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

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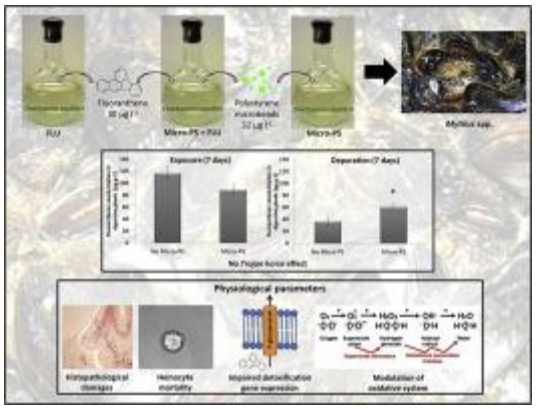
Abstract :

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.

Graphical abstract :



Highlights

► Micro-PS exhibited high sorption capacity for fluoranthene. ► Micro-PS did not modify fluoranthene bioaccumulation in marine mussels. ► Micro-PS exposure modulated oxidative and energetic processes in mussels. ► An increase in hemocyte mortality was observed in all exposed mussels. ► Combined exposure led to highest tissue alterations and anti-oxidant marker levels.

Keywords : Microplastics, Fluoranthene, Mussel, Depuration, Oxidative system

Introduction

Pollution of the oceans by microplastics, defined as plastic particles of size below < 5mm (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 (>0.33mm) 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 persistent organic pollutants (POP) from microplastics was demonstrated to be enhanced under *in vitro* simulated digestive conditions (Bakir et al., 2014). *In vivo* experiments conducted on fish (Oliveira 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

88 occurring media in marine ecosystems are needed to clarify their respective role as vector of
89 organic pollutant for marine organisms.

90 The present study aims to investigate experimentally (i) the affinity of fluoranthene (FLU) for
91 polystyrene microparticles (micro-PS) in comparison to phytoplankton by assessing its partition
92 among seawater, micro-PS, and marine algae *Chaetoceros muelleri* used as a food source for
93 mussels; (ii) whether the presence of loaded micro-PS alongside with contaminated algae and
94 seawater may affect FLU bioaccumulation and depuration in marine mussels *Mytilus* spp., a
95 common biological model in ecotoxicological studies (Kim et al., 2008); and (iii) the effects of
96 micro-PS exposure alone or in combination with FLU on various physiological parameters at
97 tissue, cellular and molecular levels to provide a comprehensive assessment of pollutant-related
98 effects (Lyons et al., 2010). Fluoranthene was selected as (i) it is a model PAH belonging to the
99 list of priority substances in water policy of the European Commission (Directive 2008/105/EC)
100 and (ii) it constitutes one of the most abundant PAH found in the aquatic environment and in
101 molluscs (Baumard et al., 1998; Bouzas et al., 2011). It is noteworthy that in most of the cited
102 studies, as well as in our work, animals were acclimatized and then reared in "clean water"
103 (seawater filtered on active carbon filters in our case) and exposures were performed in clean and
104 controlled laboratory conditions. This is far from what may happen in natural environments where
105 a wide range of confounding factors is likely to occur (and influence for instance the interaction
106 between fluoranthene and polystyrene microplastics). However, due to the high complexity
107 characterizing natural environments, controlled laboratory experiments remain necessary as a step
108 by step approach for understanding processes, to assess the weight of each factor (in this case
109 microplastics, food and fluoranthene) and sort out complexity of environmental pollution.

110 **Material and methods**

111 1. Mussel collection and acclimatization

112 Mussels (58.6 ± 9.6 mm, mean ± SD) were collected at the Pointe d'Armorique in the Bay of
113 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 *M. edulis* and *M. galloprovincialis* (Bierne, 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 100L-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. Mussel exposure

After the acclimation, mussels were transferred to 30L 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 30µg 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).

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 24h 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; Ostroumov, 2003; Sussarellu et al. 2016).

Once micro-PS and FLU were added to the algal cultures, the daily prepared mixtures were gently stirred for 45 minutes before being supplied to the tanks containing the mussels using peristaltic pumps (flow rate: 6.5 $mLmin^{-1}$; feeding duration: 5h). The food ratio for control and exposed mussels was 1.5% organic matter per gram of mussel tissue per day, corresponding to 2 10^5 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=30µg/L; Algae=9160 μgL^{-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 cleaned (*i.e.* the shells were carefully brushed and rinsed to avoid any transfer of micro-PS or FLU) and transferred to clean 30L tanks for 7 days of depuration with similar seawater and food conditions as those used during the exposure phase.

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 5h 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 was pelleted with the algae. Due to their lower size and density, the 2µm beads remained in suspension. Microscopical examinations were backed up by flow cytometry analyses to confirm that > 95% of the 2µm 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, 0.7µm mesh) to retain the 2µm micro-PS. The filter and the filtrate were separately kept to assess the quantity of fluoranthene associated with the 2µm microbeads (F2) and dissolved in seawater (Fd), respectively. The pellet (containing algae and the 6µm micro-PS) was re-suspended in 4ml 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 2µm micro-PS (F2) were directly measured using a Stir Bar Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry (SBSE-TD-GC-MS) method as described below. The fraction of FLU associated with the 6µm beads (F6) was estimated based on (i) the results of the quantity of FLU associated to 2µm beads (F2) and (ii) polymer volume ratio calculations between the 2µm and 6µm micro-PS. The quantity

of FLU associated to the algae (Fa) was calculated by subtracting the estimated quantity of FLU associated to the 6µm beads (F6) to the quantity of FLU measured in the pellet (Fp). Partition coefficients Kp (L Kg^{-1}) for algae (KpA) and micro-PS (KpMPS) were calculated by dividing the quantity of FLU in µg/kg associated to algae or micro-PS (2 and 6 µm) by the aqueous phase concentration (µg/L).

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 et al., 2002) for 48h 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.

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 whole mussel and digestive gland were expressed as µg FLU g^{-1} wet tissue weight (WW).

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 (Hégaret 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.).

6. Antioxidant enzyme activities

An aliquot of 50 mg of grounded digestive gland was homogenized (1:4, w/v) in K-phosphate buffer 100mM, pH 7.6 containing 0.15 M KCl, 1mM 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 x g for 20 min (4ºC). 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 expressed as µmol of H_2O_2 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 $protein^{-1}$.

7. Gene expression analyses

244 7.1. Total RNA extraction and cDNA synthesis

245 An aliquot of 50 mg of grounded tissue was homogenized in 0.5 ml of Tri Reagent (Ambion)
246 using a Precellys®24 grinder coupled to a Cryolys® cooling system (Bertin technologies) for total
247 RNA extraction. An aliquot of 40 µg RNA was then treated with the RTS DNase™ Kit (1U/3µg
248 total RNA, Mo Bio). RNA purity and concentration were measured using a Nanodrop
249 spectrophotometer (Thermo Scientific) and RNA integrity was assessed using RNA nanochips and
250 Agilent RNA 6000 nanoreagents (Agilent Technologies).RNA Integrity Numbers (RIN) were 8.3
251 ± 1.0 and 6.6 ± 0.6 (mean ± standard deviation) for gills and digestive gland samples, respectively.
252 2.5 µg RNA were reverse-transcribed using the RevertAidTM H Minus First Strand cDNA
253 Synthesis Kit (Fermentas) with random hexamers. A "reference" cDNA sample was made by
254 pooling the same volume of 10 cDNA samples produced similarly from 10 mussels sampled at the
255 end of the acclimation.

