Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia)

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ABSTRACT

Microplastics (MPs), plastic debris smaller than 5 mm, are widely found in both marine and freshwater ecosystems. However, few studies regarding their hazardous effects on inland water organisms, have been conducted. For this reason, the aim of our research was the evaluation of uptake and chronic toxicity of two mixtures (MIXs) of virgin polystyrene microbeads (PMs) of 10 µm and 1 µm in size (MIX 1, with 5×10^5 of 1µm size PMs/L and 5×10^5 of 10 µm size PMs/L, and MIX 2 with 2×10^6 of 1µm size PMs/L and 2×10^6 of 10 µm size PMs/L) on freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia) during 6 exposure days. The PM uptake in the mussel body and hemolymph was assessed using confocal microscopy, while the chronic toxicity of PMs was evaluated on exposed mussels using a comprehensive battery of biomarkers of cellular stress, oxidative damage and neuro- genotoxicity. Confocal microscopy analyses showed that MPs concentrated in the gut lumen of exposed mussels, absorbed and transferred firstly in the tissues and

then in the hemolymph. The results revealed that PMs do not produce oxidative stress and genetic damage, with the exception of a significant modulation of catalase and glutathione peroxidase activities in mussels exposed to MIX 1. Regarding neurotoxicity, we observed only a significant increase of dopamine concentration in mussels exposed to both MIXs, suggesting a possible implication of this neurotransmitter in an elimination process of accumulated PMs. This research represents a first study about the evaluation of virgin MP toxicity in zebra mussel and more research is warranted concerning the long term neurological effects of virgin MPs.

Keywords:
Microplastics, zebra mussel *Dreissena polymorpha*, uptake, confocal microscopy, chronic toxicity, biomarkers

1. INTRODUCTION

The problem of plastics as emerging environmental pollutants is a growing concern because the global plastic production has risen exponentially since the 1950's, reaching more than 320 millions of tons in 2014 (PlasticsEurope, 2016). China is the largest producer of plastic materials (26%), followed by Europe (20%) and NAFTA (North American Free Trade Agreement) countries (19%). It is interesting that two third of plastic demand in Europe is concentrated in only five countries: Germany (24.8%), Italy (14.3%), France (9.6%), UK (7.7%) and Spain (7.4%; PlasticsEurope, 2016). Therefore, the so-called "plastic age" carries negative consequences for aquatic and terrestrial ecosystems, biota and human health. Sutherland et al. (2010) suggested that the problem due to plastic debris must be considered, along with the climate change, as the issue which could affect the conservation of biological diversity in the short to medium-term.

Microplastics (MPs) are operationally defined as plastic fragments < 5 mm in diameter down to the μm range (Thompson et al., 2009). The release of fragments down to the nanometer range is also considered given that nanomaterials could be become more reactive owing to their

53 increased surface area/volume ratio and readily available towards cells. They are produced by
54 primary and secondary sources: the first one includes manufactured plastic products, such as
55 scrubbers in cleaning and cosmetic products or pellets used in feedstock or plastic production
56 (Fendall and Sewell, 2009; Cole et al., 2011), while the secondary origin of MPs results from the
57 breakdown of larger plastic items, such as fishing nets, line fibers, films, industrial raw materials,
58 consumer products and pellets and polymer fragments from degradable plastic (Hidalgo-Ruz et al.,
59 2012; Free et al., 2014). Although there is little information about degradation rates of plastic items
60 and their fragmentation in the environment, the spread and abundance of MPs is raising worldwide
61 (Browne et al., 2011; Law and Thompson, 2014).

62 The studies regarding the impact of MPs on aquatic environments have been focused mainly
63 on marine ecosystems, while a more limited amount of studies have been conducted on freshwater
64 habitats (Wagner et al., 2014; Horton et al., 2017). For instance, MPs have been recently found in
65 Europe in surface waters or sediments of Lake Geneva (Switzerland; Faure et al., 2012), Lake
66 Garda (Italy, Imhof et al. 2013, 2018), Danube River (Austria; Lechner et al., 2014), Tamar estuary
67 (UK; Sadri and Thompson, 2014) and in the Elbe, Mosel, Neckar and Rhine rivers (Germany;
68 Wagner et al., 2014). Other surveys were carried out on freshwater ecosystems of North America,
69 Asia and Africa (Free et al., 2014; Su et al., 2016; Wang et al., 2016, 2017; Anderson et al., 2017;
70 Di and Wang, 2018; Nel et al., 2018). The gap in the knowledge of MPs' distribution between
71 marine and freshwater ecosystems is also reflected in the knowledge of their potential toxic effects
72 on biota.

73 Indeed, while several studies were carried out in field and under laboratory conditions to
74 evaluate the ingestion and effects of MPs in marine organisms, studies regarding the impact of MPs
75 on freshwater species are wanting (e.g. Wagner et al., 2014; Guilhermino et al., 2018; Lei et al.,
76 2018). While the available results showed the ingestion capability of MPs in all the examined
77 freshwater taxa (fish, crustaceans, ostracods, gastropods and chironomids; Imhof et al., 2013; Nel et
78 al., 2018), their ecotoxicological effects remain largely unknown. However, the few available

studies seemed to suggest physical impacts similar to those observed for marine organisms (Eerkes-Medrano et al., 2015).

Another problem of MPs is related to their composition and large surface area, which make them prone to adsorb waterborne organic contaminants (Cole et al., 2011) that can be then transported in the aquatic organism trough a "Trojan-horse mechanism" as with products from nanotechnology. Moreover, also the leaching of the plasticizers (e.g., phthalates and bisphenol A) can increase the toxicity of MPs when in the organism. A review by Wagner et al. (2014) underlined several gaps of knowledge about monitoring, source, fate, exposure and effects of MPs, that need to be addressed by the near future studies on freshwater ecosystems and biota.

Therefore, in the present study, we investigated the gaps related to the evaluation of the MP exposure and effects in freshwater organisms. In particular, we choose as biological model the freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia), considering its physiological characteristics, as the high filtration rate (Binelli et al., 2014, 2015; Magni et al., 2015), its easiness in stabulation, and its key role in the European and American freshwater ecosystems, being a species that links the littoral and benthic habitats. We exposed for 6 days in static conditions zebra mussel specimens to two different mixtures (MIXs), at different concentration, of virgin polystyrene microbeads (PMs), one of the main MP class detected in the environment, with size of 10 μm and 1 μm, respectively. After the exposures, we investigated the MP ingestion and their eventual uptake and infiltration in the mussel tissues through the use of cryostat and confocal microscopy, while a wide battery of biomarkers was used to assess the potential chronic toxicity of selected contaminants. In particular, on the basis of other evidences of MP effects on oxidative status and neuro-enzyme activity on aquatic organisms (Oliveira et al., 2013; Avio et al., 2015; Ribeiro et al., 2017; Barboza et al., 2018), in the present work we choose to investigate more profoundly these aspects evaluating end-points of cellular stress, oxidative damage and neuro- genotoxicity. To the best of our knowledge, the present study represent an innovative attempt to simultaneously

104 investigate both the fate of MPs and their toxicological impact on freshwater mussels by a multiple
105 biomarker approach.
106
107 2. MATERIALS AND METHODS
108 2.1 *Mussel collection*
109 We collected zebra mussel specimens from Lake Iseo (Lovere, North Italy) in January 2017.
110 Mussels were collected from the rocks and transported in containers filled with lake water to the
111 laboratory. Before the exposure, mussels were acclimated for a period of two weeks in 15 L tanks
112 with tap and deionized water (50:50 v/v) and maintained at 20±1 °C in oxygen saturation
113 conditions, with natural photoperiod. Mussels were fed three times *per* week with phytoplankton
114 (*Spirulina* sp.), as reported in our previous work (Magni et al., 2016, 2017).
115
116 2.2 *Concentration selection and mussel exposure to polystyrene microbeads*
117 The two standard aqueous suspensions (5%) of virgin PMs with a size of 10 µm and 1 µm
118 were purchased from Sigma-Aldrich (Italy). Selected standards were diluted in ultrapure water to
119 obtain the two PM working suspensions of 50 mg/L. Since we decided to perform the exposures by
120 considering the real number of beads (and not a simple mass/volume ratio), we quantified the
121 number of 10 µm and 1 µm PMs in the 50 mg/L working suspensions using the Bürker chambers
122 (neutral beads were not subjected to aggregation phenomena), obtaining the following bead
123 numbers (mean±SD): $116\times10^6\pm33\times10^6$ of 10 µm PMs/L and $23\times10^9\pm530\times10^6$ of 1 µm PMs/L.
124 Because of the great release of MPs in the freshwater environment from Wastewater Treatment
125 Plants (WWTPs) of about 65 millions of MPs/day (Murphy et al., 2016), we chose to test the
126 toxicity of these two different PM MIXs: MIX 1, with 5×10^5 of 1µm size PMs/L and 5×10^5 of 10
127 µm size PMs/L, and MIX 2 with 2×10^6 of 1µm size PMs/L and 2×10^6 of 10 µm size PMs/L. The
128 PM exposures were conducted in triplicate (three tanks for control, three tanks both for MIX 1 and
129 MIX 2), placing in each tank (4 L) 70 mussels under static conditions for 6 days (from t = 0 to t = 6

