Plastics and biodegradable plastics: ecotoxicity comparison between polyvinylchloride and Mater-Bi® micro-debris in a freshwater biological model

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ABSTRACT

The improper release of plastic items and wastes is nowadays one of the main environmental and social problems, whose solution or mitigation represents a great challenge worldwide. In this context, the growing use of the so-called biodegradable plastics could represent a possible solution in the short to medium term. The few information known about the ecological impact of these materials on freshwater organisms, especially the ones relative to the micro-debris derived from their aging, prompted us to study the comparison of the sub-lethal effects eventually caused by plastic and biodegradable plastic micro-debris on the mussel *Dreissena polymorpha*, which represents an excellent biological model for the freshwater ecosystems. We selected two powders of polyvinylchloride (PVC) and Mater-Bi® administered at 1 mg/L to *D. polymorpha* specimens in semi-static conditions for 14 days. The presence of micro-debris was evaluated on mussel tissues and pseudo-faeces using advanced microscopy techniques. The sub-lethal effects were investigated on exposed mussels at 6 and 14 days using a suite of biomarkers of cellular stress, oxidative damage, and genotoxicity. Lastly, we compared the ecotoxicity of these two materials integrating each endpoint in the Biomarker Response Index. Microscopy observations highlighted the

surprising absence of micro-debris in the gut lumen and tissues of exposed mussels, but the presence of both PVC and Mater-Bi® micro-debris in the pseudo-faeces, suggesting a possible efficient elimination mechanism adopted by mussels to avoid the micro-debris gulping. Consequently, we did not observe significant sub-lethal effects, except for the glutathione-S-transferase activity modulation after 6 days of exposure.

Keywords:

Ecological impact, *plastics, biodegradable plastics, sub-lethal effects, freshwater ecosystems*

1. INTRODUCTION

Plastics are polymers of several elements containing additives to modulate their physico-chemical characteristics (Kale et al., 2015). In the last decades, these chip and supposed inert materials improved the quality of human life. Nevertheless, nowadays, after their indiscriminate production and uncorrected disposal, plastic pollution represents an emerging global issue whose solution and/or mitigation is one of the main challenges of modern society. Indeed, although the issue of plastics released into the environment is widely known to scientists and above all to citizens, plastic production is still following a positive trend, going from 335 million tons in 2016 (60 million tons only in Europe) to 348 million tons in 2017 (64.4 million tons only in Europe; PlasticsEurope, 2018). China is the first plastic producer worldwide, with a percentage of about 29% and Asia reached 50% of global production of plastics (PlasticsEurope, 2018). Despite their recycling increased up to 79% and their landfill storage decreased by 43% from 2006 to 2016 (PlasticsEurope, 2018), plastics continue to be detected in the marine environment (Napper and Thompson, 2019) and freshwater ecosystems of Europe, America, Asia, and Africa (Free et al., 2014; Lechner et al., 2014; Wagner et al., 2014; Su et al., 2016; Anderson et al., 2017; Wang et al., 2017; Di and Wang, 2018, Nel et al., 2018; Ding et al., 2019; Binelli et al., 2020). Oceans represent the final compartment of plastic accumulation, and both freshwaters and continental areas

55 contribute about 80% to the global marine plastic pollution, in particular with a constant release of
56 plastic micro-debris (MDs; Andrady et al., 2011). Plastic MDs were usually defined as plastic
57 debris with a dimension < 5 mm. However, Hartmann et al. (2019) recently suggested a new and
58 clearer dimensional plastic classification as follows: nanoplastics < 1 μm, microplastics 1-1000 μm,
59 mesoplastics 1-10 mm and macroplastics > 1 cm. One of the main sources of MDs toward
60 freshwaters is represented by the wastewater treatment plants (Browne et al., 2011; Mason et al.,
61 2016; Murphy et al., 2016; Leslie et al., 2017; Mintening et al., 2017; Lares et al., 2018; Magni et
62 al., 2019a), which transfer plastics derived by our daily actions from the anthropic environments to
63 the natural ones. In this regard, we can define primary MDs the plastic items contained in the
64 personal care products as abrasive agents, produced intentionally with micrometric structure, while
65 the secondary MDs originate directly in the environment from the aging of macroplastics, due to the
66 abrasion and bio- or photo-degradation (Cole et al., 2011 and citations therein). Among the plethora
67 of MDs present in freshwaters, the main detected polymer classes are polystyrene (PS),
68 polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC) and polyesters (PEST; Klein et al.,
69 2015; Di and Wang, 2018; Sighicelli et al., 2018; Binelli et al., 2020). In particular, PVC, being
70 denser than water (1.35-1.70 g/cm^3; Crawford and Quinn, 2017), seems to be more abundant at the
71 bottom of water bodies (Di and Wang, 2018). Several studies demonstrated that these contaminants
72 induce an increase of inflammation, oxidative stress, neurotoxicity, protein modulation and
73 developmental alteration in exposed freshwater organisms (Lu et al., 2016; Magni et al., 2018,
74 2019b; Parenti et al., 2019; Binelli et al., 2020; Malafaia et al., 2020). Another important point is
75 the ability of MDs to accumulate in the gut lumen and infiltrate in the circulatory system and tissues
76 of biota (Magni et al., 2018, 2019b; Parenti et al., 2019), an aspect associated to the transport of
77 plastic MDs through the food web (Wang et al., 2019). Considering that the plastic ban is probably
78 unrealistic and that the main problem related to plastic pollution is predominantly the release of
79 single-use objects in the environment, an alternative to these products could be represented by the
80 biodegradable plastics, in the short to medium term at least. Several countries worldwide banned the

use of plastic bags (UNEP, 2018), replacing the shoppers with bags of biodegradable plastics, such as starch-like polymers (Shah et al., 2008). In this context, the so-called Mater-Bi® (a biodegradable plastic produced by Novamont S.p.A. (Italy) and originally developed in the 90's) is currently used in packaging to produce shoppers or waste bags (Shen et al., 2009). At the end of its life, the Mater-Bi® is transformed in harmless compounds suitable for eventual agricultural uses in specific composting plants. A previous study conducted by Sforzini et al. (2016) on edaphic (*Eisenia andrei*) and freshwater organisms (*Pseudokirchneriella subcapitata*, *Daphnia magna*), as well as on plants (*Sorghum saccharatum*, *Lepidium sativum*), pointed out that high concentrations of the powders obtained by the Mater-Bi® films did not induce adverse effects. On the basis of this evidence and considering the few information regarding the impact of plastic MDs in freshwaters, the goal of this study was the evaluation and comparison of the sub-lethal effects induced by PVC (one of the most used plastic polymer) and the biodegradable plastic Mater-Bi® on the freshwater mussel *Dreissena polymorpha*, which is a representative species of aquatic ecosystems commonly used in the ecotoxicological studies (Binelli et al., 2015 and citations therein). We exposed several mussels to 1 mg/L of PVC or Mater-Bi® MDs for 14 days in semi-static condition. At the end of exposure, we evaluated their presence in the gut lumen, tissues and pseudo-faeces using advanced microscopy techniques. Considering some evidence on the increase of the oxidative stress/damage in various organisms (Avio et al., 2015; Espinosa et al., 2019; Magni et al., 2019b; Qiao et al., 2019; Binelli et al., 2020) caused by plastic MDs, in this study we focused on the measurement of many endpoints related to cellular stress. On account of this, the sub-lethal effects were evaluated at 6 and 14 days using a battery of biomarkers, measuring the total content of reactive oxygen species (ROS) as well as the activity of antioxidant/detoxifying enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST). In addition, we assessed biomarkers of oxidative damage, the levels of lipid peroxidation (LPO) and protein carbonylation content (PCC), and some endpoints of genotoxicity, measuring the frequencies of apoptosis, necrosis and micronuclei (MN) in mussel hemocytes. Lastly, we integrated each response

in the Biomarker Response Index to compare the ecotoxicity of tested materials, reducing the variability of the considered biomarker responses.

