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1 Effect of an environmental microplastic mixture from the Seine River and one of the main
2 associated plasticizers, dibutylphthalate, on the sentinel species *Hediste diversicolor*

3

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18

19 Abstract

20 The aim of this study was to explore the adverse effects of a microplastic (MP) mixture obtained
21 from litter accumulated in the Seine River (France) compared to those of their major co-
22 plasticizer, dibutylphthalate (DBP), on the sentinel species *Hediste diversicolor*. A suite of
23 biomarkers has been investigated to study the impacts of MPs (100 mg.kg⁻¹ sediment), DBP
24 (38 µg.kg⁻¹ sediment) on worms compared to non-exposed individuals after 4 and 21 days. The
25 antioxidant response, immunity, neurotoxicity and energy and respiratory metabolism were

26 investigated using biomarkers. After 21 days, worms exposed to MPs showed an increasing
27 aerobic metabolism, an enhancement of both antioxidant and neuroimmune responses. Energy-
28 related biomarkers demonstrated that the energy reallocated to the defence system may come
29 from proteins. A similar impact was depicted after DBP exposure, except for neurotoxicity. Our
30 results provide a better understanding of the ecotoxicological effects of environmental MPs and
31 their associated-contaminants on *H. diversicolor*.

32 Keywords: Microplastic; dibutylphthalate; *Hediste diversicolor*; Biomarker; Plastics
33 Ecotoxicology; Estuaries

34

35 **1 Introduction**

36

37 Environmental concerns have so far not led to a decrease in global plastic production reaching
38 almost 370 million tons in 2019 (PlasticsEurope 2021). It can be predicted an increase of
39 worldwide plastic production and use since 2020 due, among others, to the need of single-use
40 plastics during COVID-19 pandemic (Silva et al. 2021). Therefore, plastic wastes have
41 gradually invaded and accumulated in most ecosystem (Hohn et al. 2020), ending up inevitably
42 in aquatic environments including marine ecosystems. Upon biotic and abiotic degradation
43 processes, macroplastics can be fragmented into microplastics (MPs), nanoplastics (NPs).
44 However, their associated additives are likely to spread through the water leading to health risks
45 to organisms at all levels of biological organization.

46 Smaller than 5 mm in size, MPs result from the weathering of plastic debris discharged into the
47 environment and can release their own additives and also adsorb external contaminants like
48 POPs (persistent organic pollutants) or heavy metals from their surrounding environment,
49 concentrating them on their surface (Cao et al. 2021). Environmental MPs (EMPs) act as
50 carriers of contaminants during ingestion through the food chain increasing the risks of

51 environmental exposure (Purwiyanto et al. 2020; Mateos-Cardenas et al. 2022; Hasegawa and
52 Nakaoka, 2021). Indeed, once inside the animal's body, contaminants or additives can
53 potentially be released and cause toxic effects on organs and cells (Pannetier et al. 2019a,
54 2019b; Missawi et al. 2022). Herrera et al. (2022) compared the impact of virgin and EMPs on
55 the European seabass (*Dicentrarchus labrax*) and demonstrated that additives and chemical
56 contaminants adsorbed on EMPs bioaccumulate in fish liver after long-term (60 days) ingestion
57 of MPs.

58 Among plastic additives, phthalic acid esters (PAEs) are widely used as plasticizer to increase
59 the physical properties of polymers like durability or flexibility during plastic manufacturing.
60 As they develop only weak bonds with plastic polymers, they have been demonstrated to be
61 easily released into environmental compartments (air, water or food) by leaching (Net et al.
62 2015). Nowadays, it has been shown that all environmental compartments are contaminated by
63 phthalates esters including marine sediments (Cuvillier-Hot et al. 2018; Schmidt et al. 2021;
64 Hidalgo-Serrano et al. 2022). Cuvillier-Hot et al. (2018) reported total phthalates concentrations
65 in sediments sampled from 3 sites (Authie, Gravelines, Boulogne in France) varying from 3.63
66 to 23.88 $\mu\text{g}\cdot\text{g}^{-1}$ sediment. Hermabessiere et al. (2017) reported phthalate concentrations in sea
67 waters ranging from a few pg L^{-1} to around 10 $\mu\text{g L}^{-1}$. Among PAEs, di-n-butyl phthalate (DBP)
68 and bis-2-ethylhexyl phthalate (DEHP) were the most detected in water phase (Zhang et al.
69 2018; Paluselli et al. 2018), in sediment (Wang et al. 2021) and in certain marine organisms
70 (Hu et al., 2016, 2020; Sun et al. 2021). DBP is considered as an endocrine disruptor and also
71 as a carcinogen, a reproductive toxicant, inducing also immunotoxic effects on various marine
72 aquatic organisms as copepods (Li et al. 2020), mussels (Gu et al. 2021) or swimming crab (He
73 et al. 2022).

74 Living in sediments, the ragworm *Hediste diversicolor* is a sentinel species of estuarine habitats.
75 As a bioturbator of shallow coastal sediments, it affects the biogeochemical cycles of nutrients

76 and contaminants including MPs. In addition, *H. diversicolor* populations are important food
77 resources for higher trophic levels such as crabs, flatfish and birds (Scaps and Borot, 2000).
78 This worm has been widely used in ecotoxicological studies (Silva et al. 2020) and has been
79 previously used to assess commercial and environmental microplastic toxicity. Urban-Malinga
80 et al. (2022) exposed *H. diversicolor* during 28 days to sediment spiked with commercial MPs
81 (0.08% sediment d. wt.) leading to neurotoxicity and suppressing the worm antioxidant system
82 function. EMPs (10 and 100 mg.kg⁻¹) were shown to accumulate in *H. diversicolor* (7- and 14-
83 days exposure) in a size dependent manner, reducing survival and growth, affecting
84 neurotransmission and antioxidant pathways (Missawi et al. 2021), inducing metabolomic
85 alterations (amino acid metabolism, energy and osmoregulatory processes) and promoting
86 autophagy and cytoskeleton loss (Missawi et al. 2022). Barrick et al. (2021) underlined in their
87 review the need to investigate the adverse biological and toxic effects of both plastics particles
88 and their chemical additives. One of the challenges of environmental MPs studies is to
89 discriminate the ecotoxicity of initial plastic polymer products, their additives or adsorbed
90 contaminants. The aim of this present study was to explore the adverse effects of chemically
91 characterized EMPs *versus* one of their major co-plasticizers, dibutylphthalate (DBP), on the
92 sentinel species *H. diversicolor*. A suite of biomarkers has been investigated to study the
93 impacts of MPs and DBP on the antioxidant response (catalase and glutathione-S-transferases
94 activities), immunity (phosphatase acid activity), neurotoxicity (acetylcholinesterase activity)
95 and energy and respiratory metabolism (lactate dehydrogenase activity, protein, glycogen and
96 lipid contents and electron transport system activity).