256 7.2. Real-time quantitative PCR

257 Real-time PCR was performed using a LightCycler® 480 II (Roche) for 18 target genes and 5
258 potential reference genes in gills and digestive gland of each individual. Primer pairs and amplicon
259 characteristics (primer sequence, efficiency, product length and melting temperature) are listed in
260 Table 1. Assays were performed in triplicate according to the protocol described by Lacroix et al.
261 (2014a).PCR efficiency (E) was determined for each primer pair by performing standard curves
262 from serial dilutions. Each PCR run included positive (reference cDNA sample) and negative
263 (MilliQ water) controls. For each sample, absence of DNA contamination after DNase treatment
264 was assessed by negative reverse-transcription controls on total RNA samples. PCR analysis was
265 performed following "MIQE precis guidelines" (Bustin et al., 2009).The stability of several
266 reference genes (*ef1α*, *ef2*, *rpl7*, *αtub* and *28s*rRNA) was assessed using the excel-based
267 Normfinder software (Norman et al., 2004). Results indicated *ef1α*, *rpl7* and *αtub* were the most
268 stable genes in gills (lowest stability value of 0.241, 0.286 and 0.239, respectively) and in
269 digestive gland (lowest stability value of 0.174, 0.139 and 0.179, respectively) among the

conditions. The geometric mean of these three genes was therefore used as an index to normalize target gene expression. The normalization index exhibited a stability value of 0.109 and 0.056 in gills and digestive gland, respectively. Target gene relative expression ratios (R) were expressed according to the Pfaffl formula (Pfaffl, 2001):

$$R = \frac{Eff_{Target}^{(\Delta Cq_{Target}(\text{reference}-\text{sample}))}}{Eff_{Index}^{(\Delta Cq_{Index}(\text{reference}-\text{sample}))}}$$

8. 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.

Results

1. Fluoranthene partition in algal cultures

No difference was observed between 45 min and 5h 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

293 FLU appeared associated to the micro-PS (21%). This is reflected by a higher Log KpMPS (6.58)
294 in comparison with Log KpA (4.77) (Table 2).

295 2. Fluoranthene quantification in mussel tissues

296 At T7, mussels exposed to FLU alone or micro-PS+FLU showed similar concentrations of FLU in
297 gills (12.1 ± 0.8 µg g^{-1} and 13.5 ± 1.1 µg g^{-1}, mean ± SE, respectively) and digestive gland (117.1
298 ± 10.7 µg g^{-1} and 89.2 ± 8.4 µg g^{-1}, mean ± SE, respectively) ($p > 0.05$; Figure 1). Negligible
299 amounts of FLU (< 2 µg g^{-1}) were detected in tissues of control and micro-PS exposed mussels. At
300 T14, FLU concentration in gills was similar in mussels exposed to FLU alone or micro-PS+FLU
301 (4.5 ± 0.5 µg g^{-1} and 6.2 ± 0.7 µg g^{-1}, respectively) (Figure 1). In digestive glands, the
302 concentration of FLU was significantly lower in mussels exposed to FLU alone (36.9 ± 6.7 µg g^{-1},
303 mean ± SE) than in mussels exposed to micro-PS+FLU (61.3 ± 4.8 µg g^{-1}, mean ± SE) ($p< 0.001$;
304 Figure 1). No FLU was detected in tissues of control and micro-PS exposed mussels.

305 3. Histology and histopathology

306 At T7, micro-PS were detected exclusively inside the digestive tract and the intestine of all
307 mussels exposed to micro-PS, regardless of the exposure to FLU (Figure 2A).At T14, some
308 microbeads were still observed in the intestine of mussels exposed to micro-PS and micro-
309 PS+FLU (<1-5 beads/histological section/animal). A few micro-PS were also observed stuck in
310 the mucus on the outer side of the gills epithelium (Figure 2B). There was no observation of
311 micro-PS in any other tissue.
312 At T7, a significant increase in total histopathological lesions/abnormalities was demonstrated in
313 mussels exposed to FLU, micro-PS and Micro-PS+FLU, in comparison with controls (Figure 3).
314 Significantly higher hemocyte infiltration and ceroids (stress induced lipofuscin-pigments) were
315 observed in the stomach and digestive gland of all mussels exposed to FLU in comparison to
316 mussels exposed to micro-PS alone (Figures 2C-F, 3). At T14, significantly higher
317 histopathological lesions/abnormalities were observed in mussels exposed to micro-PS+FLU, in
318 comparison with all other treatments (Figure 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.

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 (Figure 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 (Figure 4B, Table 3). Significant interactions between both stressors were demonstrated on percentage of dead hemocytes, phagocytosis capacity and ROS production (Figure 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 (Figure 4A, Table 3). All micro-PS exposed mussels exhibited significantly lower granulocyte concentration ($1.2 \pm 0.2\ 10^5$ cells mL^{-1} and $1.1 \pm 0.2\ 10^5$ cells mL^{-1} for mussels exposed to micro-PS and micro-PS+FLU, respectively) in comparison with controls ($3.4 \pm 0.5\ 10^5$ cells mL^{-1}) and mussels exposed to FLU ($2.7 \pm 0.5\ 10^5$ cells mL^{-1}) (Table 3). This decrease was also reflected in the total hemocyte count with mean cells concentrations of $5.4 \pm 1.1\ 10^5$cells mL^{-1}, $4.8 \pm 0.8\ 10^5$ cells mL^{-1}, $2.8 \pm 0.5\ 10^5$ cells $mL^{-1}$and $2.3 \pm 0.4\ 10^5$ 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 (Figure 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. 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 (52.6 ± 3.9 and 66.6 ± 8.7 µmol min^{-1}