2. MATERIALS AND METHODS

2.1 *MD production and polymer characterization*

To compare the ecotoxicity of the two types of MDs, we selected two materials, used to produce everyday objects: PVC (density of 1.38 g/cm^3) and Mater-Bi® HF03V2 (density of 1.28 g/cm^3), a commercial biodegradable and compostable plastic produced and gently provided by Novamont S.p.A. (Italy). Mater-Bi® HF03V2 is composed by about 65% of biodegradable polyester (made with monomers biodegradable in the soil; Siotto et al., 2011), starch (about 28%) and a bio-based biodegradable polyol (about 6%), which is a natural plasticizer. Polyol is completely biodegraded within 28 days under aqueous aerobic conditions at ambient temperature (Organic Waste Systems, Belgium, data not shown). The selected plastic raw materials were coarse powder for PVC, and pellets of some mm for Mater-Bi®. Hence, a preliminary cryogenic grinding with liquid nitrogen of both materials was carried out, to obtain MDs suitable for the mussel exposure. Although *D. polymorpha* specimens ingest suspended particulate with a dimension up to ~ 40 µm (Winkel and Davids, 1982), this bivalve has an inhalant siphon with a diameter of about 1 mm that allows the entrance of debris even bigger than 40 µm in the pallial cavity (Binelli et al., 2020). To confirm the chemical nature of selected materials, we analyzed the obtained powders by infrared spectroscopy using a Fourier Transform Infrared Microscope System (µFT-IR; Spotlight 200i equipped with Spectrum two, PerkinElmer). The spectra of PVC and Mater-Bi® were obtained in attenuated total reflectance (ATR) between 500 and 4.000 cm^{-1}, acquiring 32 scans each, and using the Spectrum 10 software (PerkinElmer) for data elaboration (matching between substance and library spectra; Magni et al., 2019a). To ascertain that the obtained powders had similar physical characteristics, we measured the dimensions (major length) of MDs, analyzing images (ImageJ software; Ferreira and Rasband, 2012) acquired on suspensions of PVC or Mater-Bi® (50 mg/mL) by the Jenaval light

133 microscope endowed with a DeltaPix Invenio 3S 3M CMOS camera. In addition, we further
134 characterized MDs by determining the surface charge, morphology and reflectance properties using
135 Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM) and Confocal Microscopy
136 (CM), respectively. CM in reflection mode was fundamental to detect *non*-fluorescent debris in the
137 tissues of exposed organisms. In detail, the surface charge, Zeta potential (ζ-potential) of PVC and
138 Mater-Bi® MDs was measured using a DLS Malvern Zetasizer Nano ZS instrument (equipped with
139 a solid-state He-Ne laser operating at a wavelength of 633 nm and with the equipment for the Zeta
140 potential measurement) on MD MilliQ® water suspensions (1 mg/mL). The measurement was
141 repeated 3 times using a disposable cuvette (Folded Capillary Zeta Cell). The scattered light was
142 collected at 173° using the Zetasizer Nano Series Software 7.02 (Particular Sciences) for data
143 elaboration. For the SEM analysis, MDs were placed on aluminum stubs, gold coated and observed
144 at the SEM Leo 1430 (Zeiss, Germany) operating at 5 KV with a working distance of 15 mm.
145 Lastly, PVC and Mater-Bi® MDs were put on microscope slides and observed at the CM (Laser
146 Scanning Confocal Microscope Nikon A1) in reflection mode to assess the MD reflection.
147
148 2.2 *D. polymorpha exposure*
149 We collected *D. polymorpha* specimens on the coasts of Lake Iseo (Northern Italy) at a depth of 2-3
150 m during September 2018. Mussels were then transported to laboratory in a bag filled with lake
151 water and acclimated for 2 weeks at 20 °C under oxygen saturation, using tap and deionized water
152 (50:50), and fed 3 times *per* week with a suspension of the micro-alga *Spirulina spp.* (Magni et al.,
153 2016, 2017). We conducted the exposures for 14 days in triplicate by using 3 tanks of 4 L for each
154 experimental group (control, 1 mg/L of PVC and 1 mg/L of Mater-Bi®) and placing 30 mussels in
155 each of them. Exposures were carried out in semi-static conditions, renewing the suspensions of
156 PVC and Mater-Bi® MDs at the sixth day of exposure. During the exposure, mussels were fed 3
157 times *per* week with the suspension of *Spirulina spp*. In detail, we added 4 mg of PVC or Mater-Bi®
158 powders in the 4 L tanks to obtain the exposure concentration of 1 mg/L. We tried to calculate this

159 concentration value as number of MDs/L using the Burker chamber. However, a realistic count
160 directly made in the tanks is not feasible, due to the tank dilution as well as the high heterogeneous
161 dispersion of MDs in water, as already reported in our previous studies (Magni et al., 2018, 2019b).
162 Nevertheless, considering the need of the correspondence between the mg/L of MDs with the
163 number of MDs/L in an ecotoxicity context, we prepared two much more concentrated MD
164 suspensions (2 mg/mL) that allowed an easier and robust estimation of the number of MDs present
165 in each tank, after the appropriate proportions. Thus, we calculated a concentration of 16,875 PVC
166 MDs/L (67,500 MDs/tank) and 18,750 Mater-Bi® MDs/L (75,000 MDs/tank), respectively. The
167 diffusion of the high-density materials was assured in the entire exposure tank, avoiding the
168 presence of MDs at the surface that could have decreased the MD bioavailability towards the
169 mussels, while maintaining water under a constant magnetic stirring at the tank bottom, as carried
170 out in Magni et al. (2018). We collected the bivalves from each tank at t = 0 (basal levels), t = 6
171 days and at the end of the exposure (t = 14 days) to evaluate the ecotoxicological endpoints. Firstly,
172 we pooled 5 mussels from the acclimation tank to measure the basal levels for the cellular stress
173 analyses, while other 5 mussels were collected for the oxidative damage and genotoxicity
174 endpoints. To evaluate the effects at 6 and 14 days, the soft tissues of 3 mussels for each tank (9
175 mussels *per* treatment) were collected to assess the cellular stress and other 3 mussels for each tank
176 (9 mussels *per* treatment) were collected to measure the oxidative damage. The hemolymph from
177 these 3 latter mussels was sampled from the abductor muscle, using a hypodermic syringe with 100
178 µL of PBS-EDTA 10 mM, for the subsequent genotoxicity evaluation. Then, the soft tissues were
179 frozen in liquid nitrogen and stored at -80 °C before the assays, while the hemolymph was
180 immediately processed after the evaluation of hemocyte viability using the Trypan Blue exclusion
181 method. We also collected 2 specimens for each tank (6 mussels *per* treatment) at the end of
182 exposure to evaluate the PVC and Mater-Bi® uptake: the whole bivalves were put directly in
183 paraformaldehyde in phosphate buffer saline (PBS) solution (4%), after the injection of this fixative
184 also in the soft tissues through the mussel valves, and stored at 4 °C in the dark. At the end of

185 exposure, also the pseudo-faeces produced by mussels were collected using a clean pipette and
186 stored at -80 °C before the CM analysis. The pseudo-faeces were identified according to Juhel et al.
187 (2006).
188
189 2.3 *Uptake* and *biomarker evaluation*
190 The uptake and biomarker evaluation methods were described elsewhere (Parolini et al., 2010;
191 Magni et al 2016, 2017, 2018). Briefly, as far as the uptake evaluation is concerned, the soft tissues
192 of mussels (fixed in 4% w/v paraformaldehyde), were washed in 2 distinct sucrose solutions (15 and
193 30% w/v), as cryo-protectant agent, included in the cryostat-embedding medium (Bio Optica), and
194 stored at -80 °C. Included samples were cut at -23 °C with the CM1850 cryostat (Leica, Wetzlar,
195 Germany) to obtain transversal section of 15 µm, then placed on Superfrost Plus Microscope Slide
196 (Thermo Scientific) and stained with ProLong® Gold antifade reagent with DAPI (Invitrogen). The
197 so obtained sections, together with pseudo-faeces, were observed using the CM (Laser Scanning
198 Confocal Microscope Nikon A1) to identify in the mussel tissues the possible MDs in reflection
199 mode (Magni et al., 2018). Moving to the biomarkers, we evaluated the cellular stress on the
200 homogenates of 3 mussels for each tank, pottering the soft tissues in a 100 mM phosphate buffer at
201 pH = 7.4, 1:10 w/v ratio, with 100 mM KCl, 1 mM EDTA, 1 mM DTT and protease inhibitors
202 (1:100 v/v). Crude homogenates were centrifuged at 15,000 g for 30 min at 4 °C. As endpoints of
203 cellular stress, we measured in the S15 fraction the activity of antioxidant/detoxifying enzymes
204 SOD, CAT, GPx and GST, as well as the total content of ROS. After protein quantification using
205 the Bradford method (Bradford, 1976), we processed the S15 fractions for the kinetic measurement
206 of abovementioned enzymes using the 6715 UV/Vis spectrophotometer (Jenway, UK), as reported
207 by Orbea et al. (2002). As to the ROS quantification we used 10 mg/mL of dichlorofluorescein-
208 diacetate (DCFH-DA) in DMSO; 20 µL of S15 fraction were added to a 96-well plate and
209 incubated for 5 min at 37 °C. Subsequently, we added to each well 100 µL of PBS and 8.3 µL of
210 DCFH-DA then incubated at 37 °C for 30 min. We measured the fluorescence at 485 nm