97

98 **2 Materials and methods**

99 2.1 Organism sampling

100 Ragworms *H. diversicolor* were collected in January 2022 on an intertidal mudflat located on

101 the French Atlantic coast (1° 59' 02' 80' W, 47° 01' 49.20'' N, Port du Collet, Bay of Bourgneuf,
102 France). This site was considered a reference due to the lack of industrial activity and reduced
103 anthropogenic pressure. During the low tide (water temperature 5°C and salinity 21 practical
104 salinity unit (PSU), worms were carefully hand-collected before being transported in their
105 sediment in cool boxes to the laboratory. Worms were immediately acclimated in aquaria filled
106 with sediment from the sampling site covered with 5 cm of artificial seawater (ASW - Tropic
107 Marin®) at 21 PSU, 16 °C ± 1 °C under a photoperiod of 16h light/8h dark.

108 2.2 Preparation and characterization of environmental microplastics

109 Plastic litter was collected in the Seine estuary near Villequier, an active floodplain of 3 ha
110 located on the left riverbank of the Seine River. Presence of plastic debris on the Seine River
111 banks in estuarine zone have been reported in Tramoy et al. (2019, 2020a, 2020b). These
112 macroplastics were used to prepare a representative mixture of environmental microplastics
113 found in the sediment based on Leslie et al. (2017) and Phuong et al. (2018) field analysis. Each
114 macroplastic was first manually fragmented into pieces of around 1 cm² for polymer
115 identification using FTIR spectroscopy (VERTEX 70v, Bruker). Fragments were separated by
116 type of polymer (PE, PP, PVC, PET) and cryogrinded for 2 cycles of 4 consecutive min
117 (Freezer/mill 6770, prex Sampleprep). The powder obtained was then sieved in order to retain
118 only the fraction <100 µm (10 min, Retsch AS 200). The particle size distribution (d10, d50
119 and d90) was carried out with a Malvern-type laser particle sizer after sieving the powder at
120 800 µm. The granulometry of the different sampled and cryo-grinded plastic polymers was
121 shown in the supplementary data. The representative mixture of MPs was prepared by mixing
122 120 mg of PE (40%), 120 mg of PP (40%), 30 mg of PVC (10%) and 30 mg of PET (10%)
123 from grinded environmental macroplastics. The concentration in term of number of particles
124 was not determined. PAE (15 congeners) were analysed for this microplastic mixture, among
125 which dibutylphthalate (DBP) was currently shown as the most concentrated phthalate

126 plasticizer (126 μg of DBP.g⁻¹ of MPs) representing 60% of total phthalate concentration. The
 127 characteristics of the environmental MPs mixture used for *H. diversicolor* exposure
 128 experiments are summarised in Table 1, the whole results of the chemical analysis of the MP
 129 mixture are shown in the supplementary material. Briefly, three successive solid-liquid
 130 extractions with 40 mL of a methanol/dichloromethane/hexane (1/1/1 v/v/v) mixture were
 131 carried out. Internal standards of the three families of compounds monitored (Polycyclic
 132 aromatic hydrocarbons-PAH, Alkylphenols-AP, Phthalic acid esters-PAE) have been added
 133 beforehand. The first extraction was carried out using an orbital shaker (200 rpm) for 2 h and
 134 its fraction was recovered. The two other extractions were performed under sonication for 30
 135 min, then the fractions were collected. All three fractions were combined and filtered on pleated
 136 filters in order to remove some particles residues. For phthalates quantification, the final extract
 137 was evaporated to dryness using a rotary evaporator then taken up in 5 ml of ethyl acetate before
 138 purification on an alumina cartridge (1 g). Then, 100 μL of the purified extracts were analysed
 139 by gas chromatography coupled with mass spectrometry (GC-MS). Experimental blanks were
 140 performed by preparing samples with solvents and following all the extraction and purification
 141 procedure (Gasperi et al. 2022; Flanagan et al. 2018).

142

143 **Table 1:** Characteristics of the MP mixture: composition (in mass percentage) in polyethylene
 144 (PE), polypropylene (PP), polyvinyl chloride (PVC) and polyethylene terephthalate (PET),
 145 median size of particles d10, d50, d90 (in μm) and dibutylphthalate (DBP) concentration (in
 146 mg.kg^{-1} MPs).

MP mixture	Mass composition (%)	Median size d10 (μm)	Median size d50 (μm)	Median size d90 (μm)
PE	40	14.35	51.49	120.56
PP	40	11.51	51.82	115.46
PVC	10	18.50	62.35	110.50
PET	10	27.46	76.78	144.46

Concentration of phthalate (DBP) (mg.kg⁻¹ MPs)	126.6
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147

148

149 2.3 Experimental set up

150 Laboratory assays followed the ASTM guidelines (ASTM, 2013) for conducting sediment tests

151 with annelids with some minor adaptations. Worms were sampled outside of their reproduction

152 period and of the similar size to avoid a potential influence of sexual maturity on biomarker

153 responses. A set of 8 bioassay units consisted of 5L-aquaria filled with 3 kg of wet homogenized

154 artificial sediments (90% Quartz; 9% Kaolinite; 1% CaCO₃). The representative mixture of

155 MPs was prepared (40% PE, 40% PP, 10% PVC, 10% PET) and placed in 50 mL of 21 psu

156 artificial salt water (ASW). The MPs suspension was homogenised within 3 kg of artificial

157 sediment, corresponding to the “Microplastics” (MP) exposure condition at 100 mg.kg⁻¹ (2

158 aquaria). As DBP was the most represented additive measured in the MPs mixture (126.6 mg

159 DBP.kg⁻¹ MPs), it was used in parallel at an equivalent concentration corresponding to the

160 “Phthalate” (P) exposure condition (2 aquaria). To this end, a stock solution of DBP was

161 prepared at 1 µg.µL⁻¹ in methanol (w/v) (HPLC grade, Fisher scientific). Then, 38 µL of this

162 stock solution (corresponding to 38 µg of DBP, the quantity associated with 300 mg MPs) were

163 added to 50 mL of ASW and homogenised within 3 kg of sediment. As methanol was used as

164 a solvent for DBP powder dissolution to prepare the DBP spike solution, 38 µL of methanol

165 (same batch as the one used to dissolve DBP powder) was added to 50mL of ASW homogenised

166 with 3 kg of sediment corresponding to the “solvent” (S) exposure (2 aquaria). A “control” (C)

167 exposure condition was conducted by mixing 50 mL of ASW within 3 kg of sediment (2

168 aquaria). The appropriate spiking concentration was introduced in the corresponding aquarium

169 and manually mixed for 10 min with a stainless-steel spatula. The sediment was then covered

170 with 500 mL of 21 psu ASW under aeration. The chosen concentration of exposure (100 mg
171 MPs.kg⁻¹ sediment) represents the concentration found in sediment at the outlet of a sewage
172 treatment plant and was described by Missawi et al. (2021) as the one allowing MPs uptake in
173 *H. diversicolor* and leading to a clear response in term of biomarkers.