345 mg^{-1}, respectively) in comparison with controls and mussels exposed to FLU alone (70.4 ± 7.1
346 µmol min^{-1} mg^{-1} and 82.9 ± 4.3 µmol min^{-1} mg^{-1}, respectively) (Figure 5, Table 4). Lipid
347 peroxidation (LPO) was also significantly reduced in mussels exposed to micro-PS alone or in
348 combination with FLU (0.9 ± 0.2 and 1.1 ± 0.2 TBARS mg^{-1}, respectively) compared to controls
349 (1.4 ± 0.2 TBARS mg^{-1}) and mussels exposed to FLU alone (1.8 ± 0.1 TBARS mg^{-1}) (Figure 5,
350 Table 4). Activities of GR and SOD were significantly higher in mussels exposed to FLU (17.1 ±
351 1.7 nmol min^{-1} mg^{-1} and 61.1 ± 4.8 U min^{-1} mg^{-1}, mean ± SE, respectively) compared to control
352 mussels (9.6 ± 0.4 nmol min^{-1} mg^{-1} and 47.0 ± 2.3 U min^{-1} mg^{-1}, respectively) (Figure 5, Table 4).
353 At T14, significant effects of micro-PS and FLU exposures were observed on GST and SOD, with
354 an increase of enzyme activities in all exposed mussels. Highest GST and SOD activities (16.9 ±
355 1.4 nmol min^{-1} mg^{-1} and 50.7 ± 3.5 U min^{-1} mg^{-1}, mean ± SE, respectively) were observed in the
356 micro-PS+FLU condition in comparison with all other treatments (Figure 5, Table 4). Significant
357 effects of micro-PS were observed on LPO levels with the lowest concentration being measured in
358 micro-PS+FLU exposed mussels (Figure 5, Table 4). A significant interaction between micro-PS
359 and FLU was observed on the activity of the GR: FLU exposure led to a decrease in GR activity in
360 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
361 FLU exposed mussels, respectively), while a significantly higher activity was observed in micro-
362 PS+FLU exposed mussels (12.8 ± 0.8 nmol min^{-1} mg^{-1}) in comparison with micro-PS exposed
363 animals (9.7 ± 0.4 nmol min^{-1} mg^{-1}) (Figure 5, Table 4).

364 6. Gene expression

365 At T7, significant effects of micro-PS were observed in mRNA levels only in gills. Compared to
366 controls, mRNA level of *lys* increased 2.2 and 1.2 folds, respectively in micro-PS and micro-
367 PS+FLU exposed mussels; the mRNA levels of *cat* were 0.7 and 0.8 times lower, respectively in
368 micro-PS and micro-PS+FLU exposed mussels (Table 5). At T14 in gills, exposure to micro-PS
369 led to a significant increase in mRNA level for *pk* by 1.1 and 1.4 times in mussels exposed to
370 micro-PS and micro-PS+FLU, respectively, compared to controls (Table 6). The mRNA level of

371 *sod* increased significantly by 1.1, 1.2 and 1.5 times in mussels exposed to FLU, micro-PS and
372 micro-PS+FLU, respectively, compared to controls. The mRNA levels of *gpx* and *idp* genes were
373 significantly higher in gills of mussels exposed to FLU alone (by 2.3 and 2.4 times, respectively)
374 or in combination with micro-PS (by 2.7 and 2.6 times, respectively) in comparison with controls
375 (Tables 5, 6). A significant interaction between both factors (micro-PS and FLU) was observed on
376 *cat* mRNA level in gills: micro-PS and FLU single exposures led to a reduction by 0.9 and 0.7
377 times, respectively, in comparison with controls, while exposure to micro-PS+FLU induced an
378 increase of *cat* mRNA level by 1.2 times compared to controls (Table 5). The mRNA levels of *pk*,
379 *sod* and *cat* genes were systematically highest in the mussels exposed to micro-PS+FLU,
380 compared to the 3 other conditions. At T14 in digestive gland, micro-PS exposure induced a
381 significant decrease of *pgp* mRNA level by 0.4 and 0.7 in mussels exposed to micro-PS and
382 micro-PS+FLU, respectively, compared to controls (Tables 5, 6). A significant interaction
383 between both factors was observed for *cat* mRNA level: micro-PS and FLU single exposures led
384 to a reduction by 0.7 and 0.8 times, respectively, in comparison with controls, while the double
385 exposure micro-PS+FLU induced an increase of mRNA level by 1.7 times compared to controls
386 (Table 5). In addition, the mRNA levels of *amylase*, *pk* and *sod* were significantly higher by 1.6,
387 1.2 and 1.5 times respectively in mussels exposed to FLU compared to controls, with highest
388 inductions observed in mussels exposed to micro-PS+FLU (3.2, 1.5 and 1.7 times, respectively)
389 (Tables 5,6). Exposure to FLU induced a diminution of *σ gst* mRNA level by 0.7 times in
390 comparison with controls. Overall the experiment, no effects of micro-PS or FLU or their
391 combination were observed on *cyp11*, *cyp32*, *ωgst*, *μgst*, *gadd45α*, *gapdh*, *hk*, *p53*, *casp37-3*
392 mRNA levels.

393 **Discussion**

394 **Micro-PS exhibited high sorption capacity for fluoranthene**

395 The present study evidenced that micro-PS exhibited higher sorption capacity for fluoranthene
396 than marine algae *C. muelleri* as indicated by the partition coefficient log Kp values, and this
397 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 Kp 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.

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 to compare 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

424 the general metabolism of mussels resulting in a reduction in FLU depuration. Indeed, low
425 metabolism and activity are associated with low PAH clearance rates (Lotufo et al., 1998; Al-
426 Subiai et al., 2012)); and (iv) a possible direct impact of micro-PS on PAH detoxification
427 processes, as suggested by a decrease in P-glycoprotein mRNA levels in all mussels exposed to
428 micro-PS. Indeed, P-glycoproteins are transmembrane proteins primarily involved in the efflux of
429 a wide range of compounds including unmodified xenobiotics and PAH (Smital et al., 2003).
430 Impacts of polyethylene microbeads on detoxification mechanisms were previously demonstrated
431 in common goby *Pomatoschistus microps* juveniles and seabass *Dicentrarchus labrax* larvae
432 (Mazurais et al., 2015; Oliveira et al., 2013). The high fluoranthene concentration remaining in
433 tissues of mussels exposed to micro-PS and FLU at the end of the depuration may explain the
434 highest levels of ceroids, hemocyte infiltration and tissue lesions, known to be associated with
435 PAH (Kim et al., 2008; Al-Subiai et al., 2012), observed in this condition.

436 **Micro-PS exposure affected mussels physiology**

437 *Modulation of digestion and energy metabolism*

438 The induction of glycolysis and digestive activity upon micro-PS exposure may sign increased
439 energy requirements in response to the implementation of anti-oxidant and detoxification
440 processes (Palais et al., 2010). This mechanism would allow the animal to cope with experimental
441 stress and maintain homeostasis, as suggested in a study conducted by Van Cauwenberghe et
442 al.(2015) who demonstrated a 25% increase in energy consumption in mussels exposed to micro-
443 PS for 14 days in comparison with controls. Alternatively, the increase in digestive activity could
444 be explained by a compensatory effect on food intake and enhancement of mechanical digestion
445 upon particles exposure, as also hypothesized in oysters (Sussarellu et al., 2016). An increase in
446 absorption efficiency was for instance demonstrated in mussels exposed to moderate quantities of
447 silt in relation to an improvement of the mechanical disruption in the stomach due to the presence
448 of particles (Bayne et al., 1987). In the present study, a control condition using non-plastic
449 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.

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.

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 *lys* 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 of 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 (Donaghy 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.