130 days), feeding the animals two times with phytoplankton (*Spirulina* sp.), and maintaining a low
131 stirring to avoid PM sedimentation. Considering the high number of animals required to carry out
132 both microscopy analyses and biomarker measurements, we conducted three different PM
133 exposures in the same conditions. Before the treatment we assessed the baseline levels (t = 0) of
134 biomarkers on mussels collected from the acclimation tanks, as reported by Magni et al. (2017).
135 Every three days (t = 3 and t = 6 days) we collected mussels from each tank to measure the selected
136 end-points. To evaluate the genotoxicity on mussel hemocytes, we collected the hemolymph from 3
137 mussels *per* tank (9 mussel *per* treatment), measuring the viability of hemocytes using the Trypan
138 blue exclusion method and MN frequency. The soft tissues of these mussels were then stored at -80
139 °C to evaluate the oxidative damage. We also collected the soft tissues of other 9 mussels *per*
140 treatment, then stored at -80 °C, to perform the measurement of antioxidant/detoxifying enzyme
141 activity. Lastly, the visceral masses (containing three nerve ganglia: one in foot, one near the
142 digestive gland and one near the gonads) without gills of other 3 mussels *per* tank (9 mussel *per*
143 treatment) were stored at -80 °C for the neurotoxicity assessment (Gagné and Blaise, 2003; Gagné
144 et al., 2010). Lastly, we collected 12 mussels *per* treatment (4 mussels *per* tank) at the end of
145 exposure (t = 6) for the confocal microscopy observations.
146
147 2.3 *Evaluation of polystyrene microbead uptake: confocal microscopy analyses*
148 To establish the uptake of PMs on zebra mussel tissues, we prepared 6 mussels *per*
149 treatment (n = 6) for cryostat analyses, considering that traditional preparation for paraffin
150 inclusion, with xylene, causes a plastic dissolution (Callebaut and Meeussen, 1989). Therefore, to
151 fix the mussel tissues, we firstly injected 4% paraformaldehyde in phosphate buffer saline solution
152 (PBS) 0.1 M at pH = 7.2 into the mussel adductor muscle, then we gently opened the valves with a
153 scalpel to make easier the preservative absorbance. The whole mussel body (shell included) was
154 inserted in tubes with 4% paraformaldehyde, then preserved at 4 °C. After fixation, we opened the
155 shell, cutting the muscles and ligaments, and placed the soft body mass (without *byssus*) in PBS for

45 min. We repeated this wash for three times. After that, we placed the soft tissues in 15% sucrose solution, for 2 h at room temperature and subsequently transferred the samples in a 30% sucrose solution overnight at 4 °C under agitation (150 rpm). We gently removed the cryoprotectant solution, using filter paper, and included the samples in the cryostat embedding medium (Bio Optica), maintaining the samples on dry ice. The samples were then stored at -80 °C. We obtained 30 µm cryostat transversal section of mussels, using the CM1850 cryostat (Leica, Wetzlar, Germany) and cutting the samples at 23 °C. We collected the sections on the Superfrost® Plus microscope slide (Thermo Scientific), then stained with ProLong® Gold antifade reagent with DAPI (Invitrogen). To investigate the eventual presence of PMs into the thickness of the sections, along the Z axis, we observed the samples using the Leica SP2 laser scanning confocal microscope (Leica Microsystems) exploiting the intrinsic reflection of PMs. To facilitate the detection of 10 µm and 1 µm PMs we characterized the PM standards in the aqueous matrix at confocal microscopy. To avoid the overestimation of 1 µm PM beads, we eliminated any interference of other materials in the mussel tissues by investigating the section thickness with serial scan of 1 µm. In addition, to exclude the possibility that observed PMs in the cryostat sections were originally on the surface of the sections, we performed the orthogonal projections of selected PMs using the software of confocal microscopy. To observe the uptake of PMs in the hemolymph, we collected 6 mussels *per* treatment. Before the sampling of the hemolymph, to exclude the eventual contamination by PMs present in the mantle cavity, we washed the shell with ultrapure water and drained the pallial water on filter paper. To collect hemolymph from the mussel abductor muscle we used a hypodermic syringe contained 100 µL of 10 mM EDTA/PBS to avoid hemocyte agglutination. To conserve the hemolymph samples at -80 °C, we added 10% DMSO and fetal calf serum (FBS) in PBS medium (90%) as cryoprotectant agent. To observe PMs in the hemolymph exploiting their intrinsic reflection, we added 500 µL of hemolymph-EDTA-FBS complex on the microscope glasses previously covered with poly-lysine, to facilitate the cell attachment. In addition, considering that

coccus bacteria present in the mussel hemolymph have the same size, shape and reflection of 1 µm PMs, we stained the hemolymph with 1 µg/mL Hoechst to exclude bacteria from PM detection.

2.4 *Evaluation of polystyrene microbead chronic toxicity: biomarker analyses*

Since the procedure for cellular stress, oxidative damage and genotoxicity biomarkers is highlighted in previous studies (Magni et al., 2016, 2017), in this paper we reported only a brief description of these methods.

2.4.1 *Biomarkers of cellular stress*

The kinetics of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the activity of detoxifying enzyme glutathione-S-transferase (GST) were evaluated in triplicate on the homogenate of 3 mussels collected from each tank (n = 3 pools of three mussels *per* treatment; 9 mussels *per* treatment). The soft tissues of mussels were homogenized in 100 mM phosphate buffer (pH = 7.4), 1:10 W/V ratio, with 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and protease inhibitors (1:100 v/v). The homogenates were centrifuged at 15,000 g (S15 fraction) for 30 min at 4 °C. Subsequently, after S15 protein quantification (Bradford, 1976), to normalize the enzyme kinetics, we processed the homogenates for the absorbance measurement of the enzymatic activity, using the 6715 UV/Vis spectrophotometer (Jenway), as reported by Orbea et al. (2002). The results were expressed as SOD units (U) mg $prot^{-1}$ (1 SOD unit = 50% inhibition of the xanthine oxidase reaction), nM of hydrogen peroxide (H_2O_2) consumption min^{-1} mg $prot^{-1}$ for CAT activity, µM of H_2O_2 consumption min^{-1} mg $prot^{-1}$ for GPx activity and mM of gluthatione and1-chloro-2,4-dinitrobenzene (GSH-CDNB) conjugated min^{-1} mg $prot^{-1}$ for GST activity.

2.4.2 *Biomarkers of oxidative damage*

206 The level of lipid peroxidation (LPO) and protein carbonyl content (PCC) were determined
207 in triplicate on the homogenate of 3 mussels collected from each tank (n = 3 pools of three mussels
208 *per* treatment; 9 mussels *per* treatment). The homogenates were obtained by homogenizing the soft
209 tissues of mussels in 100 mM phosphate buffer (pH = 7.4), 1:10 W/V ratio, with 100 mM KCl, 1
210 mM EDTA, 1 mM DTT and protease inhibitors (1:100 v/v). Subsequently, after homogenate
211 protein quantification (Bradford, 1976) to normalize the PCC, we processed the samples for LPO
212 and PCC assays (Ohkawa, 1979; Mecocci, 1999), measuring spectrophotometrically the
213 absorbance. The results for LPO were expressed as nM of thiobarbituric acid (TBA) reactants/g wet
214 weight (ww), while the results for PCC were expressed as nM of carbonyls mg $prot^{-1}$.
215
216 2.4.3 *Biomarkers of neuro- genotoxicity*