174 A total of 192 organisms *Hediste diversicolor* were washed in ASW and then randomly
175 distributed into each aquarium and exposed for 4 and 21 days to the 4 conditions prior to
176 biomarker and energy reserves analysis. When adding worms in the aquaria, aeration was
177 stopped for 15 min to allow organisms to settle undisturbed in the sediment. A constant aeration
178 was then applied to oxygenate the water of each aquarium before endpoints covering them with
179 glass plates to avoid MPs contamination from the ambient air and evaporation. During
180 exposure, controlled temperature of 16 °C ± 1 °C and photoperiod of 16h light:8h dark were
181 applied. *H. diversicolor* individuals were fed every 2 days at 2% of their biomass with "Goldfish
182 Tetra ©" fish food (Madeira et al. 2021). After 4 and 21 days of exposure to the 4
183 conditions, worms for biomarkers (n=9) and energy reserves (n=15) analysis were gently
184 collected from sediment, rapidly rinsed in clean ASW to remove adsorbed particles, weighed
185 and immediately frozen in liquid nitrogen and then stored at -80 °C until investigation.

186

187 2.4 Biomarker analysis

188 Enzyme activities (catalase-CAT, Glutathione-S-transferase-GST, Lactate dehydrogenase-
189 LDH, acetylcholinesterase-AChE, phosphatase acid-PA) were quantified in whole individuals
190 (n=9). First, the worms were thawed, weighed and homogenized, on ice, in Tris buffer at pH
191 7.2 (Tris-HCl 0.008M (Trizma-base Sigma® T6791); NaCl 0.02M (Sigma® S9888); DTT
192 1mM (Sigma® 8349-12-3); CIP 1/1000 (Protease Inhibitor Cocktail, Sigma® P8340)). The
193 homogenates were then centrifuged either at 3300 g at 4°C for 5 min for LDH analysis or at
194 9000 g at 4°C for 30 min for the other enzymatic analysis (CAT, GST, AChE, PA). The amount

195 of total protein was quantified using Bradford method (Bradford, 1976). CAT activity was
196 measured spectrophotometrically at 240 nm ($\epsilon = 0.04 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; UviLine® 9600) at 25°C,
197 following the dismutation of hydrogen peroxide (H_2O_2) (Claiborne et al. 1985). Specific
198 activity was expressed in $\text{nmoles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. GST activity was measured
199 spectrophotometrically at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ TECAN® Sunrise) at 25°C by observing
200 the conjugation of the 1-chloro-2,4-dinitrobenzene (CDNB) and the L-glutathione reduced
201 (GSH) (Habig et al. 1974). Specific activity was expressed in $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein.
202 AChE activity was determined spectrophotometrically at 412 nm (TECAN® Sunrise) at 25°C,
203 monitoring the conversion of acetylthiocholine into thiocholine in the presence of 5,5-dithiobis
204 (2-nitrobenzoic acid) called DTNB (Ellman et al. 1961; Galgani and Bocquene 1991). The
205 specific activity is expressed in $\text{nmoles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. Phosphatase acid (PA) activity was
206 determined spectrophotometrically at 340 nm ($\epsilon = 18.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; TECAN® Sunrise; Acid
207 phosphatase Assay kit Sigma® CS0740), measuring the appearance of p-nitrophenol (pNP) by
208 the hydrolysis of p-nitrophenylphosphate (4-NPP) at 37°C and expressed in $\text{nmoles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$
209 of protein.

210

211 2.5 Energy-related biomarkers

212 LDH activity was measured spectrophotometrically at 340nm ($\epsilon = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$ TECAN®
213 Sunrise) at 30°C, following oxidized NAD appearance (Diamantino et al. 2001). Specific
214 activity was expressed in $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. Energy reserves were quantified as
215 lipids, glycogen and proteins content of the organism. For each condition, 15 worms were
216 individually ground in liquid nitrogen, then the powder was homogenized with a motorized
217 grinder in a Tris magnesium buffer (0.1 M Tris-HCl pH 8.5, 0.4 M MgSO_4 , 15%
218 polyvinylpyrrolidone and 0.2% (w/v) Triton X-100). Total lipids were determined by the sulfo-
219 phospho-vanillin reaction and measured spectrophotometrically at 540 nm (TECAN® Sunrise)

220 (Frings et al. 1972). Total lipids concentration was expressed as mg. g⁻¹ of fresh weight (FW).
221 Glycogen content was estimated using enzymatic digestion by amyloglucosidase and measured
222 spectrophotometrically (TECAN® Sunrise) at 420 nm (Carr and Neff, 1984). Glycogen
223 concentration was expressed as mg. g⁻¹ of FW. For protein quantification and ETS (Electron
224 Transport System) activity, the homogenates were centrifuged at 3000 g, 10 min at 4 °C. Protein
225 concentration was quantified using Bradford method (Bradford, 1976) and expressed as mg. g⁻¹
226 of FW. ETS activity was determined according to a protocol adapted from De Coen and
227 Janssen (1997), based on the reduction of p-iodonitrotetrazolium as the final electron acceptor
228 of the mitochondrial respiratory chain. 50 µL of supernatant was mixed to 150 µL of buffer
229 solution (0.13 M Tris base; 0.27% (v/v) Triton X-100; 1.7 mM NADH; 274 µM NADPH) and
230 the reaction was started by adding 100 µL of INT solution (p-iodonitrotetrazolium; 8 mM). The
231 absorbance was measured spectrophotometrically at 20 °C for 10 min at 490 nm (TECAN®
232 Sunrise). Oxygen consumption rate conversion was calculated based on the stoichiometric
233 relationship, whereby for every 2 mmoles of formazan formed, 1 mmole of oxygen is
234 consumed. The oxygen consumed was then quantify related to the formazan formed ($\epsilon_{\text{INT formazan}}$
235 = 15,900 M⁻¹ cm⁻¹). The potential Energy consumption (Ec) after four days (T4) and 21 days
236 exposure (T21) was calculated based on ETS activity using an average oxyenthalpic equivalents
237 of 484 kJ.mol⁻¹ O₂ for combustion of an average mixture of protein, lipid and glycogen
238 (Gnaiger, 1983) and expressed as mJ.mg⁻¹ FW.h⁻¹ (De Coen and Janssen, 1997). The energy
239 reserve fractions: available energy (Ea) was calculated as the sum of lipid, glycogen and protein
240 contents transformed into energy equivalents and expressed in mJ mg⁻¹ FW using their energy
241 of combustion, respectively 39.5 J mg⁻¹, 17.5 J mg⁻¹ and 24 J mg⁻¹ (Gnaiger, 1983). Thus,
242 CEA (cellular energy allocation) value, representing the total energy budget, and expressed in
243 h⁻¹ (Smolders et al. 2004), was calculated by the ratio Ea /Ec, with: Ea = E_{Lipids} + E_{Glycogen} + E_{Proteins}
244 (mJ mg⁻¹ FW) and Ec = ETS activity (mJ mg⁻¹ FW h⁻¹).