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 to 2500 mg L^{-1}) (Avio et al., 2015; Besseling et al., 2013; Wegner et al., 2012; Von Moos et al., 2012) and was in the range of the highest estimated field concentration (most exclusively for particles > 330 µm) based on the assumption of a steady fragmentation of plastic debris as detailed in Sussarellu et al. (2016). This, in addition with recent published papers and reports (GESAMP, 2015; Herzke et al., 2016; Koelmans et al., 2016) suggest that given the current microplastics concentration in oceans the bioaccumulation of POP from microplastics is likely to be minor in regards with uptake *via* natural pathways (waterborne and foodborne contamination). Nevertheless, marine plastic litter is expected to increase over the next decades, leading to ever increasing microplastic pollution in oceans, and the concentrations below which no effect can be seen on POP bioaccumulation could locally be exceeded.

Despite no effect observed on fluoranthene bioaccumulation, the ingestion of contaminated micro-PS may have modified bioavailability and fluoranthene kinetics in mussel tissues due to the nature and the assimilation or not of the carrier (algae *vs.* plastic). This was suggested by a reduction in the fluoranthene depuration and significant interactions between micro-PS and fluoranthene observed on some cellular and molecular biomarkers. It is noteworthy that the bioformation of fluoranthene metabolites upon micro-PS exposure were not investigated in the present study. This aspect should be considered in further experiments as it could potentially have contributed to the additional and interactive effects observed in micro-PS+FLU exposed mussels. Finally, micro-PS alone triggered substantial modulation of cellular oxidative 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

528 and/or experimental studies that more closely mimic complex *in situ* conditions, in particular by
529 using different particulate matter and chemical mixtures representative of those found in the field.

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Figures

Figure 1. Concentrations (µg g^{-1}, w.w.) of fluoranthene in gills and digestive gland of mussels after exposure (T7) and depuration (T14) phases. Micro-PS: microplastics; FLU: fluoranthene. Results are expressed as the mean concentration ± standard error (SE) (n=9).

Figure 2. Histological observations in mussels after 7 days of exposure to fluoranthene and micro-PS. A: Fluorescent 6µm and 2µm micro-PS in the intestine (INT) (arrows); B: Micro-PS (arrows) in gills (G), Ep: epithelium; C: Ceroids (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.

Figure 3. Sum ofhistopathological observations detected in mussel tissues (gills, gonads and digestive gland including stomach, digestive tubules and intestine) after exposure (T7) and depuration (T14).TA: tissue alterations (degeneration, sloughing, tear); INF: Hemocyte infiltration; DIA: Hemocyte in diapedesis (observed only in digestive gland including stomach, digestive tubules and intestine); CER: Ceroids; VAC: Vacuolation. Letters (a, b, c) indicate statistical differences (Mann-Whitney U-test, $p < 0.05$).

Figure 4. Percentage of dead hemocytes (%) (A), ROS production capacity (B) andphagocytosis capacity (C) after exposure (T7) and depuration (T14) phases. Micro-PS: microplastics; FLU: fluoranthene. Results are expressed as mean percentage of mortality ± standard error (SE) (n=12).

Figure 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). Results are expressed as mean percentage of mortality ± standard error (SE) (n=9).

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785 **Figure 1**

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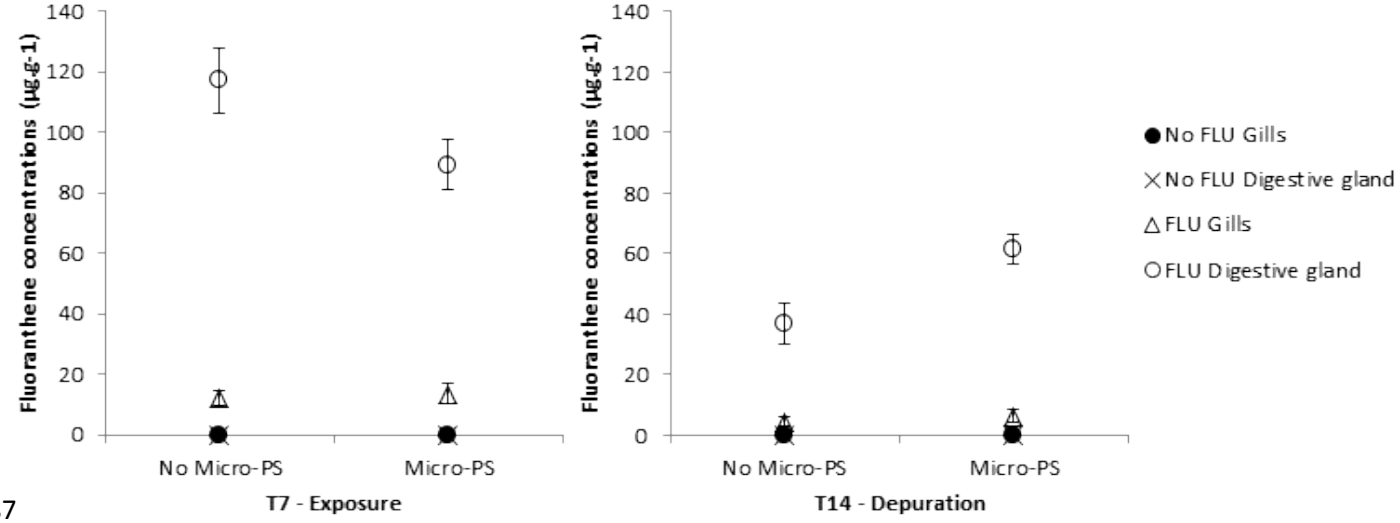