217 We evaluated the neurotoxicity on the homogenate of 3 mussels took from each tank (n = 3
218 pools of three mussels *per* treatment; 9 mussels *pe*r treatment), as described by Gagné (2014). The
219 homogenates were obtained pottering the mussel soft tissues in 25 mM HEPES-NaOH buffer (pH =
220 7.4), 1:5 W/V ratio, with 100 mM NaCl, 0.1 mM DTT and aprotinin as protease inhibitor.
221 Subsequently, we centrifuged a first aliquot of the homogenates at 15,000 g (S15) for 20 min at 4
222 °C and a second aliquot of the homogenates at 1,000 (S1) g for 20 min at 4 °C, and performed in
223 each sample the protein quantification (Bradford, 1976) to normalize all the biomarkers. Analyses
224 were performed using the multi-plate readers BioTek-Synergy 4 and BioTek-EON. For the
225 evaluation of the levels of neurotransmitters dopamine (DOP) and serotonin (SER) in mussel
226 tissues, we used a competitive enzyme-linked immunosorbent assay (ELISA). We quantified DOP
227 in S15 fraction; we coated the wells with 100 µL of 10 µg/mL conjugated BSA-DOP and incubated
228 the plate at 4 °C overnight. We emptied and washed the wells for three times with PBS;
229 subsequently we added in each well 250 µL of blocking buffer (1% fat dry milk in PBS) and
230 incubated the plate for 90 min at room temperature under agitation (150 rpm) and the wells were

231 washed once with PBS and added 50 µL of samples, or DOP standards for the calibration curve, to
232 the wells and 50 µL of primary antibody (rabbit IgG polyclonal to DOP glutaraldehyde BSA;
233 Abcam) and incubated the plate for 90 min at room temperature under agitation (150 rpm). The
234 wells were emptied and washed three times with PBS, 100 µL of secondary antibody (anti-rabbit
235 IgG, polyclonal antibody, conjugated with horseradish peroxidase; Enzo) was added and incubated
236 for 60 min at room temperature under agitation (150 rpm). The wells were washed three times with
237 PBS and added 100 µL of peroxidase substrate (BM Chemiluminescence ELISA Substrate POD;
238 Sigma-Aldrich) to the wells and incubated the plate for 3 min at room temperature in dark
239 condition. Luminescence was measured using a microplate luminescence reader (Synergy
240 microplate reader, USA). The results were expressed as µmol DOP mg $prot^{-1}$. For the quantification
241 of SER in the S1 fraction we used the Serotonin ELISA Kit (Enzo), reading the absorbance at 405
242 nm at room temperature. The results were expressed as ng SER mg $prot^{-1}$. For the quantification of
243 the neurotransmitter glutamate (GLU) in the S1 fraction, we used the Amplex® Red Glutamic
244 Acid/Glutamate Oxidase Assay Kit (Invitrogen), reading the fluorescence at 37 °C with excitation
245 at 540 nm and emission at 600 nm. The results were expressed as nmol GLU mg $prot^{-1}$. We
246 measured the activity of acetylcholinesterase (AChE) in the S15 fraction using the Ellman reagent
247 (Ellman et al., 1961), which contains 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM
248 Tris-acetate pH = 7.4, and 1 mM acetylthiocholine in 50 mM Tris-HCl as substrate. We assessed
249 the absorbance at 30 °C for 28 min (read interval of 1 min) at 412 nm. The results were expressed
250 as µmol of thiocholine formed min^{-1} mg $prot^{-1}$. The kinetics of monoamine oxidase (MAO) was
251 measured in S1 fraction using 1 mM tyramine as substrate, 10 µM dichlorofluorescein diacetate in a
252 140 mM NaCl, 10 mM HEPES-NaOH buffer, pH = 7.4, 1 mg/mL peroxidase and 10 mM of 3-
253 amino-1,2,4-triazole (catalase inhibitor). We measured the fluorescence for 3 min (read interval of
254 42 sec) with excitation at 485 nm and emission at 528 nm. The results were expressed as fluorescein
255 produced min^{-1} mg $prot^{-1}$. Regarding genotoxicity, we measured the frequency of micronuclei (MN)
256 in 9 mussels *per* treatment. The MN test was conducted as reported by Pavlica et al. (2000). In

particular, 400 hemocytes for each slide were counted (9 slides *per* each treatment; 1 slide per specimens) and micronuclei were checked using the criteria suggested by Kirsch-Volders et al. (2000). The results were expressed as frequency of micronuclei (‰).

2.4.4 *Statistical analyses*

To perform the statistical analyses, we used STATISTICA 7.0 software package. We verified the data normality and homoscedasticity using the Shapiro-Wilk and Levene tests respectively and identified the differences between treated and control performing a two-way analysis of variance (two-way ANOVA), where treatment (control, MIX 1 and MIX 2), time (t = 3 and t = 6) and their interaction (treatment *per* time) were predictor factors, and each biomarker was a dependent variable. To evaluate the differences (* $p < 0.05$; ** $p < 0.01$) between treated and control, time *versus* time, we used the Fisher LSD post-hoc test. Lastly, to observe the eventual covariation between tested biomarkers, we performed the Pearson's correlation considering all end-points.

3. RESULTS

3.1 *Microscopy observations*

Figure 1 illustrates how both 10 µm (A) and 1 µm (B) PMs can be view in bright field and in reflection mode, without aggregation phenomena.

Confocal observations of cryostat sections stained with DAPI highlighted the ingestion of the two types of PMs (Fig. 2A, B, C) and their concentration in the gut lumen, completely saturated mainly by the larger PMs. Although the identification of the smaller MPs was much more complicated in the mussel tissues than standards, considering the different matrices, their co-presence with 10 µm PMs in the gut lumen was observed (Fig. 2C). The merging of fluorescence, reflection and bright field acquisition at confocal microscope showed the capability at least of the 10 µm PMs to pass through the biological barriers, moving to the basal lamina of the gut epithelium

283 (Figs. 2B, 3A) and to reach the tissues of the digestive gland (Fig. 3B). Lastly, it was observed the
284 presence of 10 µm and 1 µm PMs (Fig. 4A, B, C) in the mussel hemolymph, close to the
285 hemocytes, demonstrating the translocation of these beads inside the organism. The PM uptake and
286 their infiltration in mussel tissues was confirmed by the orthogonal projections (Fig. 3) conducted
287 both at gut level and in digestive gland tissues.
288
289 3.2 *Biomarker responses*
290 During the exposures, the percentage of hemocyte viability was 93±6% for control group,
291 89±7% for MIX 1 and 91±1% for MIX 2, values much higher than the required 70% for
292 genotoxicity tests (Kirkland et al., 2007).
293 The two PM MIXs did not modulate the activity of the detoxifying enzyme GST since only
294 a marginally non-significant ($p = 0.087$, with $n = 3$ pools) increase of about 28% was noticed after
295 the MIX 1 exposure in comparison with the relative control (Fig. 5A). Regarding the antioxidant
296 enzymes, for SOD we obtained only a significant effect of treatment ($F_{2,12} = 6.46$; $p < 0.05$), and no
297 significant ($p > 0.05$) variations in the SOD activity during the exposure was observed (Fig. 5B),
298 while a significant ($p < 0.01$) increase of CAT was measured in mussels at the end of the MIX 1
299 exposure (Fig. 5C). For CAT activity we obtained indeed a significant effect of treatment ($F_{2,12} =$
300 5.59; $p < 0.05$) and of the interaction between time and treatment ($F_{2,12} = 4.22$; $p < 0.05$). MIX 1
301 caused a significant decrease ($p < 0.01$) of the GPx activity in mussels at $t = 3$ days with a recovery
302 to the control activity at the end of exposure, while MIX 2 did not influence GPx (Fig. 5D). We
303 obtained for GPx activity a significant effect of time ($F_{1,12} = 16.01$; $p < 0.01$) and of interaction time
304 to treatment ($F_{2,12} = 11.66$; $p < 0.01$). The low effects observed for the antioxidant activities were
305 also reflected by the lack of significant ($p > 0.05$) oxidative damage measured by LPO (Fig. 5E) and
306 PCC (Fig. 5F). For LPO we obtained indeed only a marginally non-significant effect of the
307 interaction time to treatment ($F_{2,12} = 3.70$; $p = 0.056$) and only a significant effect of time ($F_{1,12} =$
308 7.79; $p < 0.01$) and treatment ($F_{2,12} = 6.56$; $p < 0.01$), but a marginally non-significant effect of their

interaction ($F_{2,12}$ = 3.66; p = 0.057), for PCC. Regarding the neurotoxicity biomarkers, DOP amount was increased by both PM MIXs (Fig. 6A), with a significant interaction time and treatment ($F_{2,12}$ = 6.57; $p < 0.05$). In detail, we measured a significant ($p < 0.05$) increase of DOP after 3 days (+47% than relative control) when mussels were exposed to MIX 1, while the highest significant ($p < 0.01$) increase was obtained at the end of MIX 2 exposure (+65% than relative control). By contrast, the other measured neurotransmitters (SER and GLU) did not shown any significant ($p > 0.05$) variation (Fig. 6B, C); only for SER we observed a marginally non-significant effect of interaction time to treatment ($F_{2,12}$ = 3.59; p = 0.060). Even the activities of the two-selected neuro-enzymes (AChE and MAO) were not changed by MIX 1 and MIX 2 exposures (Fig. 6D, E); indeed, we obtained only a marginally non-significant effect of interaction time to treatment ($F_{2,12}$ = 3.70; p = 0.056) for AChE. The measurement of micronucleus frequency showed the lack of irreversible effects on the genetic material (Fig. 6F).