211 (wavelength of excitation) and 530 nm (wavelength of emission) using the EnSight™ multimode
212 plate reader (PerkinElmer), as reported by Parenti et al. (2019). We evaluated the oxidative damage
213 on the homogenate of 3 mussels for each tank, pottering the soft tissues in a 100 mM phosphate
214 buffer at pH = 7.4, 1:10 w/v ratio, with 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and
215 protease inhibitors (1:100 v/v). We processed the crude homogenates, after protein quantification
216 (Bradford, 1976), to evaluate the LPO and PCC (Ohkawa, 1979; Mecocci, 1999), measuring the
217 absorbance using the 6715 UV/Vis spectrophotometer (Jenway, UK). Regarding genotoxicity, we
218 measured the apoptotic and necrotic frequencies as well as the frequency of micronuclei (MN) on
219 hemocytes of *D. polymorpha* in 9 mussels *per* treatment. For apoptotic and necrotic frequencies, we
220 used the method reported by Singh (2000), considering 300 cells for each slide (9 slides for each
221 treatment, 1 slide *per* mussel) while for the evaluation of MN frequency we used the method
222 reported by Pavlica et al. (2000), considering 400 cells for each slide (9 slides for each treatment, 1
223 slide *per* mussel). The micronuclei were identified according to Kirsch-Volders et al. (2000).
224
225 2.4 *Statistical analyses and Biomarker Response Index (BRI)*
226 The significant differences (*$p < 0.05$; **$p < 0.01$) between treated (PVC and Mater-Bi®) and
227 control (time *versus* time) were evaluated through the two-way analysis of variance (two-way
228 ANOVA) followed by the Fisher LSD *post-hoc* test. All statistical analyses were performed using
229 STATISTICA 7.0 Software. To compare the ecotoxicity of considered materials on *D. polymorpha*,
230 on the basis of the biological trends of biomarkers, we used the Biomarker Response Index (BRI),
231 proposed by Hagger et al. (2008) and modified and described in detail in our previous studies
232 (Magni et al., 2016, 2017, Binelli et al., 2020).
233
234 3 RESULTS AND DISCUSSION
235 The μFT-IR analysis confirmed that the powders derived from cryogenic grinding do are still
236 composed of PVC and Mater-Bi® materials, with a score of coverage of 0.85 and 0.92 between

measured and reference infrared spectra, respectively (Figure 1). ζ-potential analysis showed that MDs have the same negative small surface charge (-11.7 ± 2.2 mV and -13.5 ± 2.1 mV for PVC and Mater-Bi®, respectively), suggesting a similar poor electrostatic repulsion (Figure 1). The ultrastructural analysis achieved by SEM revealed a globular-like shape with superficial depressions and protrusions for PVC MDs, whilst a flake and lamellar shape for Mater-Bi® MDs (Figure 1). These structures are observed also by CM, which confirmed the reflection propriety of these substances (Figure 1), a very important aspect to detect *non*-fluorescent MDs inside the organisms (Magni et al., 2018). The size measurement showed a mean value of 56 ± 35 μm and 41 ± 36 μm for PVC and Mater-Bi® MDs respectively, a dimension compatible with the ingestion range of *D. polymorpha*, of ~ 40 μm (Winkel and Davids, 1982). However, we did observe neither PVC nor Mater-Bi® MDs in the gut lumen and consequently their accumulation in the mussel tissues (n = 6 mussels *per* treatment) even after 14 days of exposure, as shown in Figure 2 in which a transversal section of *D. polymorpha* revealed the principal anatomical structure free from MDs. This evidence was very surprising, also in the light of our previous evidence that showed the presence of smaller PS MDs (microbeads of 1 and 10 μm) in the digestive gland and hemolymph of *D. polymorpha* only after 6 days of exposure (Magni et al., 2018). Furthermore, other studies carried out on marine mussel *Mytilus spp.*, exposed to PS MDs of 2, 3, 6 and 45 μm showed their presence in the hemolymphs and digestive tract of mussels (Paul-Pont et al., 2016; Franzellitti et al., 2019). Another study on *Mytilus galloprovincialis* exposed to 1, 10 and 90 μm PS MDs highlighted that different sizes of ingested MDs were retained in a different manner in the gut, before their excretion with faeces (Kinjo et al., 2019). More in detail, smaller MDs were excreted quickly by mussels, although some MDs were retained by organisms, whilst larger MDs were excreted slowly but, after excretion, no debris were retained. Therefore, the absence of PVC and Mater-Bi® MDs in *D. polymorpha* could be due to both the different retention time and sizes of debris compared to those reported in Magni et al. (2018). Moreover, while the above-mentioned studies were conducted by using only spherical microbeads, our MDs had different shapes extremely heterogeneous that can be

recognized as possible dangerous materials by the defensive mechanism of *D. polymorpha* to eliminate before the ingestion in the gut. In any case, this aspect needs major clarifications, considering that different species of bivalves could have different elimination processes as well as retention times of MDs. On the other hand, bivalves can select the food, possessing different fields of sorting at the level of the labial palps, gills, and stomach, and using siphons to manage the water flows (Ruppert et al., 2004). For instance, *D. polymorpha* introduces the water and suspended material through the inhalant (ventral) siphon to branchial lamellae in which gas exchanges take place. Then, the smallest suspended particles are directed to the digestive tract by the branchial cilia, while the rough material is thrown again into the pallial cavity and eliminated as pseudo-faeces by the inhalant siphon (Nalepa and Schloesser, 1992; Baker et al., 1998; Baker et al., 2000). The presence of MDs in the pseudo-faeces of *D. polymorpha* (Figure 3) supports this hypothesis, as also found in our previous study (Binelli et al., 2020) in which we observed the presence of larger plastics (up to 3 mm) in the pallial cavity of *D. polymorpha*. It is important to note that the methods of sample preparation for advanced microscopy observations do not allow the conservation of possible MDs in the pallial cavity. An alternative procedure could be the application of μFT-IR on the whole soft tissue homogenates, but this method does not allow the detection of plastics in the different anatomical districts. For these reasons, in some cases, the evaluation of plastic uptake in mussels is affected by uncertainty and further investigations are needed. Coherently with the absence of MD uptake in mussels, the results of biomarkers did not show adverse effects in *D. polymorpha* for both the tested materials, at least for the measured endpoints. As to the cellular stress, we did not observe for SOD and CAT activities a significant effect of time, treatment and time/treatment interaction on mussels exposed to PVC and Mater-Bi® MDs. Only a marginally *non*-significant effect of treatment ($p < 0.069$; $F_{2,12} = 3.4$) and time/treatment interaction ($p < 0.074$; $F_{2,12} = 3.3$) for SOD activity was observed. Considering the GPx activity, we observed a significant effect of time ($p < 0.05$; $F_{1,12} = 6.5$) and treatment ($p < 0.05$; $F_{2,12} = 5.4$), but only a marginally *non*-significant effect of their interaction ($p < 0.07$; $F_{2,12} = 3.3$); an increasing *non*-significant trend was