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Figure 2

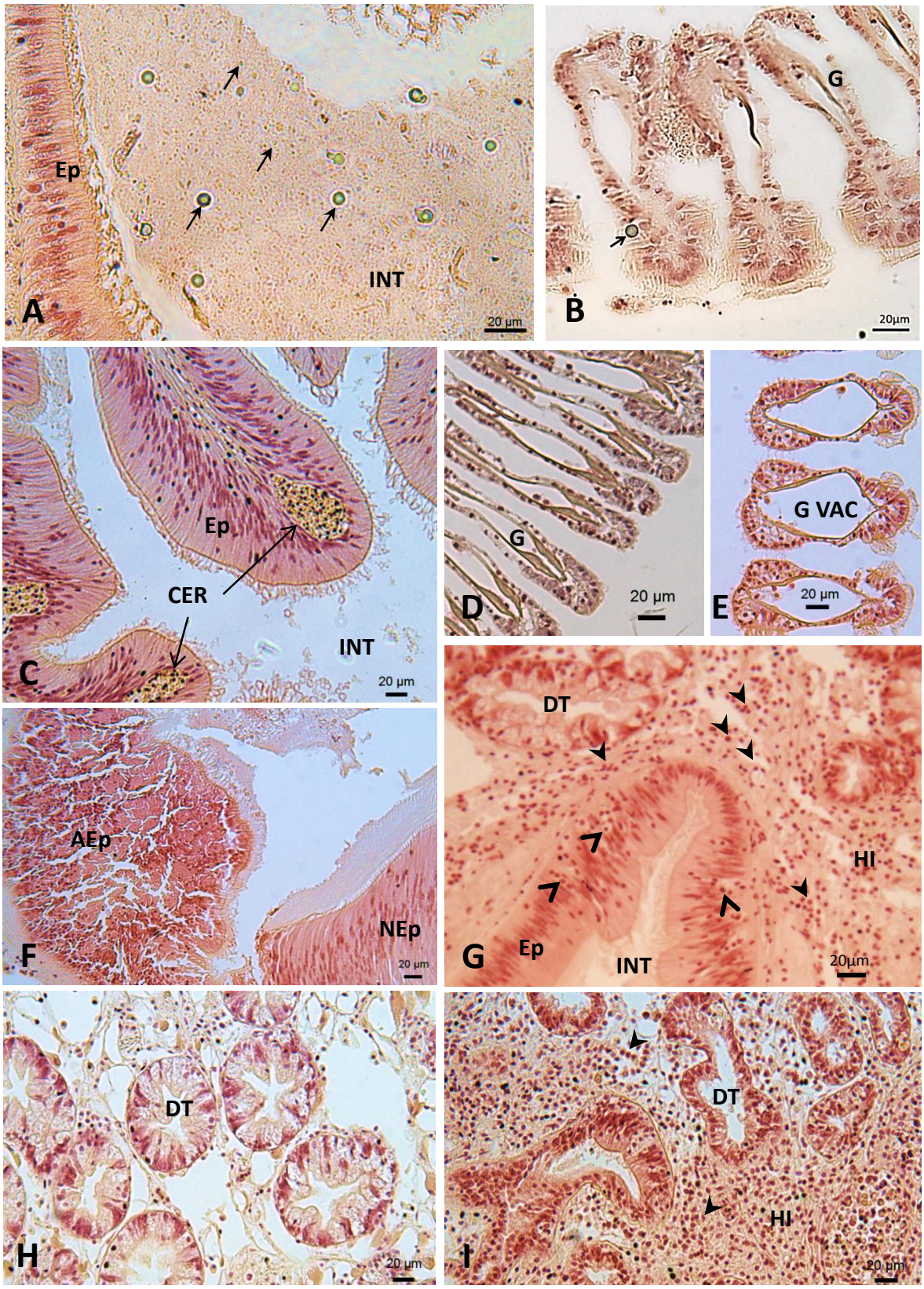
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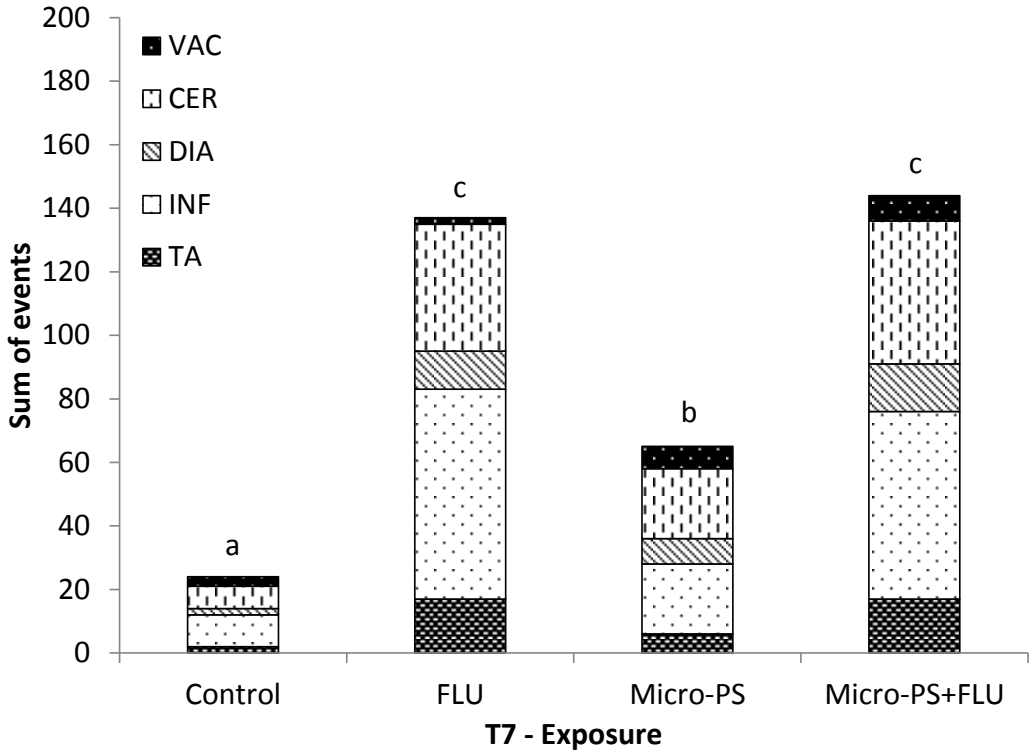
Figure 3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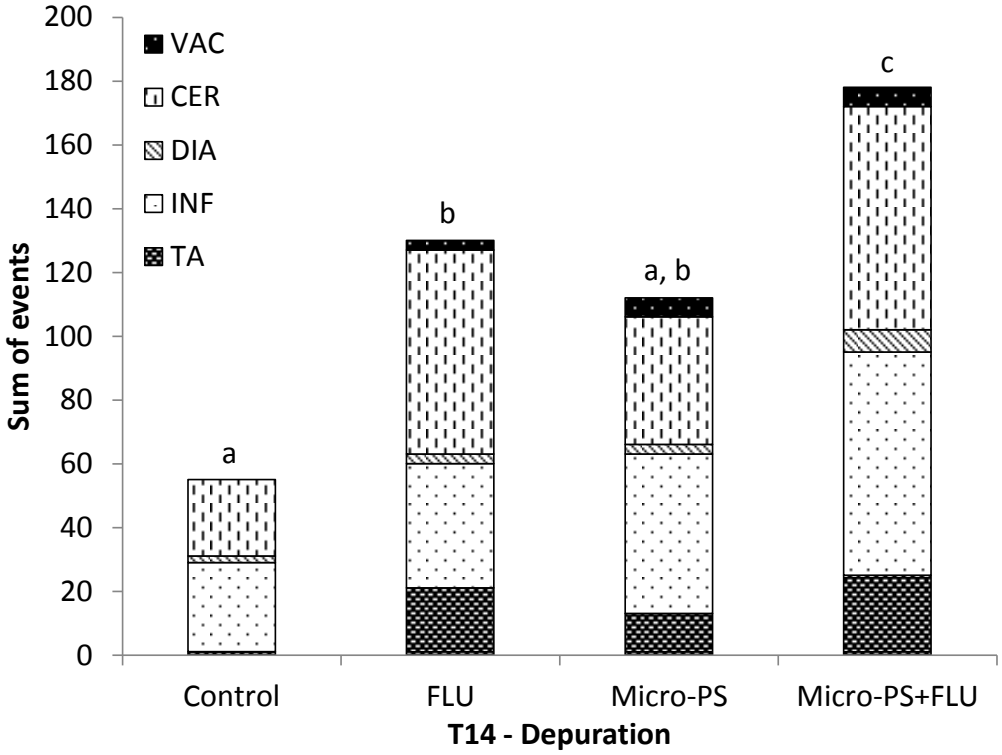
Micro-PS+FLU
T7 - Exposure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Micro-PS+FLU
T14 - Depuration