Lastly, we observed correlations (Tab.1) between: GST and CAT (r = 0.7074; p = 0.001), GST and GPx (r = 0.5805; p = 0.012), GST and SER (r = 0.4929; p = 0.038), CAT and SER (r = 0.4703; p = 0.049), CAT and MAO (r = 0.5377; p = 0.021), DOP and GLU (r = 0.5961; p = 0.009), MAO and SER (r = 0.7145; p = 0.001), MAO and AChE (r = 0.5126; p = 0.030).

4. DISCUSSION

The present study firstly highlights the capability of PMs to enter in the gastrointestinal tract of zebra mussel and to be transferred in the mussel tissues and hemolymph. The characterization of standard aqueous suspensions (Fig. 1A, B) facilitated the detection of PMs in the tissues of mussels, exploiting the intrinsic reflection of beads. However, Figure 2 shows that within the mussel tissues the PM reflection is much lower compared to standards. This aspect is probably due to the different matrix where beads are suspended, which may interfere with their reflection; in this context, the merging of collected images (Fig. 2B, C) can facilitate the localization of beads in the mussel tissues. Indeed, we observed a great concentration of MPs in the gut lumen of mussels exposed to

335 both MIXs (Fig. 2B), as previously observed also for other animals, such as vertebrates (Derraik,
336 2002) and invertebrates (Murphy and Quinn, 2018). A large amount of 10 μm PMs found in the gut
337 lumen (Fig. 2B) in comparison to the low presence of 1 μm PMs (Fig. 2C), could be only associated
338 to the difficulties in the detection of 1 μm beads in mussels, as previously described. Nevertheless,
339 the presence of MPs in the gut lumen have only a limited relevance in the light of toxicity since the
340 gastrointestinal tract is considered outer to the body and the fast elimination of MPs can cause only
341 mechanical effects on the gut structures. The study highlighted that even the 10 μm PMs are able to
342 penetrate the epithelium (Fig. 3A) but also the digestive gland tissues (Fig. 3B), close to the mantle
343 cavity. This observation clearly demonstrates that at least the 10 μm PMs are able to pass the
344 biological membranes and move into the mussel tissues, while this phenomenon needs to be further
345 investigated for 1 μm PMs due to the limited instrumental resolution.

346 Our results regarding the PM uptake in the gut lumen and in the tissues of zebra mussel are
347 in accordance with other observations in *Mytilus* spp. and *Crassostrea gigas* exposed to polystyrene
348 and polyethylene beads with a variable range < 100 μm (von Moos et al., 2012; Avio et al., 2015;
349 Paul-Pont et al., 2016; Sussarellu et al., 2016). Therefore, the capability of MPs to penetrate
350 bivalves' tissues suggests that zebra mussel can be a useful bio-indicator of MP pollution in
351 freshwater. Probably the size of MPs, with similar dimension of plankton and suspended matter,
352 and the high filtration rate of zebra mussel (200 mL/bivalve/h; Ackerman, 1999), are the main
353 factors involved in the considerable MP uptake of this filter-feeding organism.

354 Another important finding is the detection of both 10 μm and 1 μm PMs in the mussel's
355 hemolymph (Fig. 4B, C). Indeed, the PM presence in the circulatory system confirms that these
356 MPs are able to pass-through the gut epithelium interface, translocate in the soft tissues (Fig. 2B, C)
357 and then in hemolymph (Fig. 4B, C). Our observations are supported by Browne et al. (2008) who
358 reported the presence of 3 μm and 9.6 μm PMs in the hemolymph of *Mytilus edulis*. Moreover, MPs
359 with a dimension < 100 μm were also found in the hemolymph of *Mytilus galloprovincialis* by Avio
360 et al. (2015). Recently, Farrell and Nelson (2013) highlighted the transfer of MPs from *Mytilus*

361 *edulis* to the hemolymph of its predator *Carcinus maenas*, showing the capability of MPs to pass
362 through the food chain and this poses a serious threat to human food safety especially with edible
363 marine mussels (Van Cauwenberghe and Janssen, 2014). Indeed, microparticulates with a range
364 from 0.03 to 150 μm have been demonstrated to be transferred in humans, dogs and rodents across
365 the gut lumen towards other tissues, as lymphatic system (Hussain et al., 2001), due to the
366 microfold cells (M cells) in the Peyer's patches of gut epithelium (Van Cauwenberghe and Janssen,
367 2014).

368 Apparently, the important concentration of PMs in the gut lumen of zebra mussel and the
369 following infiltration in tissues and hemolymph do not cause neither particular biological responses
370 nor affect the cellular functionality at least within the duration of the experiment. We observed
371 significant ($p < 0.01$) alterations only for CAT and GPx activity (Fig. 5C, D), as well as for DOP
372 levels (Fig. 6A). The DOP is a catecholamine mainly found in nerve ganglia and gonads that acts as
373 neuro-hormone in mollusks, regulating gametogenesis and spawning (Fong et al., 1993), with the
374 indolamine SER which is more involved in the final maturation stage and spawning. More
375 specifically, DOP regulates oogenesis (Khotimchenko, 1991) but also the inhibition of the cilia
376 movement in bivalves (Stefano and Aiello, 1975; Smith, 1982), especially in gills (Fong et al.,
377 1993). Therefore, the significant increase of DOP, which is observed in mussels exposed to both
378 MIX 1 ($p < 0.05$ at $t = 3$) and MIX 2 ($p < 0.01$ at $t = 6$), could be explained as a defense mechanism
379 of zebra mussel due to the reduction of the entrance of PMs across the inhalant siphon. Perhaps, the
380 presence of PMs in the gut lumen (Fig. 2B, C), and probably in the gills (several 10 μm PMs were
381 observed in fresh gills by optical microscope, data not shown), can induce mussels to reduce the
382 filtration rate. Indeed, the recent study of Murphy and Quinn (2018) reported that polyethylene
383 flakes fill the gastric cavity of *Hydra attenuata*, reducing its feeding rate. This is further
384 corroborated by the observation of increasing DOP concentrations increasingly inhibited food
385 uptake in *Mytilus edulis* veliger larvae while SER and norepinephrine increased food uptake (Beiras

386 et al., 1995). Hence, the increase of DOP levels could have been involved in feeding activity as part
387 of defence mechanisms in mussels.
388 On the contrary, Gardiner et al. (1991) reported that DOP increases the cilia activity in unionids.
389 Therefore, another hypothesis might be that the significant increase of DOP could be associated to
390 an enhancement of cilia movement in the gut epithelium and gills to eliminate the PMs, in a manner
391 similar to pseudofaeces. In this context, the eventual increase of filtration/respiration rate could be
392 associated to a major energy consumption (Van Cauwenberghe et al., 2015) and, perhaps, to the
393 decrease of energy storage, as observed in lugworms exposed to MPs (Wright et al., 2013). More
394 experiments would be required to examine more closely ciliary activity in mussels exposed to small
395 MPs.
396 Since the autoxidation or enzyme degradation processes of DOP produce reactive oxygen
397 species (ROS; Luo and Roth, 2000), it is possible that the significant increase ($p < 0.01$ at $t = 6$) of
398 CAT and the significant decrease ($p < 0.01$ at $t = 3$) of GPx (substrate inhibition) activities in
399 mussels exposed to MIX 1 is due to the formation of the H_2O_2 during the DOP deamination reaction
400 catalyzed by MAO, as suggested by Spina and Cohen (1989). Indeed, the specimens exposed to the
401 lowest concentration of PMs (MIX 1) could adapt themselves during the exposure, and the
402 significant increase of DOP at t = 3 days is consequently reduced by MAO at the end of exposure (t
403 = 6; Fig. 6A), causing the alteration of the oxidative balance. On the other hand, we did not obtain
404 an oscillatory trend for DOP in the mussels exposed to MIX 2 since the higher amount of PMs
405 could produce a high generic stress in exposed specimens, showing that the high levels of DOP are
406 fundamental in the reduction of PM uptake in zebra mussels, as described above. In this context, as
407 suggested by Rist et al. (2016) in a study on *Perna viridis* exposed for 91 days to polyvinylchloride
408 MPs, the stress exerted by MPs leads to valve closure and a reduction of respiration rate with no
409 immediate consequence given the relatively short exposure times in the present study. At the same
410 time, it is important to consider that we did not obtain a significant increase of MAO activity during
411 the exposure and the level of DOP was not correlated with MAO activity ($p = 0.648$), even if the

activities of CAT and MAO show similar trends and significant positive correlation ($p = 0.021$), as reported in Table 1. According to our results, low levels of oxidative stress/damage were obtained also by Avio et al. (2015) on *Mytilus galloprovincialis* exposed to MPs for 7 days.