245 No difference of worm biometric parameters (size and weight) were noticed at the end of
246 experiment between conditions.

247

248

249 2.6 Statistical analysis

250 Normality of data distribution and homogeneity of variance were tested using the Shapiro-Wilk
251 test and Bartlett test, respectively. Data analysis was performed at 5% alpha level using
252 XLSTAT 2019 software (4.2.63762). If the data did not meet the conditions for parametric
253 tests, Kruskal-Wallis tests were therefore used to test for differences between the treatments. If
254 the overall test was significant, a Dunn procedure was performed to determine which means
255 were significantly different. If the data followed the conditions for application of parametric
256 tests, an ANOVA was used to test for differences between the treatments. If the overall test was
257 significant, a Tukey post hoc test was performed to determine which means were significantly
258 different. The means are given with their standard deviations (SD). The statistical analysis
259 using the Principal Components Analysis (PCA) was carried out with XLSTAT 2019 software
260 (4.2.63762) and the significance of the correlation was tested by the Pearson test ($\alpha = 0.05$).
261 The PCAs were performed on a matrix containing all the studied parameters for the four
262 treatments (C, S, P and MP).

263

264 **3 Results**

265 3.1 Oxidative stress and detoxication

266 After 4 days (T4), no significant difference of *H. diversicolor* catalase (CAT) specific activity
267 (SA) was observed after microplastic (MP) exposure compared to control (C) and after
268 phthalates (P) exposure compared to the solvent (Figure 1A). The S and P treatments led to a
269 significant CAT decrease when compared to the C and MP treatments. After 21 days (T21) of

270 exposure, a significant inhibition of CAT activity was observed only for worms exposed to
271 solvent compared to the other treatments (Figure 1B). A CAT SA increase was depicted
272 between both time of exposure for all the conditions (T4 and T21).

273 Concerning Glutathione-S-Transferase (GST) activity, after 4 days of exposure, a significant
274 inhibition was depicted in the worms exposed to microplastic (MP) compared to the control one
275 (Figure 1A). No significant difference was observed comparing worms exposed to DBP (P) to
276 those exposed to solvent (S), both activities were significantly lower than the control (C) one.
277 After 21 days of exposure (Figure 1B), a significant increase of GST activity was depicted in
278 the all exposed *H. diversicolor* compared to control: the increase was significant comparing MP
279 exposed worms to the control, but not significant comparing P exposed worms to the solvent
280 treatment. Comparing both time exposures (T4 and T21), the mean value of GST activities
281 increased for MP, S and P treatments, while it decreased for the control one.

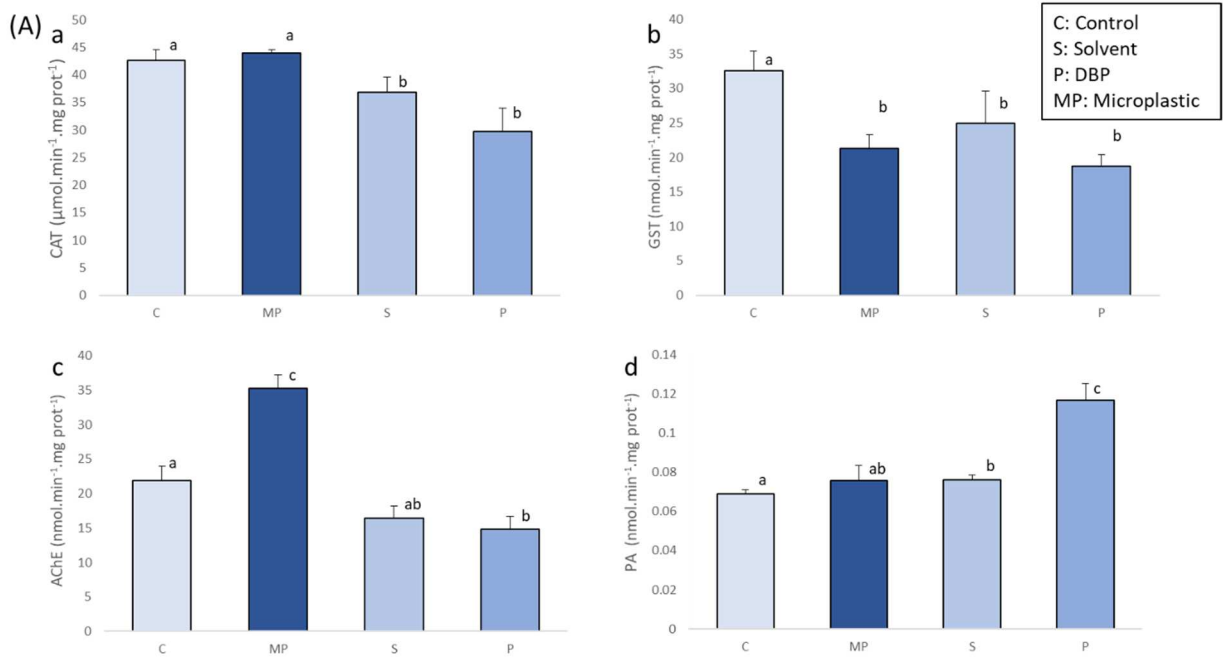
282 3.2 Neurotoxicity

283 After 4 days of exposure (Figure 1A), compared to the control, a significant increase of the
284 acetylcholinesterase (AChE) activity was observed in worms exposed to MP. No significant
285 difference was depicted for the S and P treatments. After 21 days of exposure, a significant
286 increase of AChE activity was depicted after worm exposure to MP compared to the control
287 (Figure 1B). AChE activity was significantly lower in the worms exposed to P compared to S.