800 **Figure 4**

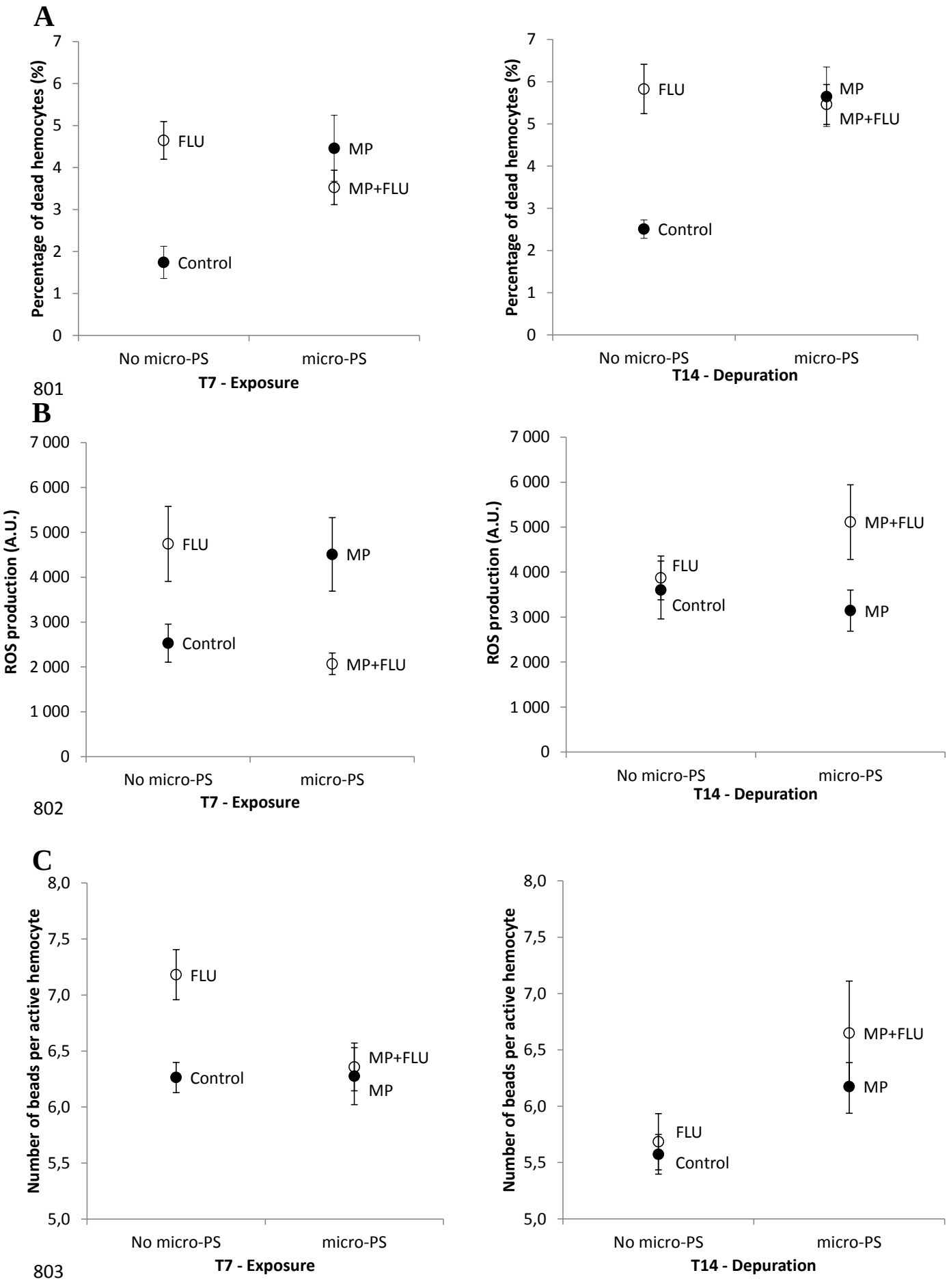

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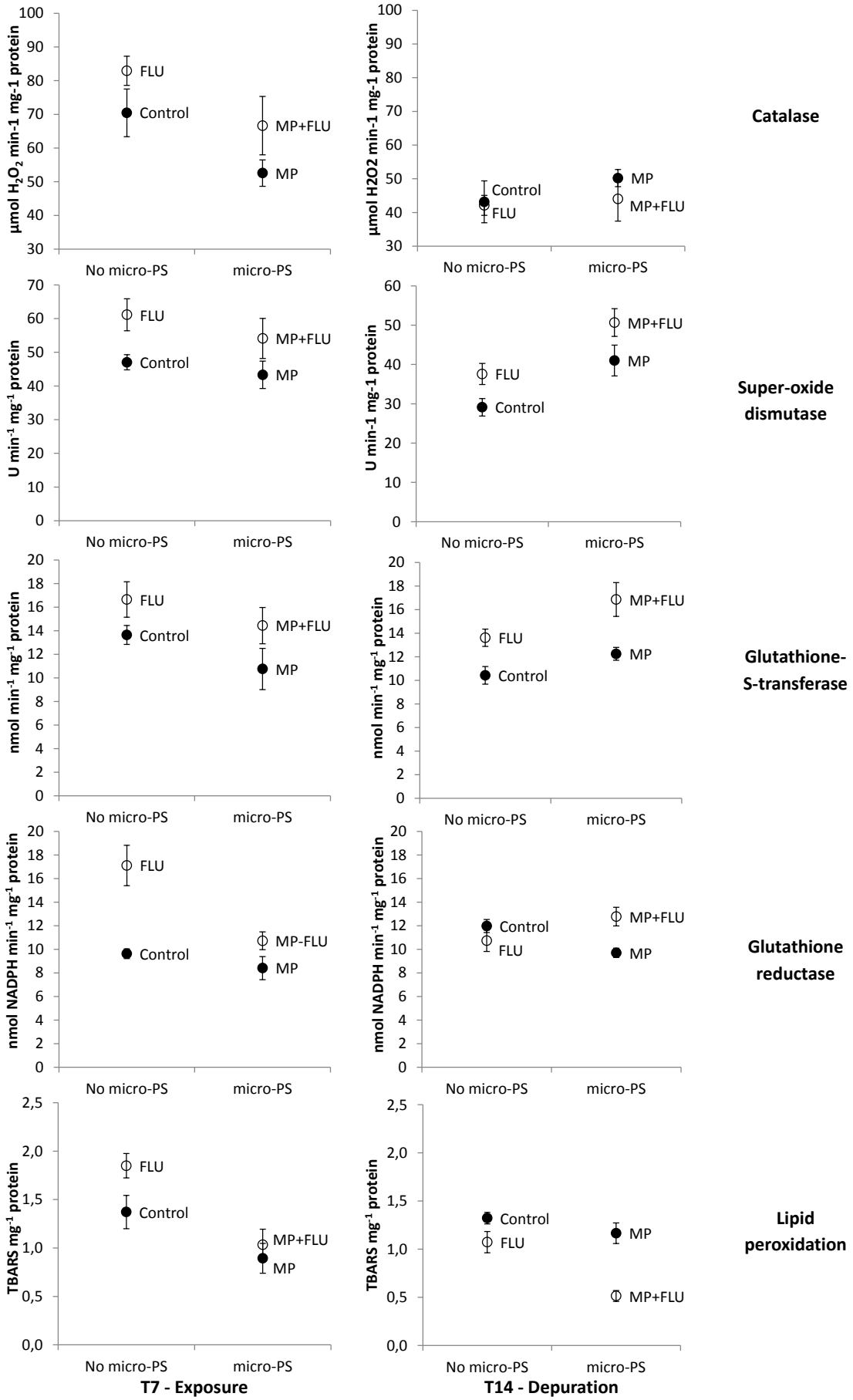
Figure 5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TBARS mg-1 protein
FLU
Control
MP+FLU
MP
MP-FLU
No micro-PS
micro-PS
Catalase
Super-oxide dismutase
Glutathione-S-transferase
Glutathione reductase
Lipid peroxidation
T7 - Exposure
T14 - Depuration