Since we debated two contrary hypotheses regarding the phenomenon associated with DOP increase, in future studies will be necessary the evaluation of other physiological end-points, as the filtration rate, to validate one of these suggestions. Given that SER levels were not affected, it appears that former hypothesis is more likely i.e., DOP increase decrease feeding rate activity since an increase of SER would have followed feeding activity. The lack of significant alteration of AChE activity in treated mussels, in contrast with other studies on fishes (Oliveira et al., 2013; Barboza et al., 2018) and bivalves (Avio et al., 2015; Ribeiro et al., 2017), could be associated to the difference in the selected tissues for the analyses. Indeed, while Avio et al. (2015) and Ribeiro et al. (2017) evaluated AChE activity in gills of *Mytilus galloprovincialis* and *Scrobicularia plana* respectively, we assessed all neurotoxicity end-points in the whole visceral mass (without gills), which could have decreased the responses to PMs. The lack of increased AChE activity and SER with increased DOP is consistent with the cilio-inhibitory action of DOP (Aiello et al., 1986) as explained above. However, DOP levels were significantly correlated ($p = 0.009$) with GLU levels in the visceral mass which suggests a neuro-excitatory state induced by MPs concentration in the gut lumen. This suggests that mussels are actively involved in the "digestion/assimilation" process when exposed to MPs at first which is followed by decreased GLU levels as the concentrations in MPs increase.

Lastly, considering that the significant increase of DOP can be a defense mechanism towards PMs through reduced intake and elimination phenomena, we can assert that PMs, contextually to our experimental design, do not have direct adverse effects on the nervous system of zebra mussel. Probably the bivalves, being a suspension feeder, are adapted to fight more abrasive items than plastic beads, such as sands and diatom frustules. As suggested by Rist et al. (2016), MPs should be currently considered as a new component of seston and their effects on bivalves

438 could be comparable to natural suspended matter. Therefore, in future studies it will be pivotal to
439 investigate better these factors, prolonging the exposure to observe eventual adverse effects on
440 longer times, as well as to deepen the knowledge regarding possible oscillatory behavior (wave
441 trend) of assessed end-points, which represents an interesting aspect about the biomarker
442 interpretation in ecotoxicology (Gagné, 2016; André and Gagné, 2017).
443
444 5. CONCLUSIONS
445 Our results confirm the uptake of 10 μm and 1 μm PMs in the gut lumen, tissues and
446 circulatory system of zebra mussel after only 6 days of exposure. However, despite the
447 accumulation of PMs in the exposed mussels, our results highlight that PMs did not induce great
448 alteration of both oxidative balance and neuro- genotoxicity in zebra mussel, for the selected end-
449 points and exposure time. Significant increase of DOP level can be considered only an indirect
450 neurotoxic effect of PMs, to either promote their elimination or reduce intake of MPs in exposed
451 organisms. However, considering the important uptake of PMs in the tissues and gut lumen of
452 bivalves, it will be interesting to evaluate the inflammation and the modulation of the energy
453 storage in the exposed mussels. At the same time, it will be important to investigate also the carrier
454 role of MPs towards chemical pollutants, as well as the effect of plastic debris using the "omics"
455 techniques, to completely characterize the MP toxic action on freshwater species. Lastly, this is a
456 first study about the evaluation of oxidative stress/damage and neuro- genotoxicity of virgin MP in
457 zebra mussel where the presence findings will stimulate further investigation on the influence of
458 MPs on feeding behavior and energy reserves.
459
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465

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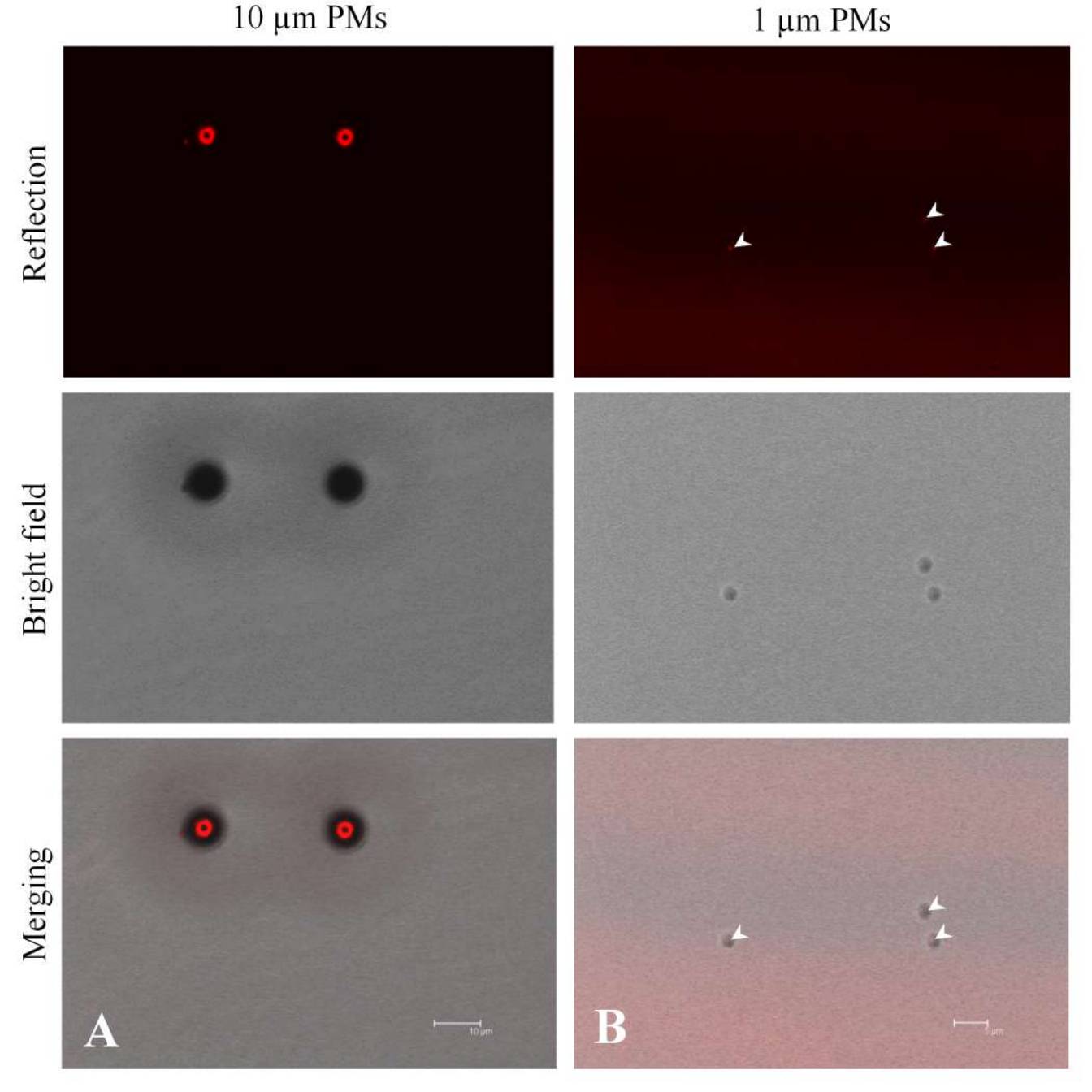
657 Captions:
658 Figure 1: Characterization of standard aqueous suspensions of 10 μm (A) and 1 μm (B; see the
659 white arrows) PMs at confocal microscopy in reflection mode (red reflection).
660
661 Figure 2: 30 μm cryostat transversal sections of zebra mussel specimens (n = 6 mussels *per*
662 treatment) observed at confocal microscopy at the end of exposure (t = 6). In blue are shown the
663 cell nuclei stained with DAPI and in red the PM reflection. It is possible to observe the control (A)
664 and the uptake of both 10 μm (B) and 1 μm (C; see the white arrows and zoom) PMs in the gut
665 lumen and tissues of exposed mussels.