observed for this enzyme only at 6 days in mussels exposed to both materials. According to the results on oxidative stress, also the ROS measurements did not show significant differences between exposed and control groups (Figure 4). Only for GST activity we observed a significant effect on its modulation during the exposure; indeed, a significant effect of time ($p < 0.01$; $F_{1,12} = 9.6$) and time/treatment interaction ($p < 0.01$; $F_{2,12} = 8.8$), with a significant increase of GST activity in mussels exposed to both PVC ($p < 0.01$) and Mater-Bi® ($p < 0.05$) MDs was measured at t = 6 days (Figure 5). The activation of the phase II detoxification mechanisms seems to be a contradictory response because of the lack of the MD entrance in mussels. The modulation of this enzyme after plastic MD exposure was reported in other studies (Prokić et al., 2019 and citations therein), and one of the possible hypotheses to justify this modulation could be associated to the release in the exposure tanks of additives from plastics. A significant alteration of GST activity was detected also in our previous study on *D. polymorpha* exposed to plastics collected in the Italian subalpine great lakes and explained as the release of chemicals and/or plasticizers adsorbed on the plastics (Binelli et al., 2020). Moving to the results of biomarkers of oxidative damage, we observed a significant effect of time ($p < 0.05$; $F_{1,12} = 8.5$), but not of treatment and time/treatment interaction for PCC in organism exposed to the contaminants, while for LPO we did not observe significant effects of time, treatment and time/treatment interaction (Figure 5). Regarding the biomarkers of genotoxicity, the hemocyte viability was higher than the value of 70% required to perform genotoxicity biomarkers, as reported by Kirkland et al. (2007), with values of 95.2 ± 1.7 (t = 6) and 87.3 ± 8.2 (t = 14) for controls, 96.1 ± 2.7 (t = 6) and 95.5 ± 2.2 (t = 14) for PVC group, 96.7 ± 1.8 (t = 6) and 93.7 ± 6.8 (t = 14) for Mater-Bi® group. Since genotoxicity is often an indirect consequence of the increase in oxidative stress, the lack of significant genotoxic effects induced by PVC and Mater-Bi® MDs was not surprising (Figure 6). Even if no significant differences were noticed between treated and controls for quite all the selected biomarkers, with the exclusion of GST, there is another important summary endpoint to be considered, bearing in mind the limitation of the tests carried out at laboratory conditions mainly due to the limited time of exposure, which

315 makes only a partial picture of the real (eco)toxicological effect which can occur in the
316 environment, especially for the sub-chronic endpoints. Thus, to compare the effects of tested
317 materials on *D. polymorpha*, we integrated each response in the BRI, which considers only the
318 biological trends, without the statistical significance that was related to the specific selected
319 exposure period with a limited ecological realism. As reported in Figure 7A, PVC and Mater-Bi®
320 MDs showed a comparable total ecotoxic effect on the exposed mussels and the biological trends
321 (Figure 7B) seems due for 50% to the oxidative stress/damage (blue and green strips) and for the
322 other 50% to genotoxicity (brown strips). The cellular effects (as genotoxicity and oxidative
323 damage) have indeed a weight in the BRI calculation double than molecular endpoints (as the
324 enzyme activities) because they have an impact on a higher level of the biological organization. It is
325 important to note that the results obtained in this study are not exhaustive because of several other
326 variables should be considered, such as longer exposure time, other endpoints belonging to neuro-
327 toxicity or mechanical damage on the gill tissues, and the use of omics approaches (proteomics,
328 metabolomics, and genomics). In our opinion, the crucial point to be clarified should be the reason
329 for the lack of the MD intake in the mussel gut that can shed light on a possible very performing
330 mechanism of defense against these physical contaminants executed by this species.
331
332 CONCLUSIONS
333 Considering the dimension of plastic pollution worldwide, as well as the potential ecological impact
334 of plastics on the aquatic environment, in this study we evaluated and compared the effects of one
335 of the most of common plastics (PVC) with a biodegradable plastic (Mater-Bi®) MDs. Our results
336 highlighted that the studied materials did not induce adverse effects on exposed organisms. This can
337 be ascribed to the absence of uptake by *D. polymorpha* of PVC and Mater-Bi® MDs, possibly
338 thanks to its very effective protective mechanisms. Therefore, other studies are necessary to clarify
339 the processes of MD uptake in mussels, from the implication of faeces/pseudo-faeces production to
340 the role of debris sizes/shapes in the MD retention mechanism. This behavior clearly shows once

more that the study of these emerging contaminants is much more complex than those carried out by chemical pollutants because several variables (size, shape, color, contaminant adsorption) are involved in their uptake and infiltration, and as a consequence, in ecotoxicity. Despite we did not observe differences and adverse effects of neither PVC nor Mater-Bi® MDs on exposed mussels, it is absolutely urgent the identification of a more sustainable way in the enormous problem of plastic production and disposal, considering the longtime of resilience of ecosystems contaminated by plastics, as well as the impact of these pollutants on some ecosystem services, which directly or indirectly satisfy the human necessities and guaranty the life of all the natural species.

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534

535 Captions:

536 Figure 1: Physical-chemical characterization of the two powders of PVC and Mater-Bi® MDs,
537 obtained by cryogenic gridding, using an integrated approach of μFT-IR, DLS (Zeta potential
538 expressed as mean ± standard deviation), SEM and CM.

539

540 Figure 2: Transversal cryostat section of *D. polymorpha* (n = 6 mussels *per* treatment), stained with
541 DAPI (blue fluorescence), observed using the CM. At the end of exposure (t = 14 days), the gut
542 lumen and soft tissues of mussels were completely free by both PVC and Mater-Bi® MDs.

543

544 Figure 3: PVC and Mater-Bi® MDs detected in the *D. polymorpha* pseudo-faeces at the end of
545 exposure (t = 14 days) using the CM in reflection mode (white reflection).

546

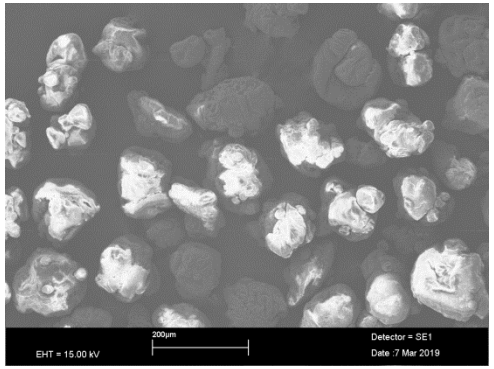
547 Figure 4: Cellular stress (mean ± standard deviation; SOD, CAT and GPx activities and ROS levels)
548 observed in *D. polymorpha* soft tissues (n = 3 pools of 3 mussels *per* treatment; 9 mussels *per*
549 treatment) during 14 exposure days to 1 mg/L of PVC and Mater-Bi® MDs. The red line indicates
550 the baseline level (t = 0) of each biomarker. Asterisks indicate the significant differences, time
551 *versus* time (6 and 14 days), between treated and control (two-way ANOVA, Fisher LSD post-hoc
552 test: * $p < 0.05$, ** $p < 0.01$).

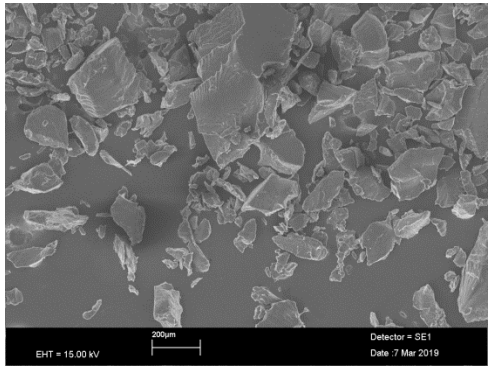
553

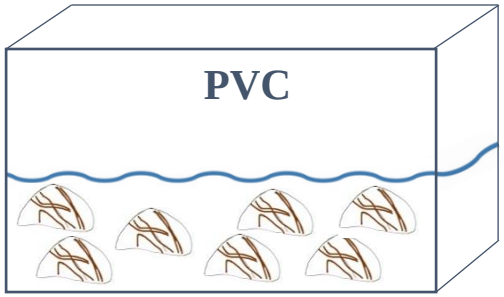
554 Figure 5: Cellular stress (GST activity) and oxidative damage (mean ± standard deviation; PCC and
555 LPO levels) observed in *D. polymorpha* soft tissues (n = 3 pools of 3 mussels *per* treatment; 9
556 mussels per treatment) during 14 exposure days to 1 mg/L of PVC and Mater-Bi® MDs. The red
557 line indicates the baseline level (t = 0) of each biomarker. Asterisks indicate the significant
558 differences, time *versus* time (6 and 14 days), between treated and control (two-way ANOVA,
559 Fisher LSD post-hoc test: * $p < 0.05$, ** $p < 0.01$).