Tables

Table 1. Name, abbreviation, primer pair, efficiency, linear range, product length, melting temperature (Tm), and Genbank accession numbers for reference and target genes analyzed in mussel tissues exposed to micro-PS and FLU. G: gills, DG: digestive glands, bp: base pair.

Transcript name	Abreviation	Forward (5'-3')	Reverse (5'-3')	Efficiency (G/DG)	Length (bp)	Tm (°C)	Accession number (species)	Reference
28S rRNA *	*28s*	GGAGGTCCGTAGCGATTCTG	CGTCCCCAAGGCCTCTAATC	1.96/1.94	174	81.8	AB103129 (mg)	Lacroix et al, 2014a
Elongation factor 1 alpha *	*ef1α*	ACCCAAGGGAGCCAAAAGTT	TGTCAACGATACCAGCATCC	1.92/1.95	212	79.2	AF063420 (me) / ABA62021 (mg)	Lacroix et al, 2014a
Elongation factor 2 *	*ef2*	GCAGTACATCACCCAGCAAA	GTCAACAAGGCCAAGTCCAT	1.89/1.88	249	80.1	FL497408 (mg)	Lacroix et al, 2014a
Ribosomal protein L7 *	*rpl7*	CAGAGACAGGCCAAGAAAGG	TGGGTAGCCCCATGTAATGT	1.95/1.95	227	81.9	AJ516457 (mg)	Lacroix et al, 2014a
alpha-tubuline *	*αtub*	GGATTCAAGGTCGGAATCAA	ACGTACCAATGGACGAAAGC	1.92/1.97	179	83.6	DQ174100 (me) / HM537081 (mg)	Lacroix et al, 2014a
Catalase	*cat*	CACCAGGTGTCCTTCCTGTT	CTTCCGAGATGGCGTTGTAT	1.86/1.85	235	81.5	AY580271 (me) / AY743716 (mg)	Lacroix et al, 2014a
Cu/Zn-Superoxide Dismutase	*sod*	CATTTCCCAGATCACCAACA	GGAACAGTCGCTTTCAGTCA	1.93/1.90	214	82.2	AJ581746 (me) / FM177867 (mg)	Lacroix et al, 2014a
Se-dependant-Glutathione peroxidase	*gpx*	ACGGTAAAGACGCTCATCCAA	TCTTGTCACAGGTTCCCATATGAT	2.00/2.06	119	79.7	HQ891311 (mg)	Lacroix et al, 2014a
Cytochrome P450-1-like-1	*cyp11*	TGGTTGCGATTTGTTATGCCCTGGA	GGCGGAAAGCAATCCATCCGTGA	ND/1.95	150	77.5	JX885878 (me)	Zanette et al., 2013
Cytochrome P450-3-like-2	*cyp32*	CAGACGCGCCAAAAGTGATA	GTCCCAAGCCAAAAGGAAGG	1.87/1.85	194	80.1	AB479539 (me)	Lacroix et al, 2014a
ω-Glutathione-S-transferase	*ωgst*	CGACTCTATAGCATGCGATTATG	AGAACCGGAACCATACCAAGAGG	1.92/2.04	152	77.5	Locus 38757 [a] (me)	Lacroix et al, 2014a
μ-Glutathione-S-transferase	*μgst*	AGAGGCCTAGCACAGCCAGTGAG	CACTCTCTGCTGAATCCTGGACC	2.05/1.95	104	78.4	Locus 42054 [a] (me)	Lacroix et al, 2014a
σ-Glutathione-S-transferase	*σgst*	CCTGTTCGCGGAAGAGCTGAACT	GTTGGCATCTGTCCTGTTGGTAT	1.92/NA	131	78.0	FL494070 (mg)	Lacroix et al, 2014a
Growth arrest and DNA damage inducible	*gadd45α*	CCATTCCCTTCAACCTCCTC	GCCGAAACAGACGTAACAGT	1.96/1.89	140	78.7	AJ623737 (mg)	Ruiz et al, 2012
α-Amylase	*amylase*	CCTCGGGGTAGCTGGTTTTA	TCCAAAGTTACGGGCTCCTT	ND/1.91	232	79.2	EU336958 (me)	-
Pyruvate kinase	*pk*	AGACTTGGAGCTGCCTTCAG	GGAATGCACAGAGGGTTCAT	1.83/1.88	228	81.6	Locus22823[a] (me)	-
ocitrate dehydrogenase [NADP] cytoplasm	*idp*	GGAGGTACTGTATTTCGTGAGGC	TGATCTCCATAAGCATGACGTCC	1.93/1.97	104	76.9	Locus2855[a] (me)	-
Glyceraldehyde 3 phosphate dehydrogenase	*gapdh*	GTCTGGTGATGAGAGCTGCC	GCGTCTCCCCATTTGATAGCT	1.84/1.84	220	78.7	FL496349 (mg)	Lacroix et al, 2014a
Hexokinase	*hk*	CCAATATGACAATTGCCGTTGA	GCAGCACCTTTACCACTACCATCA	1.91/1.91	148	78.2	JN595865 (mg)	-
P-53 tumor supressor-like	*p53*	CAACAACTTGCCCAATCCGA	GGCGGCTGGTATATGGATCT	1.85/1.89	228	80.1	AY579472 (me) / DQ158079 (mg)	Lacroix et al, 2014a
ABCB/P-glycoprotein-like protein	*pgp*	CACTAGTTGGAGAGCGTGGA	TGTTCTTCCCTGTCTTGCCT	1.92/1.86	116	82.7	AF159717 (me) / EF057747 (mg)	Lacroix et al, 2014a
Lysosyme	*lys*	AGGGTTTGTGCATCCTCTTG	TCGACTGTGGACAACCAAAA	1.94/1.92	173	81.6	AF334662 (me) / AF334665 (mg)	-
Caspase 3/7-3	*casp37-3*	CAATGTGTAAAAACGAGAGACATTG	GTTAGTATATGCCCACTGTCCATTC	1.84/1.93	146	76.5	HQ424453 (mg)	Romero et al., 2011