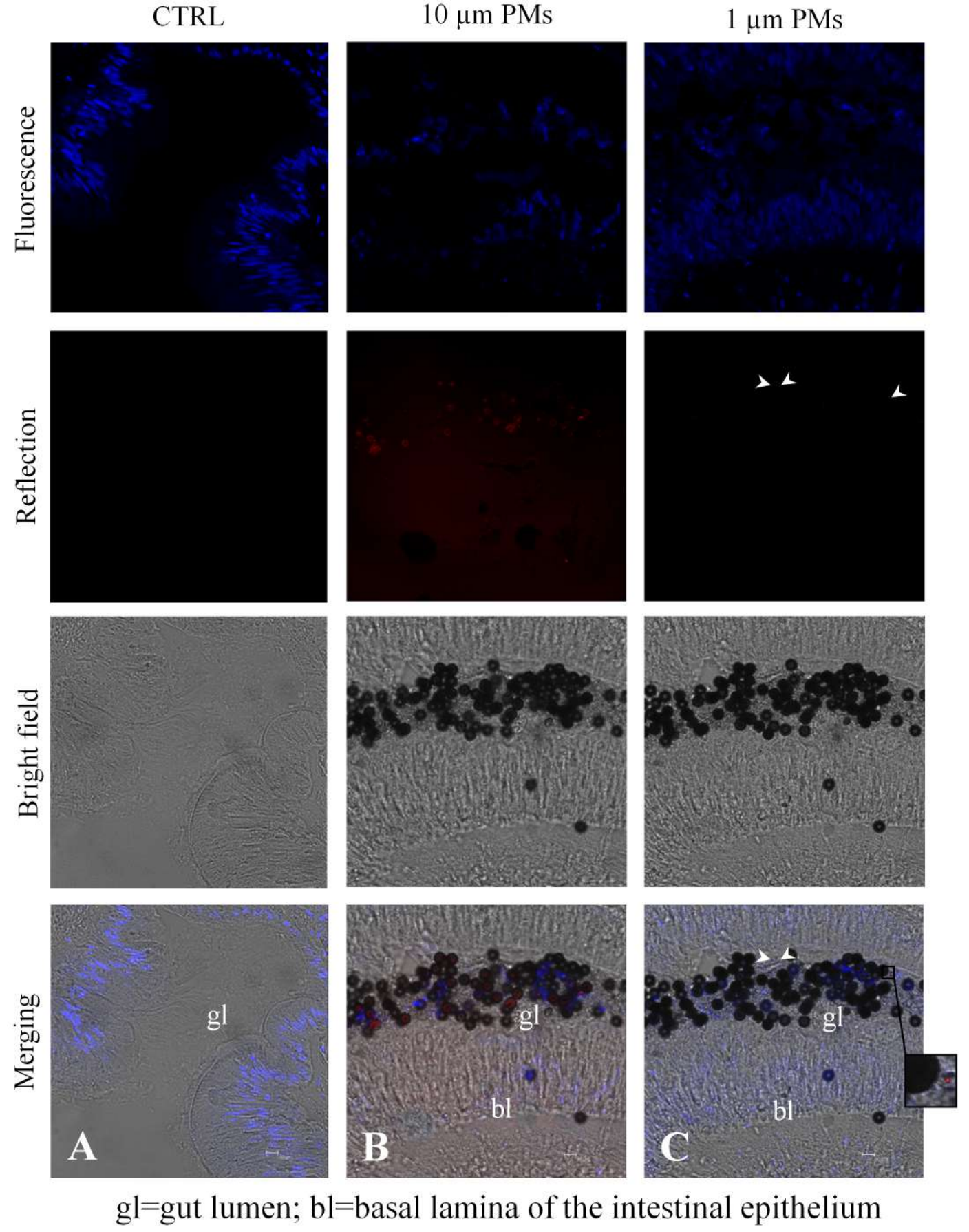
Figure 3: Orthogonal projection of 10 µm PMs in the 30 µm cryostat transversal sections of zebra mussel. In blue are shown the cell nuclei stained with DAPI. It is possible to observe that 10 µm PM at the level of the basal lamina epithelium (A) and in the digestive gland tissue (B) are into the section thickness, as highlighted by the white arrows in the orthogonal projection line intersection.

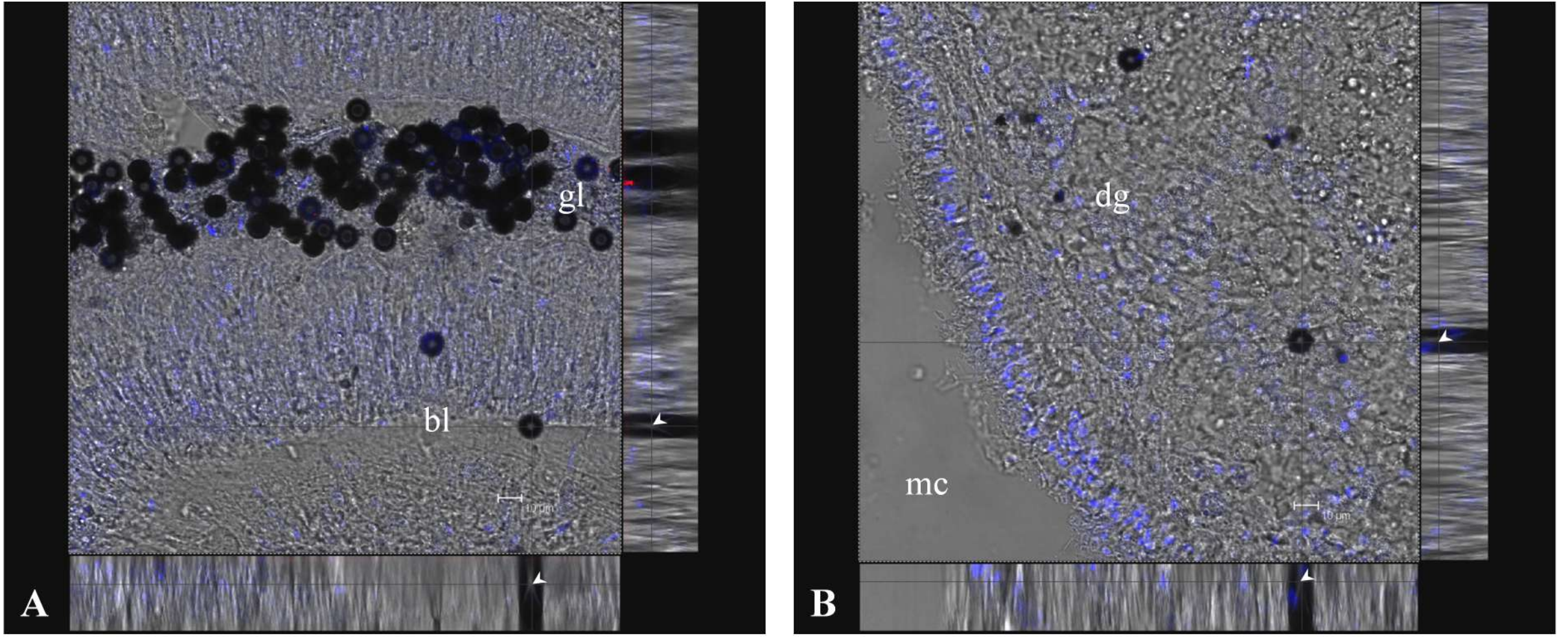
Figure 4: Zebra mussel hemolymph (n = 6 mussels *per* treatment) observed at confocal microscopy in reflection mode (red reflection) at the end of exposure (t = 6). It is possible to observe the control (A) and the uptake of 10 µm (B) and 1 µm (C; see the white arrow and zoom) PMs in the mussel hemolymph close to the hemocytes.

Figure 5: Cellular stress (activity of detoxifying/antioxidant enzymes A-GST, B-SOD, C-CAT and D-GPx) and oxidative damage (levels of LPO and PCC) in zebra mussel soft tissues (n=3 pools of three mussels *per* treatment; 9 mussels *per* treatment) during 6 days of exposure (from t = 0 to t = 6 days) at 10 µm and 1 µm PM MIXs (mean±SD). Asterisks indicate the significant differences, time *versus* time, between MIXs and control (two-way ANOVA, Fisher LSD post-hoc test: * $p < 0.05$, ** $p < 0.01$). The green line indicates the baseline level (t = 0) of considered end-point.