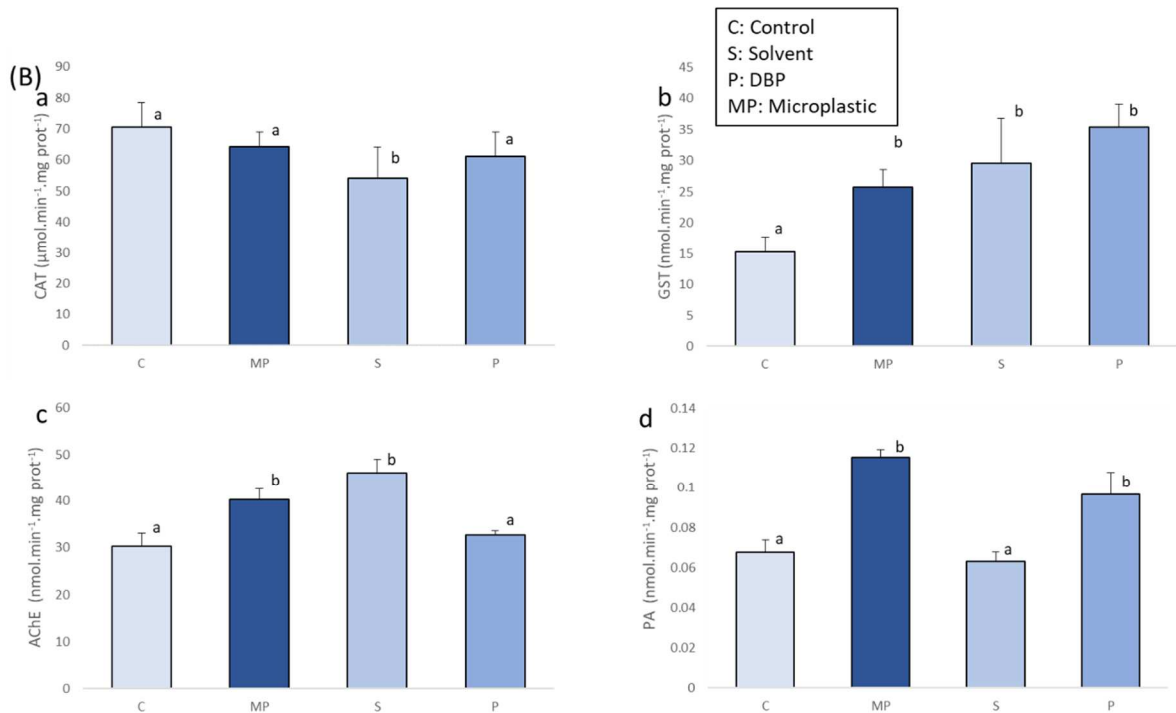
288

289 3.3 Immunity

290 After 4 days of exposure, a significant increase in the phosphatase acid (PA) activity after worm
291 exposure to Phthalate (P) and compared to methanol (S) (Figure 1A). After 21 days (Figure
292 1B), a significant increase of the PA activity was depicted in worms exposed to MP and P
293 compared to the control and the solvent exposures respectively.



294



295

296 Figure 1 Mean (a) catalase (CAT), (b) glutathione-S-transferase (GST), (c) acetylcholinesterase
 297 (AChE) and (d) phosphatase acid (PA) specific activities (mean \pm standard deviation) after 4
 298 days (A) and 21 days (B) of *H. diversicolor* exposure to 4 conditions: C: Control; S: Solvent;
 299 P: Phthalate (dibutylphthalate $0.126 \mu\text{g}\cdot\text{g MP}^{-1}$); MP: Microplastic ($100\text{mg}\cdot\text{kg}^{-1}$ sediment).
 300 N=9 for each condition. For each time exposure, different letters indicate significant differences

301 between conditions ($p < 0.05$), while the same letter indicates no significant difference between
302 treatments.

303

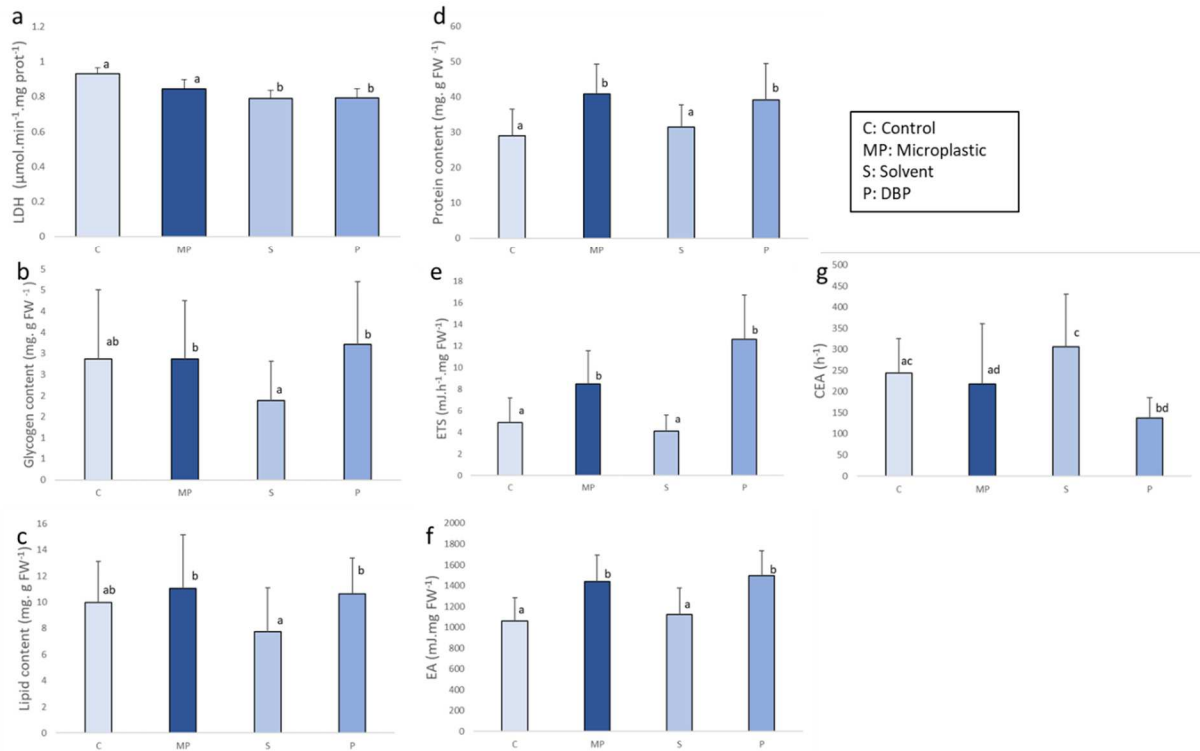
304 3.4 Energy-related biomarkers

305 After 4 days of exposure, no difference of the lactate dehydrogenase (LDH) activity was
306 depicted between worms exposed to MP vs C and to P vs S. A significant inhibition was
307 demonstrated after exposure to solvent and phthalate compared to control and MP (Figure 2).