560

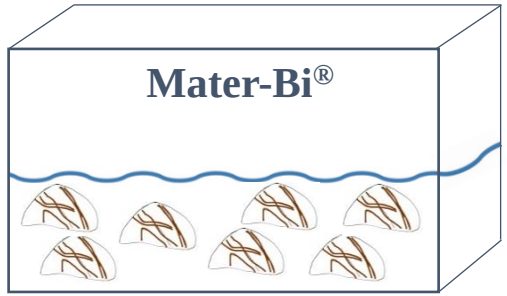
561 Figure 6: Genotoxicity (mean ± standard deviation; frequencies of apoptosis, necrosis and MN)
562 observed in *D. polymorpha* hemocytes (n = 9 mussels *per* treatment) during 14 exposure days to 1
563 mg/L of PVC and Mater-Bi® MDs. The red line indicates the baseline level (t = 0) of each
564 biomarker. Asterisks indicate the significant differences, time *versus* time (6 and 14 days), between
565 treated and control (two-way ANOVA, Fisher LSD post-hoc test: * $p < 0.05$, ** $p < 0.01$).
566
567 Figure 7: (A) Ecotoxicity comparison between 1 mg/L of PVC and Mater-Bi® in *D. polymorpha*.
568 Each histogram derives from the integration of considered biomarkers into the BRI. (B)
569 Contribution of considered biomarkers in the total ecotoxic effect of 1 mg/L of PVC and Mater-Bi®
570 in *D. polymorpha* during 14 days of exposure.







Exposure tank with *D. polymorpha*

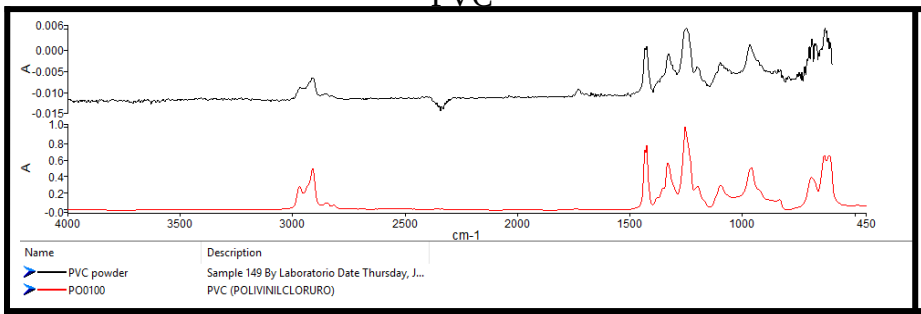
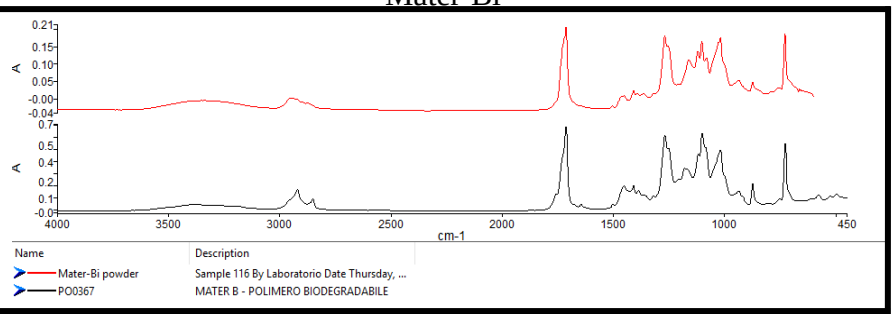
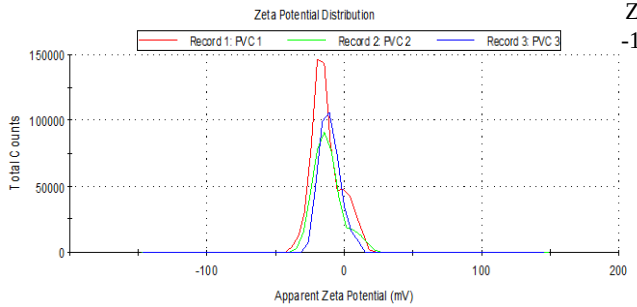
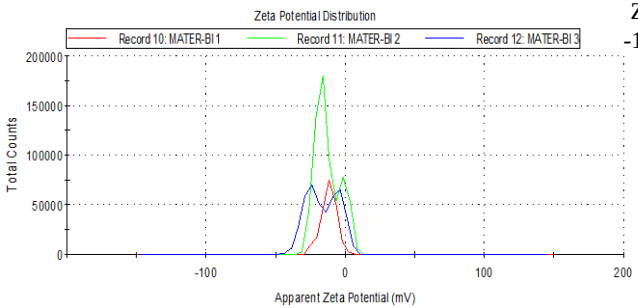
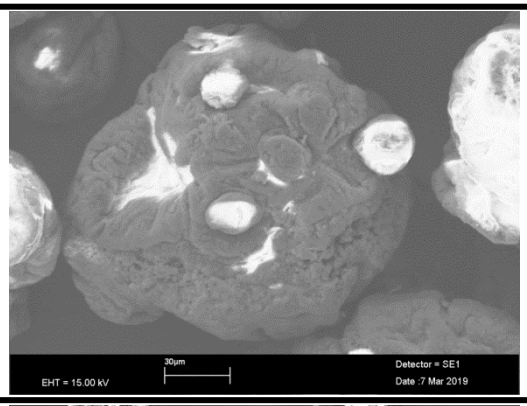
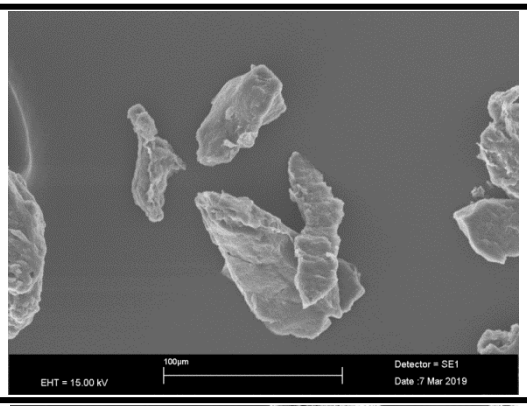
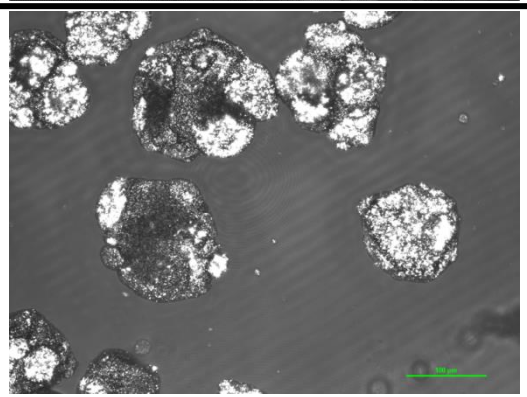
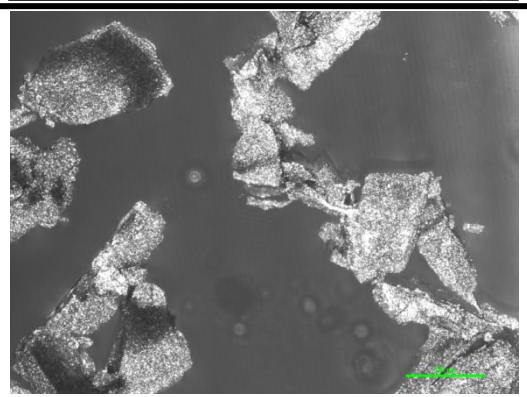