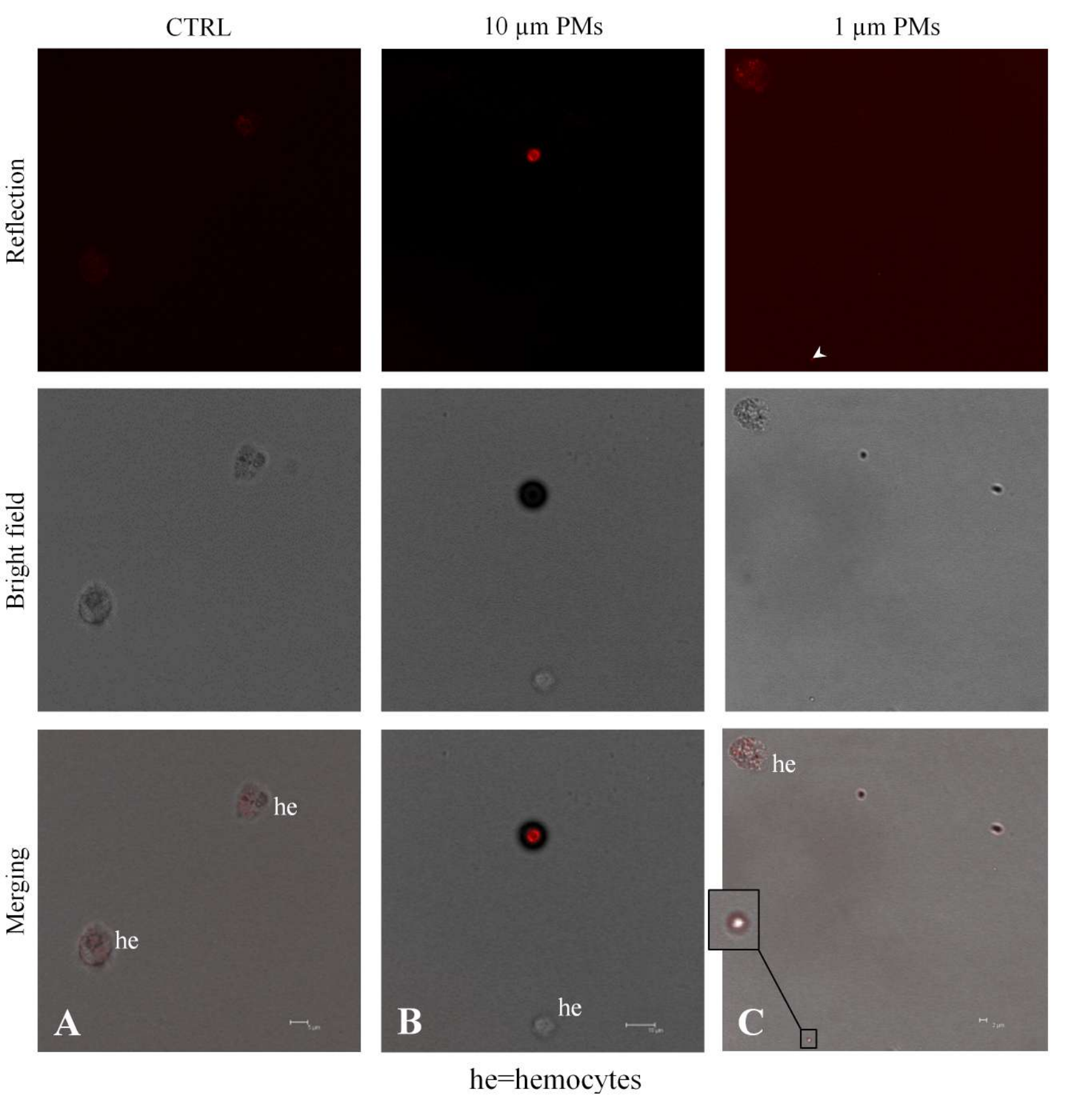
Figure 6: Neurotoxicity (levels of neurotransmitters A-DOP, B-SER and C-GLU and activity of neuro-enzymes D-AChE and E-MAO) in zebra mussel soft tissues without gills (n = 3 pools of three mussels *per* treatment; 9 mussels *per* treatment) and genotoxicity (frequency of F-MN) in zebra mussel hemocytes (n = 9 mussels *per* treatment) during 6 days of exposure (from t = 0 to t = 6 days) at 10 µm and 1 µm PM MIXs (mean±SD). Asterisks indicate the significant differences, time versus time, between MIXs and control (two-way ANOVA, Fisher LSD post-hoc test: * $p < 0.05$, ** $p < 0.01$). The green line indicates the baseline level (t = 0) of considered end-point.

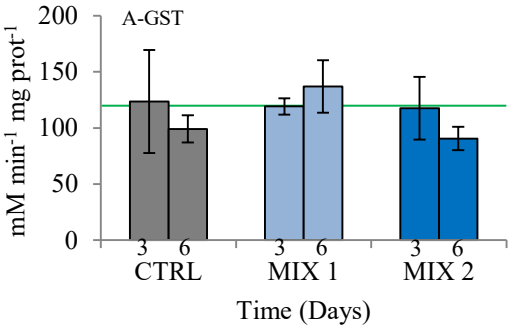

10 µm PMs
1 µm PMs
Reflection
Bright field
Merging
A
B

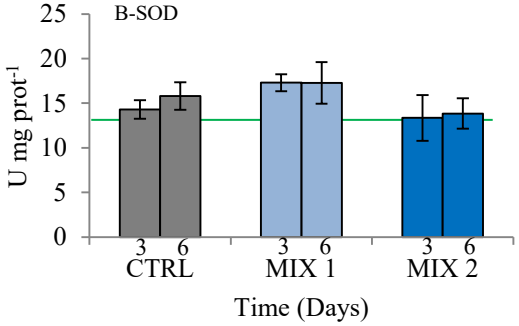

CTRL
10 µm PMs
1 µm PMs
Fluorescence
Reflection
Bright field
Merging
gl
bl
A
B
C
gl=gut lumen; bl=basal lamina of the intestinal epithelium

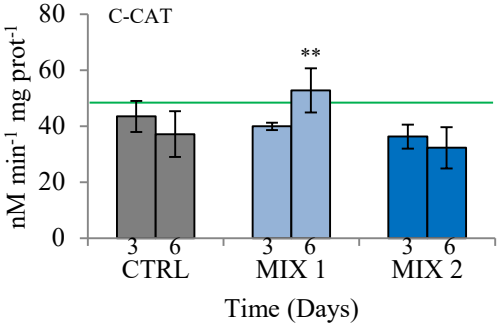


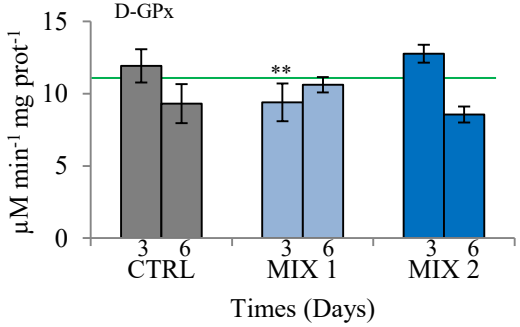
gl=gut lumen; mc=mantle cavity; bl=basal lamina of the intestinal epithelium; dg=digestive gland

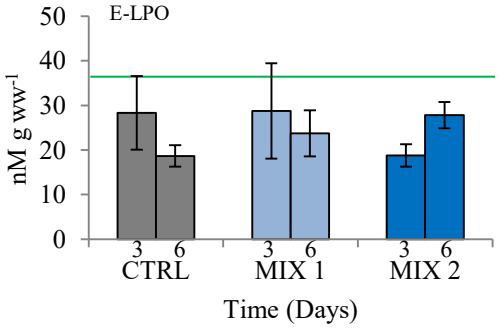

CTRL
10 µm PMs
1 µm PMs
Reflection
Bright field
Merging
he
he
A
he
B
he
C
he=hemocytes

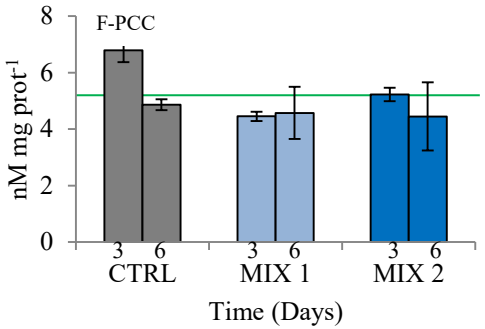

A-GST
mM min-1 mg prot-1
200
150
100
50
0
3 6
CTRL
MIX 1
MIX 2
Time (Days)

