must be further evaluated in the context of in situ exposure and/or experimental studies that more closely mimic complex in situ conditions, in particular by using different particulate matter and chemical mixtures representative of those found in the field.

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