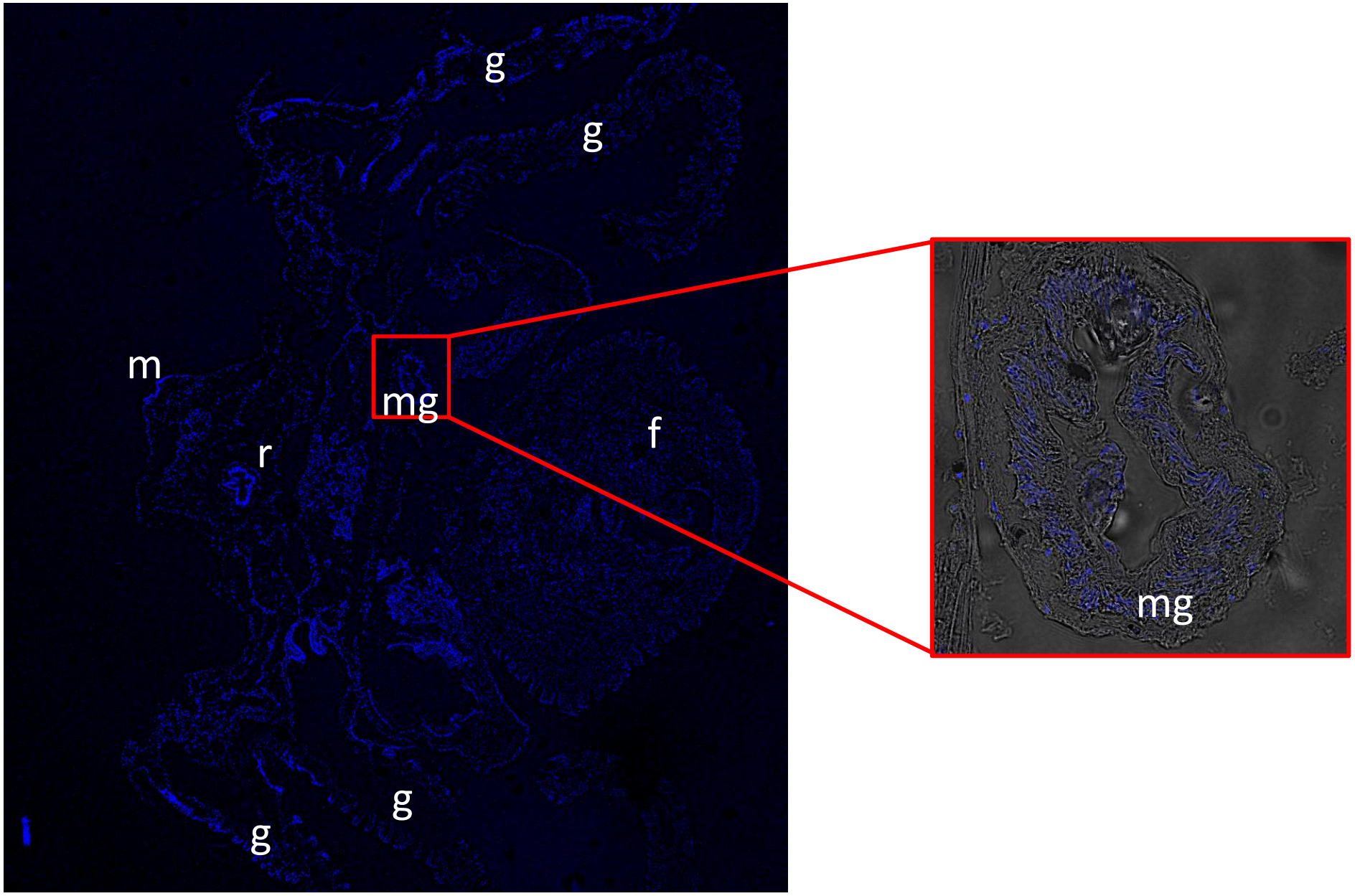
Exposure tank with *D. polymorpha*

After 14 days of exposure

Elimination of PVC and Mater-Bi® micro-debris with pseudo-faeces by inhalant (ventral) siphon

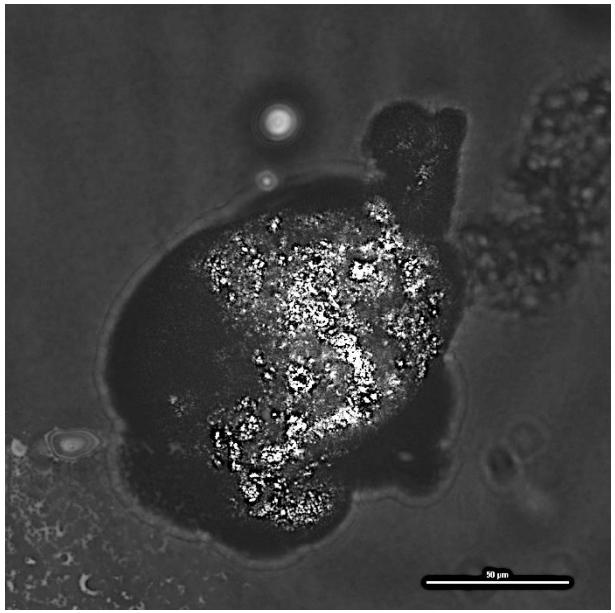
NO UPTAKE NO EFFECTS

	PVC	Mater-Bi®
FT-IR		
DLS	 Zeta potential -11.7 ± 2.2 mV	 Zeta potential -13.5 ± 2.1 mV
SEM	 Dimension 56.3 ± 34.6 µm	 Dimension 41.3 ± 36.4 µm
CM		

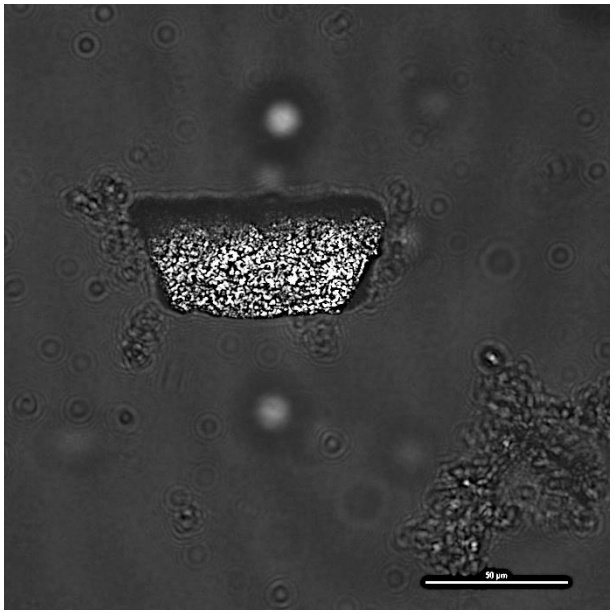


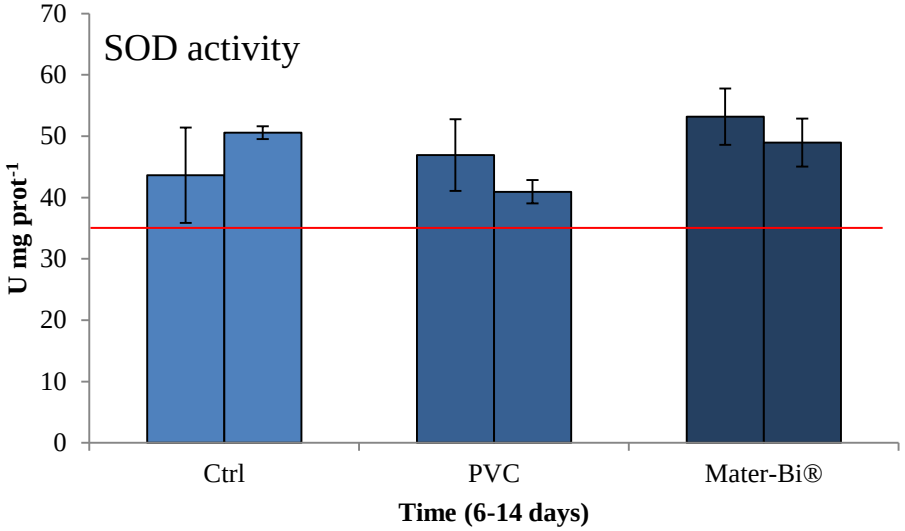
m = mantle; r = rectum (inside the pericardium); mg = midgut; g = gills; f = foot