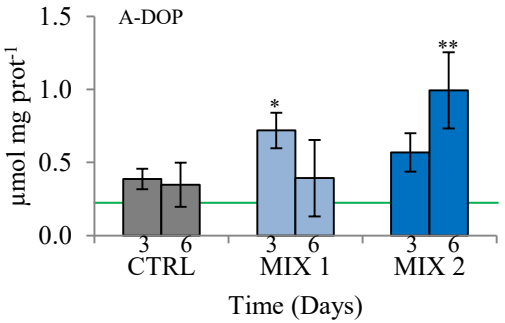

B-SOD
U mg prot-1
25
20
15
10
5
0
3 6
CTRL
MIX 1
MIX 2
Time (Days)

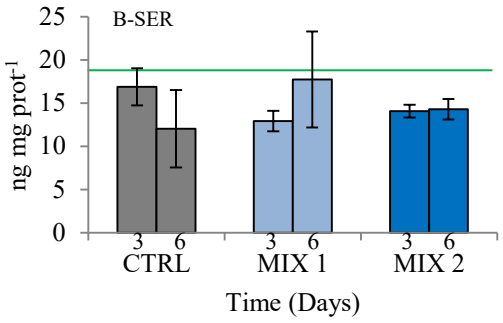

C-CAT
nM min-1 mg prot-1
80
60
40
20
0
**
3 6
CTRL
MIX 1
MIX 2
Time (Days)

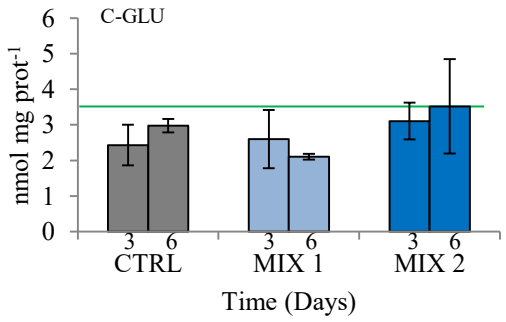

D-GPx
µM min-1 mg prot-1
15
10
5
0
**
3 6
CTRL
MIX 1
MIX 2
Times (Days)

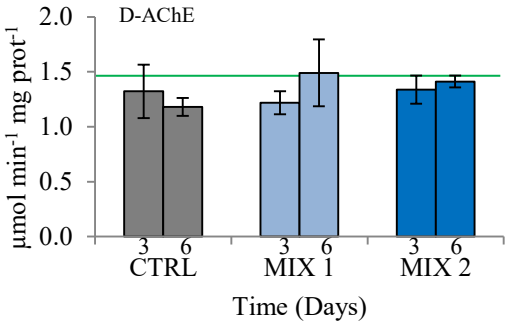

E-LPO
nM g ww-1
50
40
30
20
10
0
3 6
CTRL
MIX 1
MIX 2
Time (Days)

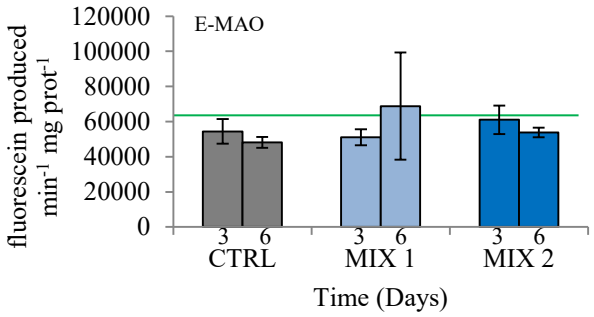

F-PCC
nM mg prot-1
8
6
4
2
0
3 6
CTRL
MIX 1
MIX 2
Time (Days)

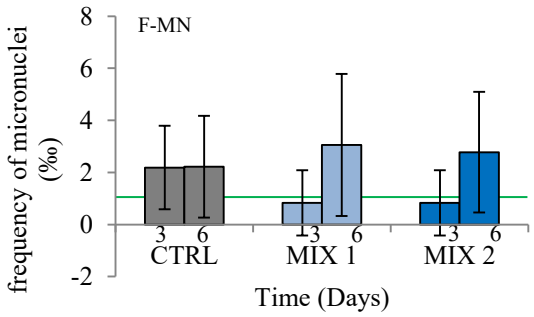

A-DOP
µmol mg prot-1
1.5
1.0
0.5
0.0
*
**
3 6
CTRL
3 6
MIX 1
3 6
MIX 2
Time (Days)


B-SER
ng mg prot-1
25
20
15
10
5
0
3 6
CTRL
3 6
MIX 1
3 6
MIX 2
Time (Days)


C-GLU
nmol mg prot-1
6
5
4
3
2
1
0
3 6
CTRL
3 6
MIX 1
3 6
MIX 2
Time (Days)


D-AChE
µmol min-1 mg prot-1
2.0
1.5
1.0
0.5
0.0
3 6
CTRL
3 6
MIX 1
3 6
MIX 2
Time (Days)


E-MAO
fluorescein produced min-1 mg prot-1
120000
100000
80000
60000
40000
20000
0
3 6
CTRL
3 6
MIX 1
3 6
MIX 2
Time (Days)


F-MN
frequency of micronuclei (‰)
8
6
4
2
0
-2
3 6
CTRL
3 6
MIX 1
3 6
MIX 2
Time (Days)

	SOD	CAT	GPx	GST	LPO	PCC	MN	DOP	SER	GLUT	MAO	AChE
SOD	1.0000 p= ---	0.3547 p=0.149	-0.1874 p=0.457	0.2195 p=0.382	0.3203 p=0.195	-0.4129 p=0.089	-0.1728 p=0.493	-0.2021 p=0.421	0.1187 p=0.639	-0.3256 p=0.187	0.3013 p=0.224	0.1954 p=0.437
CAT		1.0000 p= ---	0.2525 p=0.312	**0.7074** **p=0.001**	0.0232 p=0.927	-0.0651 p=0.797	0.1664 p=0.509	-0.3048 p=0.219	**0.4703** **p=0.049**	-0.2499 p=0.317	**0.5377** **p=0.021**	0.2682 p=0.282
GPx			1.0000 p= ---	**0.5805** **p=0.012**	-0.0793 p=0.755	0.4360 p=0.070	-0.2585 p=0.300	-0.3730 p=0.127	0.3479 p=0.157	-0.1364 p=0.589	0.3148 p=0.203	0.0355 p=0.889
GST				1.0000 p= ---	-0.0567 p=0.823	0.0492 p=0.846	-0.2023 p=0.421	-0.1951 p=0.438	**0.4929** **p=0.038**	-0.1749 p=0.488	0.4436 p=0.065	0.0010 p=0.997
LPO					1.0000 p= ---	0.0593 p=0.815	-0.0526 p=0.836	0.2917 p=0.240	0.2441 p=0.329	-0.1574 p=0.533	0.2158 p=0.390	-0.0332 p=0.896
PCC						1.0000 p= ---	0.2327 p=0.353	-0.3776 p=0.122	0.0596 p=0.814	-0.2529 p=0.311	-0.2592 p=0.299	-0.2517 p=0.314
MN							1.0000 p= ---	0.0398 p=0.875	-0.0784 p=0.757	-0.0834 p=0.742	-0.0841 p=0.740	0.2812 p=0.258
DOP								1.0000 p= ---	-0.1676 p=0.506	**0.5961** **p=0.009**	-0.1154 p=0.648	-0.1291 p=0.610
SER									1.0000 p= ---	-0.2377 p=0.342	**0.7145** **p=0.001**	0.3162 p=0.201
GLUT										1.0000 p= ---	-0.1090 p=0.667	-0.1747 p=0.488
MAO											1.0000 p= ---	**0.5126** **p=0.030**
AChE												1.0000 p= ---

Table 1: Matrix of Pearson's correlation for all considered end-points: activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), activity of detoxifying enzyme glutathione-S-transferase (GST), level of lipid peroxidation (LPO) and protein carbonyl content (PCC), frequency of micronuclei (MN), levels of neurotransmitters dopamine (DOP), serotonin (SER) and glutamate (GLU), activity of neuro-enzymes acetylcholinesterase (AChE) and monoamine oxidase (MAO). Significant correlations ($p < 0.05$) are reported in bold.