***** indicates reference genes; [a] indicates sequences obtained from Illumina technology sequencing (Courtesy of Sleiman Bassim and Arnaud Tanguy (Bassim et al, 2014)); (me): M. edulis; (mg): M. galloprovincialis. ND: not detected; NA: not analyzed

Table 2. Concentrations of fluoranthene in water, on micro-algae *Chaetoceros muelleri* and on polystyrene microbeads (micro-PS) expressed as ng mL^{-1}, percentage (%) or partition coefficient (log Kp) in the FLU and micro-PS+FLU algal cultures.

	FLU alone			Micro-PS+FLU		
	(ng mL^{-1})	%	Log Kp	(ng mL^{-1})	%	Log Kp
Micro-algae	325.59	89	4.84	234.66	67	4.77
Water	39.30	11	/	40.19	12	/
2 µm micro-PS	/	/	/	18.35	5	6.58
6 µm micro-PS	/	/	/	55.05	16	6.59

Table 3. Results of the two-way ANOVA performed on the hemocyte parameters 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.

	Hemocyte mortality		**ROS production**		**Phagocytosis capacity**		**Hemocyte concentration**		**Granulocyte concentration**	
Source of variation	T7 p-value	T14 p-value	T7 p-value	T14 p-value	T7 p-value	T14 p-value	T7 p-value	T14 p-value	T7 p-value	T14 p-value
FLU	***0.013***	***0.001***	***0.048***	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Micro-PS	***0.049***	***0.003***	***0.041***	> 0.05	> 0.05	***0.01***	> 0.05	***0.001***	> 0.05	***<0.0001***
Micro-PSxFLU	***0.001***	***<0.0001***	***0.001***	> 0.05	***0.018***	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

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.

	Catalase		**Superoxide dismutase**		**Glutathione-S-transferase**		**Glutathione reductase**		**Lipid peroxidation**	
Source of variation	T7 p-value	T14 p-value	T7 p-value	T14 p-value	T7 p-value	T14 p-value	T7 p-value	T14 p-value	T7 p-value	T14 p-value
FLU	> 0.05	> 0.05	***0.021***	***0.016***	> 0.05	***0.001***	***<0.0001***	> 0.05	> 0.05	***<0.0001***
Micro-PS	***0.025***	> 0.05	> 0.05	***0.004***	> 0.05	***0.041***	***0.003***	> 0.05	***0.001***	***0.003***
Micro-PSxFLU	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	***0.021***	> 0.05	***0.021***

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 highlighted in grey. Arrows represent the way of induction, ↗: up-regulation; ↘: down-regulation; ↕: interaction.

Function	Gene	Time	Tissue	Experimental conditions	Relative gene expression			p-value	
					Mean	SD	SE		
Anti-oxidant	*cat*	T7	G	Control	1,35	0,33	0,11		
				FLU	1,37	0,36	0,12	> 0.05	
				Micro-PS	0,97	0,26	0,09	***0,006***	↘
				Micro-PS x FLU	1,13	0,31	0,10	> 0.05	
	cat	T14	G	Control	0,97	0,29	0,10		
				FLU	0,70	0,23	0,08	> 0.05	
				Micro-PS	0,92	0,27	0,09	***0,029***	↘
				Micro-PS x FLU	1,14	0,19	0,06	***0,006***	↕
	sod	T14	G	Control	0,97	0,29	0,10		
				FLU	0,70	0,23	0,08	***0,002***	↗
				Micro-PS	0,92	0,27	0,09	***0,036***	↗
				Micro-PS x FLU	1,14	0,19	0,06	> 0.05	
	gpx	T14	G	Control	0,81	0,68	0,23		
				FLU	1,87	1,76	0,62	***0,042***	↗
				Micro-PS	1,48	0,38	0,13	> 0.05	
				Micro-PS x FLU	2,22	1,49	0,50	> 0.05	
	σ-*gst*	T14	DG	Control	1,55	0,97	0,32		
				FLU	1,05	0,70	0,23	***0,014***	↘
				Micro-PS	1,84	0,96	0,36	> 0.05	
				Micro-PS x FLU	0,87	0,42	0,15	> 0.05	
	cat	T14	DG	Control	1,26	0,96	0,32		
				FLU	0,90	0,38	0,13	> 0.05	
				Micro-PS	0,87	0,37	0,14	> 0.05	
				Micro-PS x FLU	2,09	1,29	0,46	***0,016***	↕
	sod	T14	DG	Control	0,68	0,24	0,08		
				FLU	1,05	0,15	0,05	***<0,0001***	↗
				Micro-PS	0,77	0,28	0,11	> 0.05	
				Micro-PS x FLU	1,12	0,17	0,06	> 0.05	

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.

Function	Gene	Time	Tissue	Experimental conditions	Relative gene expression Mean	SD	SE	p-value
Generation of reducing equivalents	*idp*	T14	G	Control	0,20	0,08	0,03	
				FLU	0,48	0,20	0,07	***0,008*** ↗
				Micro-PS	0,39	0,29	0,10	> 0.05
				Micro-PS x FLU	0,51	0,21	0,07	> 0.05
Detoxication	*pgp*	T14	DG	Control	1,45	0,51	0,17	
				FLU	1,12	0,29	0,10	> 0.05
				Micro-PS	0,62	0,28	0,11	***0,001*** ↘
				Micro-PS x FLU	0,98	0,33	0,12	***0,015*** ↕
Digestion	*pk*	T14	G	Control	0,72	0,20	0,07	
				FLU	0,75	0,27	0,09	> 0.05
				Micro-PS	0,82	0,32	0,11	***0,023*** ↗
				Micro-PS x FLU	1,07	0,24	0,08	> 0.05
	pk	T14	DG	Control	1,06	0,41	0,14	
				FLU	1,22	0,43	0,14	***0,012*** ↗
				Micro-PS	0,89	0,36	0,14	> 0.05
				Micro-PS x FLU	1,54	0,48	0,17	> 0.05
	amylase	T14	DG	Control	0,31	0,23	0,08	
				FLU	0,50	0,35	0,12	***0,006*** ↗
				Micro-PS	0,23	0,22	0,08	> 0.05
				Micro-PS x FLU	1,02	0,79	0,28	> 0.05
Immunity	*lys*	T7	G	Control	0,99	0,96	0,32	
				FLU	1,07	0,64	0,21	> 0.05
				Micro-PS	2,18	1,27	0,42	***0,044*** ↗
				Micro-PS x FLU	1,18	0,74	0,25	> 0.05