308 After 21 days (Figure 3), only a significant inhibition of LDH was observed for worms exposed
309 to MP compared to C. No significant difference in glycogen and lipid content was observed in
310 MP exposed worms compared to the control whatever the time of exposure (Figures 2 and 3).

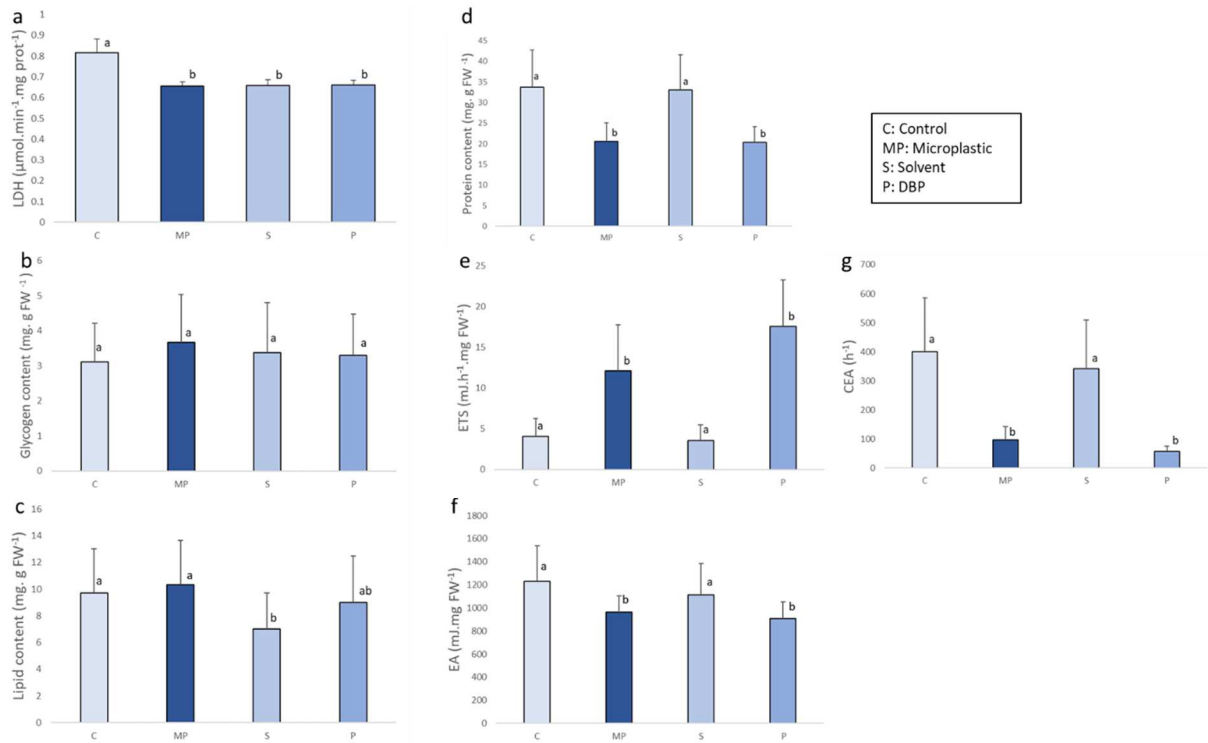
311 At T4 and T21, both contents were significantly higher in P exposed worms compared to S
312 treatment. Concerning the protein content, significant higher levels were observed after 4 days
313 of worm exposure to MP and P compared, respectively, to C and S treatments, whereas after
314 21 days, a significant decrease was observed comparing MP to C and P to S treatments (Figures
315 2 and 3). ETS, corresponding to the consumption energy (E_c), was significantly higher after
316 both time exposures for MP and P conditions when compared respectively to C and S treatments
317 (Figure 2 and 3). The available energy (E_a) was significantly higher at T4 (Figure 2) and lower
318 at T21 (Figure 3) for both MP and P exposures compared, respectively, to C and S treatments.
319 CEA was significantly lower at T4 (Figure 2) for worms exposed to P compared to S treatment,
320 and at T21 (Figure 3) for MP and P condition compared, respectively, to C and S conditions.

321



322

323 Figure 2 Mean (a) lactate dehydrogenase activity (LDH), (b) glycogen, (c) lipid and (d) protein
 324 contents, (e) ETS (Electron Transport System) activity *e.g* Energy consumption (Ec), (f) Energy
 325 available (Ea) and (g) cellular energy allocation (CEA) in *H. diversicolor* (mean \pm standard
 326 deviation) after 4 days exposure to 4 conditions: C: Control; S: Solvent; P: Phthalate
 327 (dibutylphthalate $0.126 \mu\text{g}\cdot\text{g MP}^{-1}$); MP: Microplastic ($100\text{mg}\cdot\text{kg}^{-1}$ sediment). N=15 for each
 328 condition. For each time exposure, different letters indicate significant differences between
 329 conditions ($p < 0.05$), while the same letter indicates no significant difference between
 330 treatments.



331

332 Figure 3 Mean (a) lactate dehydrogenase activity (LDH), (b) glycogen, (c) lipid and (d) protein
 333 contents, (e) ETS (Electron Transport System) activity *e.g* Energy consumption (Ec), (f) Energy
 334 available (Ea) and (g) cellular energy allocation (CEA) in *H. diversicolor* (mean \pm standard
 335 deviation) after 21 days exposure to 4 conditions: C: Control; S: Solvent; P: Phthalate
 336 (dibutylphthalate $0.126\ \mu\text{g}\cdot\text{g}\cdot\text{MP}^{-1}$); MP: Microplastic ($100\text{mg}\cdot\text{kg}^{-1}$ sediment). N=15 for each
 337 condition. For each time exposure, different letters indicate significant differences between
 338 conditions ($p < 0.05$), while the same letter indicates no significant difference between
 339 treatments.