PVC



Mater-Bi®




SOD activity
U mg prot-1
70
60
50
40
30
20
10
0
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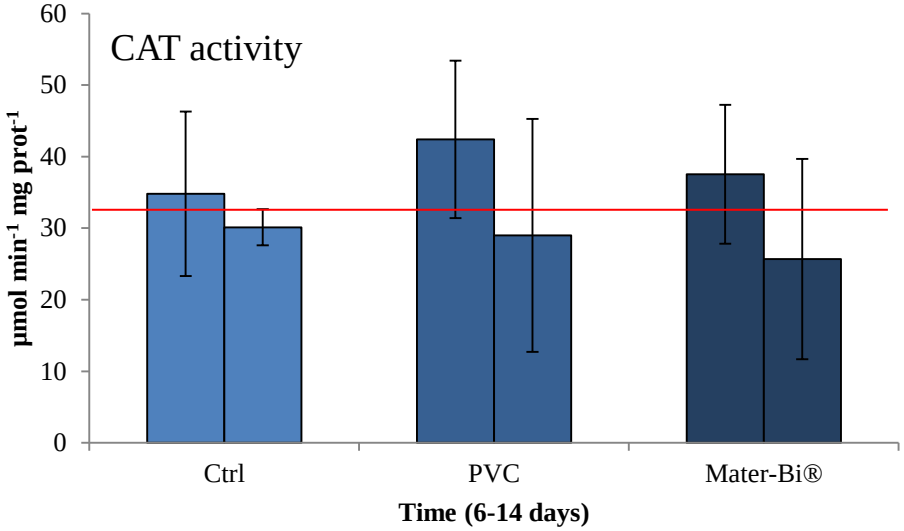
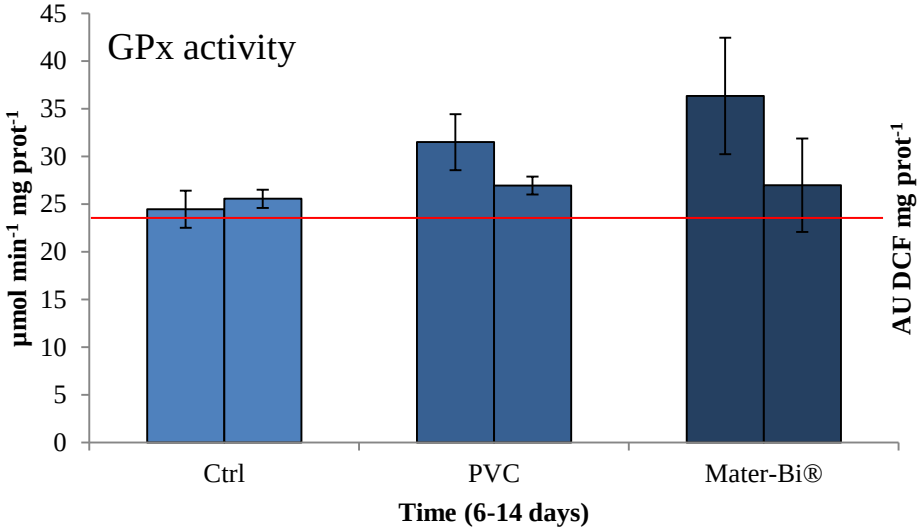
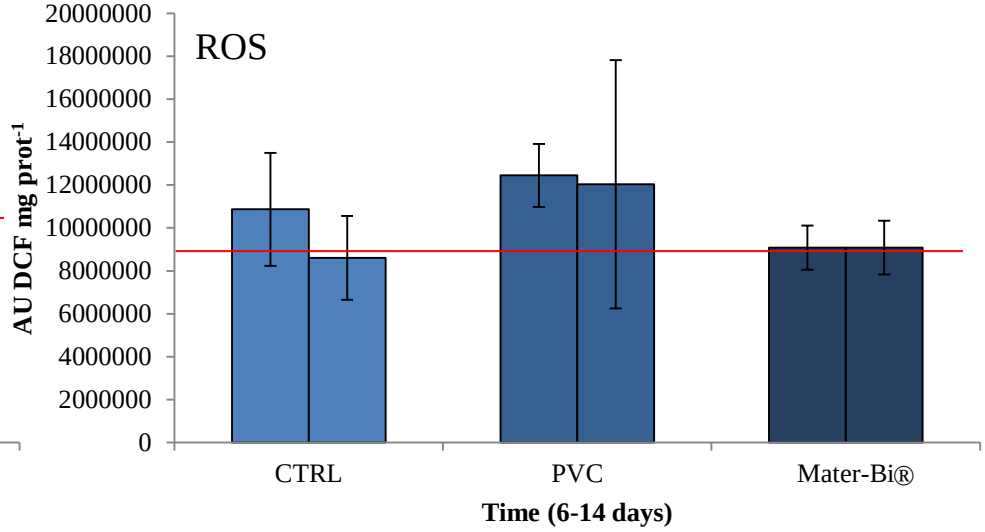
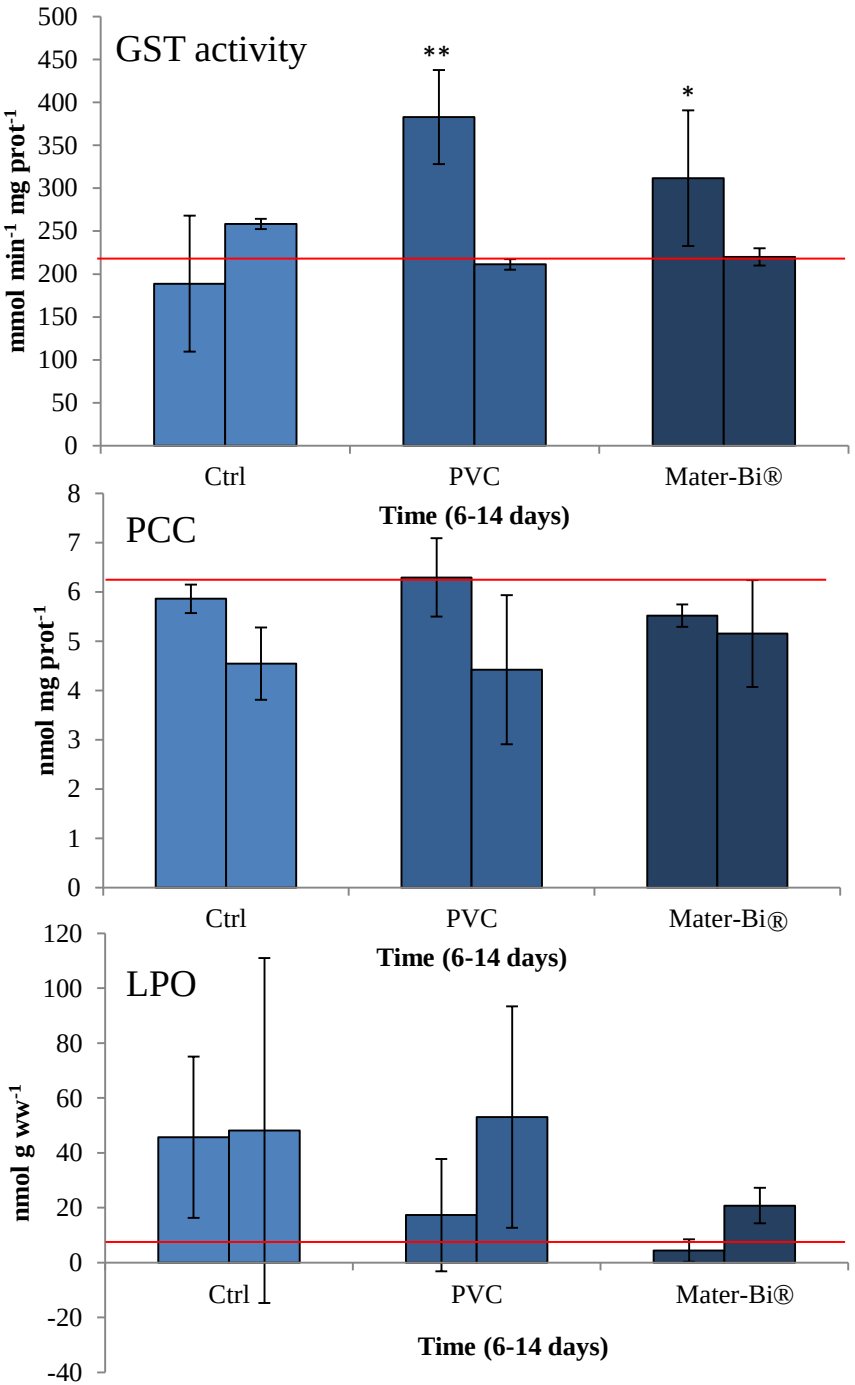
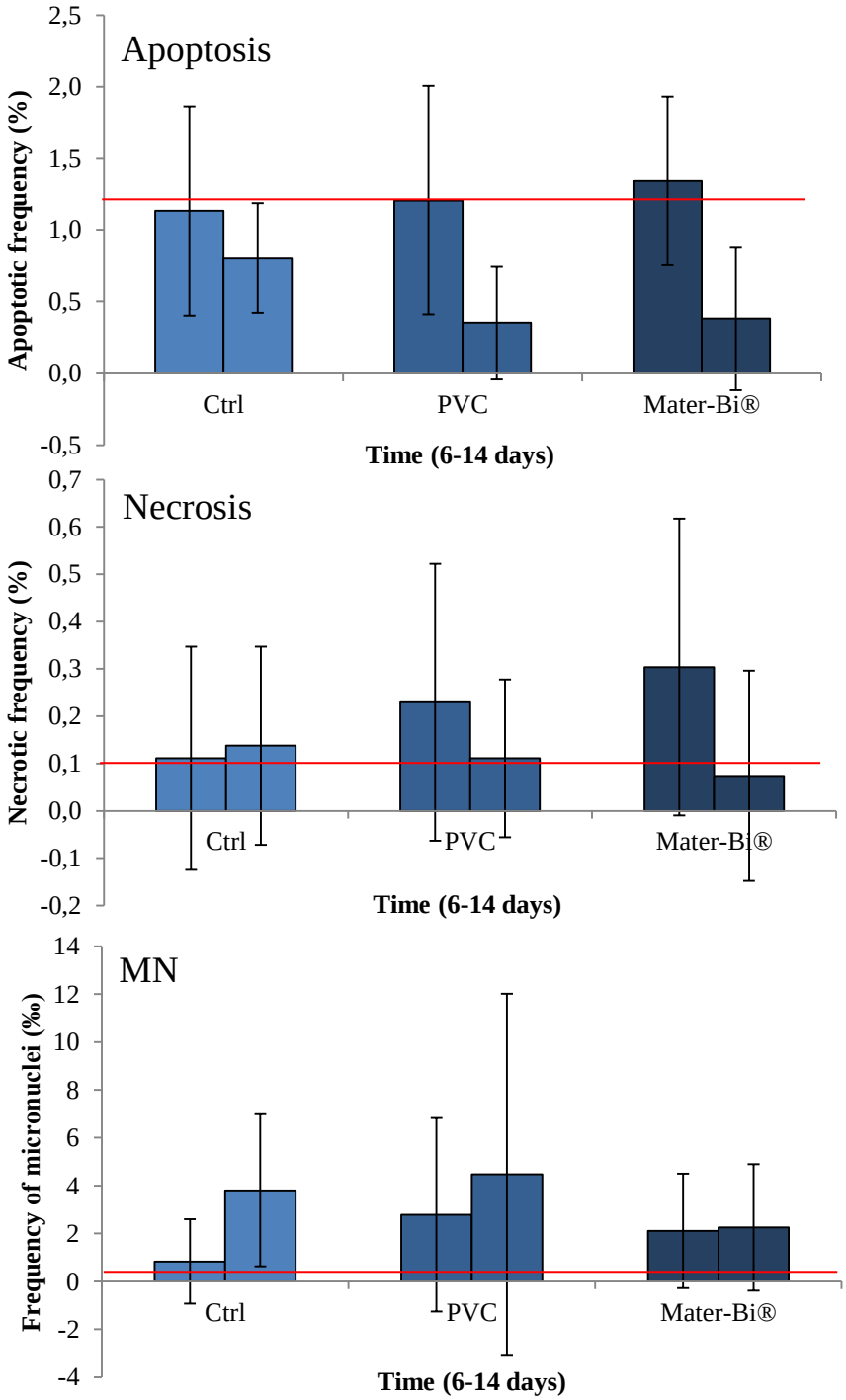
PVC
Mater-Bi®
Time (6-14 days)