340

341 3.5 Comparison of average changes in biomarkers

342 The average variations in significant specific biomarker activities between the control and MP,
 343 P and S treatments (Table 2) showed that MPs do not have a significant impact on CAT activity,
 344 glycogen and lipid contents, but modified all other studied biomarker activities especially after
 345 21 days. The main increase was ETS activity (197%), PA (71%) and GST (68.6%) activities.
 346 DBP caused also an increase in ETS activity (332%) and PA (43.1%) activities. Both MP and

347 DBP impacted protein content (-38.9 and -39.8% respectively) and CEA (-75.4% and -85.7%
 348 respectively).

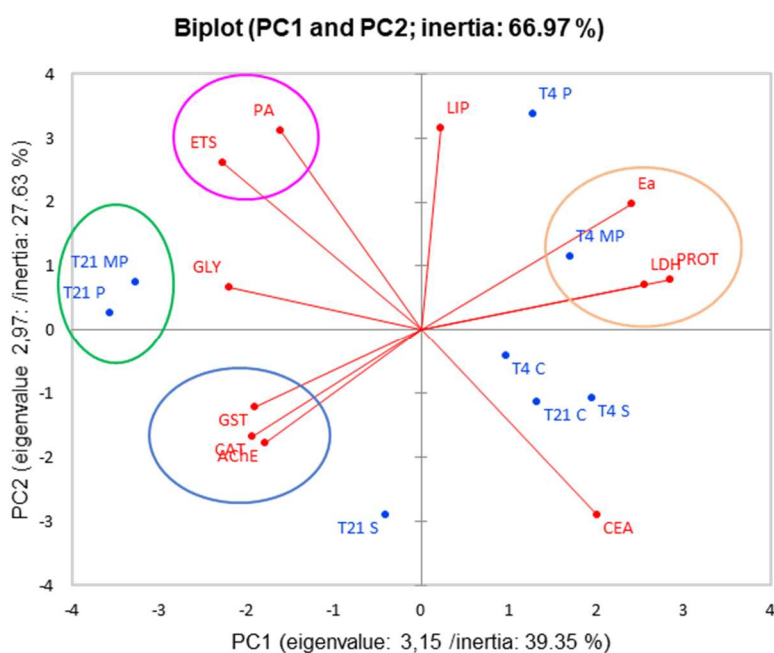
349
 350 Table 2 Significant difference ($p < 0.05$) in *H. diversicolor* specific activities of biomarkers
 351 (CAT: catalase; GST: glutathione-S-transferase; LDH: lactate dehydrogenase; AChE:
 352 acetylcholinesterase; PA: phosphatase acid; LIP: lipid content; GLY: glycogen content; PROT:
 353 protein content; ETS (Electron Transport System) activity e.g. Energy consumption (Ec); Ea:
 354 available energy; CEA: cellular energy allocation) after 4 days (T4) and 21 days (T21) of
 355 exposure to Solvent (S), Phthalate (P), Microplastic (MP), at T4 and T21, expressed as
 356 percentages as compared to control (C). Only significant differences are represented (light green
 357 to dark green: increase and yellow to red: decrease).

	T4			T21		
	S	P	MP	S	P	MP
CAT	-0.136	-0.300		-0.234		
GST	-0.237	-0.426	-0.347	0.935	1.314	0.686
AChE		-0.324	0.614	0.516		0.325
PA		0.700			0.431	0.710
LDH	-0.151	-0.148		-0.194	-0.188	-0.195
GLY						
LIP				-0.278		
PROT		0.348	0.104		-0.398	-0.389
ETS		1.565	0.725		3.325	1.979
Ea		0.413	0.359		-0.263	-0.219
CEA		-0.465			-0.857	-0.754

373
 374 3.6 Principal component analysis

375 PCA was conducted using two dimensions, representing 67% of the total inertia (39.35 for PC1
 376 and 27.63 for PC2) (Figure 4 and details in supplementary material). PC1 is mainly explained
 377 (>10%) by five biomarkers (LDH, Glycogen, Protein, ETS activity, Ea) and PC2 by four (PA,
 378 Lipid, ETS activity and CEA). A positive significant correlation (Pearson test, $\alpha = 0.05$,
 379 supplementary material) was established between Ea and PROT and between PA and ETS

380 activity. A significant negative correlation (Pearson test, $\alpha=0.05$) was depicted between GST
 381 and Ea, and between CEA, PA and ETS activity. GST, CAT and AChE were grouped, showing
 382 low contribution to both axis as ETS activity and PA, which were also grouped together. LDH
 383 was grouped to PROT and Ea. On the biplot, two conditions of exposure corresponding to T21
 384 P and T21 MP were clearly associated together and contributed to PC1 axis (>30%).



385

	GST	LDH	CAT	AChE	PA	LIP	GLY	PROT	ETS	Ea	CEA
Contribution to PC1 (%)	7.58	13.73	7.82	6.70	5.43	0.11	10.14	17.03	10.72	12.20	8.53
Contribution to PC2 (%)	3.06	1.055	5.88	6.62	20.33	20.92	0.96	1.28	14.20	8.11	17.58

386

387 Figure 4: Principal component analysis (PCA) of the effect of microplastic (MP),
 388 dibutylphthalate (P), methanol (S) and control exposure (C) for 4 days (T4) and 21 days (T21)
 389 on oxidative stress (GST, CAT), neurotoxicity (AChE), immunity (PA), and energy-related
 390 metabolism: LDH, protein (PROT), lipids (LIP), glycogen (GLY), ETS (Energy consumption),
 391 CA and CEA on *H. diversicolor*. For each axis, eigenvalues and inertia values are indicated.
 392 The table below summarizes the contributions to the axes of each variable (percentage).

393

394 **4 Discussion**

395 The Seine estuary is highly impacted by human activities and therefore by plastic pollution.
396 Tramoy et al. (2019, 2020a, 2020b) referred to the Seine estuary as a “microplastic factory”
397 describing the dynamics of plastics between land and ocean, leading to the slow degradation of
398 macroplastics into MPs before reaching the ocean. Once in the marine environment, most MPs
399 tend to sink and accumulate on the seabed and in the sediments, acting as MPs tanks (Van
400 Cauwenberghe et al. 2015b). The chemical analysis of the MP mixture used in this study
401 showed dibutylphthalate (DBP) as the most representative phthalic acid esters (PAE) associated
402 with macroplastics debris originated from the Seine estuary. DBP is known to be used as a
403 plasticizer representing 10% to 35% of the typical concentration associated with PUR and soft
404 PVC polymers (ECHA, 2022). The toxic effects of environmental MPs obtained from a mixture
405 of those debris and the equivalent content in DBP were investigated on the ragworm *H.*
406 *diversicolor*.