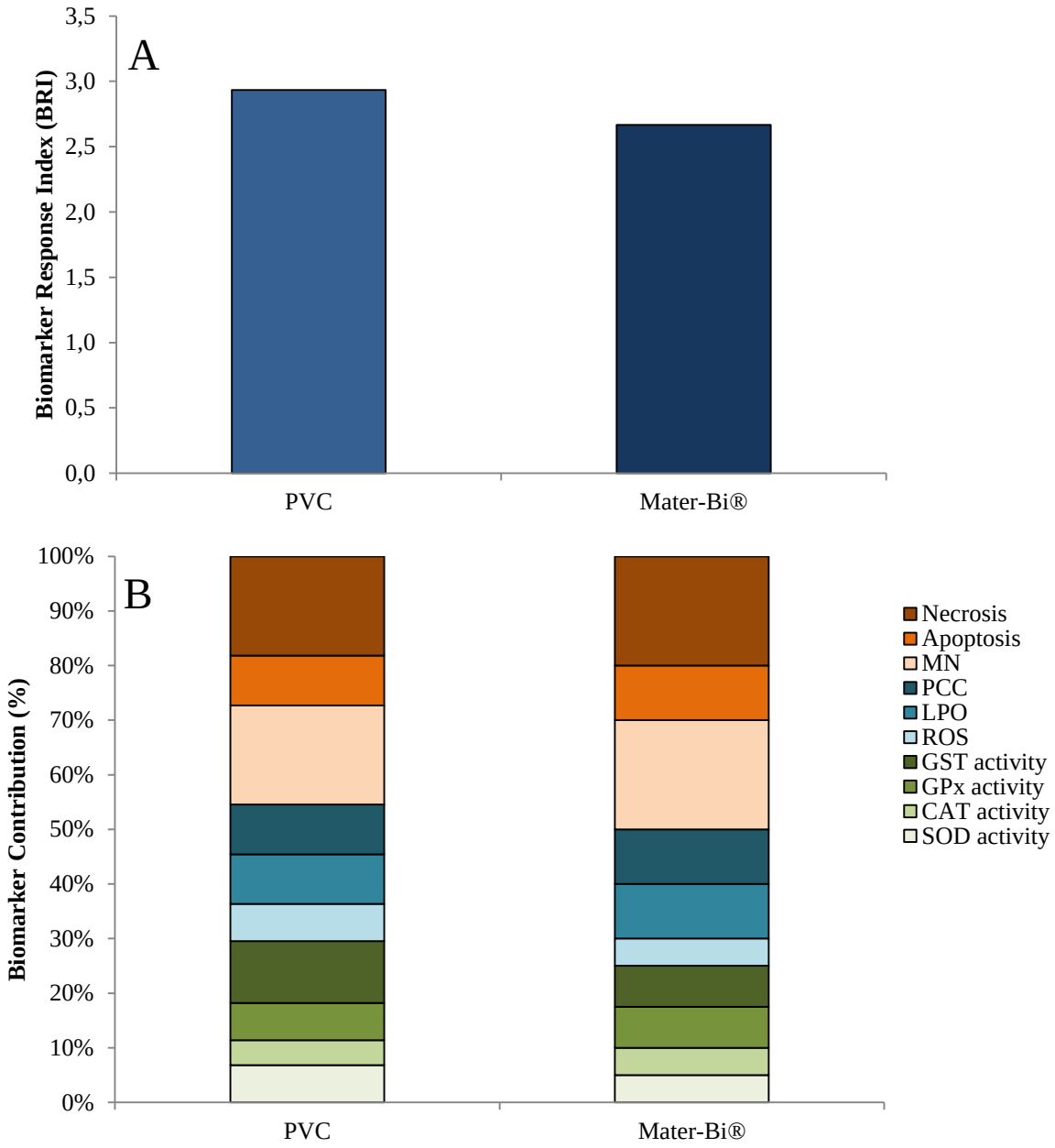

CAT activity
µmol min-1 mg prot-1
60
50
40
30
20
10
0
Ctrl
PVC
Mater-Bi®
Time (6-14 days)


GPx activity
µmol min-1 mg prot-1
45
40
35
30
25
20
15
10
5
0
Ctrl
PVC
Mater-Bi®
Time (6-14 days)


ROS
AU DCF mg prot-1
20000000
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
CTRL
PVC
Mater-Bi®
Time (6-14 days)


GST activity
mmol min-1 mg prot-1
500
450
400
350
300
250
200
150
100
50
0
**
*
Ctrl
PVC
Mater-Bi®
Time (6-14 days)
PCC
nmol mg prot-1
8
7
6
5
4
3
2
1
0
Ctrl
PVC
Mater-Bi®
Time (6-14 days)
LPO
nmol g ww-1
120
100
80
60
40
20
0
-20
-40
Ctrl
PVC
Mater-Bi®
Time (6-14 days)


Apoptosis
Apoptotic frequency (%)
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
Ctrl
PVC
Mater-Bi®
Time (6-14 days)
Necrosis
Necrotic frequency (%)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
-0,1
-0,2
Ctrl
PVC
Mater-Bi®
Time (6-14 days)
MN
Frequency of micronuclei (‰)
14
12
10
8
6
4
2
0
-2
-4
Ctrl
PVC
Mater-Bi®
Time (6-14 days)


A
Biomarker Response Index (BRI)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
PVC
Mater-Bi®
B
Biomarker Contribution (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
PVC
Mater-Bi®
Necrosis
Apoptosis
MN
PCC
LPO
ROS
GST activity
GPx activity
CAT activity
SOD activity