407

408 4.1 Microplastic effects on worms

409 Our results first showed that the mixture of environmental MPs used for exposure, at the
410 concentration of 100 mg. kg⁻¹ of sediment, caused metabolic disorders on *H. diversicolor*. The
411 duration of the exposure to environmental MPs caused different impacts with the longer
412 exposure (21 days) leading to significant level changes for 8 biomarkers out of the 11 studied,
413 whereas only 5 had their levels impacted after 4 days of exposure (Table 2). The 4 days
414 exposure to EMPs impacted the phase II antioxidant system of worms and induced a weak
415 neurotoxic response. These results are in accordance with the study of Missawi et al. (2021)
416 which showed impairments of neurotransmission and antioxidant pathways on *H. diversicolor*
417 exposed to an equivalent concentration of an environmental MPs mixture (100 mg. kg⁻¹
418 sediment). Concerning the energy metabolism, after 4 days exposure, the protein content

419 increased, together with an increase in ETS and Ea. After 21 days of exposure to MPs, an
420 enhancement of the antioxidant system (GST), neurotoxicity (AChE), immunity (PA) was
421 depicted while energy metabolism was also strongly impacted. No change in the level of
422 antioxidant enzyme catalase (CAT) was observed even though its level increased during the
423 exposure. The high level of CAT activity and its increase between both times (30-40 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$
424 $\cdot\text{mg}^{-1}$ protein after 4 days and 60-70 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein after 21 days exposure) could be
425 the result of a stress induced by the experimental conditions of exposure as previously reported
426 on *Hediste diversicolor* by Pedro et al. (2022). Studies conducted on *H. diversicolor* previously
427 demonstrated that MPs entered the body of worm when exposed through sediment (Revel et al.
428 2018; Missawi et al. 2021). It has been shown that the feeding mode of this worm as deposit-
429 feeder could enhance the accumulation of MPs through ingestion (Pires et al. 2022). Once into
430 the gut, it could disrupt essential intestinal function promoting cellular metabolism disorders
431 (Hirt and Body-Malapel, 2020). Defence mechanisms are then set up at the cellular level to
432 counter the excess of ROS. Generally, both catalase and GST are part of the antioxidant system
433 reducing excess of ROS (reactive oxygen species), whose increase was triggered by several
434 pollutants in invertebrates (De Marco et al. 2022). In our study, ROS excess did not seem to be
435 eliminated by catalase, which usually acts as an antioxidant enzyme, directly scavenging ROS
436 by neutralising acute burst of H_2O_2 . Another pathway seems to be used involving GST, a phase
437 II enzyme of detoxification and excretion of endogenous compounds and oxidative stress
438 products (Trestrail et al. 2020). However, these mechanisms didn't seem sufficient to reduce
439 the ROS effects and after 21 days of exposure, the energy metabolism was also impaired.
440 Lactate dehydrogenase, as an enzyme of anaerobic metabolism, is mainly involved in the
441 energetic homeostasis equilibrium of the cells and its level assess the metabolic state of the
442 organism (Moreira et al. 2006). After 21 days of exposure, its inhibition may demonstrate that
443 the anaerobic pathways of energy production were also inhibited when the worms faced the

444 effect of MPs exposure. Concomitantly, the ETS activity increase (197.9% vs control) in worms
445 exposed to MPs is related to an enhancement of the cellular aerobic metabolism and energy
446 consumption (Ec). Increase in ETS activity were previously reported in *H. diversicolor* exposed
447 to PS (polystyrene) nanoparticles (0.005 to 50 mg.L⁻¹) (Silva et al. 2020), to arsenic (50 µg.L⁻¹)
448 ¹) after a parental exposure to 100 nm PS nanoparticles (28 days; 0.005 mg.L⁻¹) (Silva et al.
449 2022), to mercury (Freitas et al. 2017), and to multi-walled carbon nanoparticles (De Marchi et
450 al. 2018), but also in mussels *Mytilus edulis* exposed to PS microplastics on the field (Van
451 Cauwenberghe et al. 2015a). ETS activity measures the metabolic capacity throughout the
452 oxygen consumption and stands out as an even more relevant biomarker of stress when
453 combined with the quantification of energy reserves (Pedro et al. 2022). ETS activity can be
454 the reflect of the metabolic need in energy by the worm to detoxify its body. Since the glycogen
455 and lipid levels were not impacted by exposure to MPs in our study, the energy reallocated to
456 the defence system may come from proteins. Previous studies showed that glycogen alone
457 (Silva et al. 2022) or in combination with protein (Freitas et al. 2017, De Marchi et al. 2018)
458 were the main energy source of *H. diversicolor* to face a stress caused by contaminant exposure.
459 In contrast, Silva et al. (2020) demonstrate that glycogen content was not modified by the tested
460 exposure conditions of *H. diversicolor* to polystyrene nanoplastics at 0, 0.005, 0.05, 0.5 and 50
461 mg.L⁻¹. Worm's fitness could be impacted by the energy cost related to repairing cells and
462 maintaining homeostasis, by promoting defence mechanisms to the detriment of other
463 physiological functions such as growth or reproduction (Smolders et al. 2004, Mouneyrac et al.
464 2010). The negative correlation highlighted between CEA and ETS activity and the positive
465 one between Ea and protein level were in accordance with the results obtained by Pham et al.
466 (2023), who studied the additive effect of copper and warming on *Hediste diversicolor*. CEA
467 was shown as an ecologically relevant biomarker to study environmental stressors on worms.
468 The conditions of exposure combining feeding and aeration could allow the worms to maintain

469 their energy reserves. Under the effect of stress, CEA decreased representing the time of
470 survival for the worms under energy reserves depletion (Pham et al 2023). The strong
471 contribution of the longer time of exposure (21 days) to PC1 on the PCA highlighted the
472 necessity of using long-term exposure as underlined by Li et al (2020). Moreover, the aerobic
473 respiration rate increase causes an elevation of ROS levels, which is consistent with the GST
474 increase depicted in our study (Trestrail et al. 2020). A somewhat unexpected result concerned
475 neurotoxicity. Indeed, AChE is considered to be a biomarker of neurotoxicity, whose activity
476 has, according to many chemicals' exposure studies (Deidda et al 2021, Parrino et al. 2021, De
477 Marco et al. 2022), a tendency to be inhibited by contaminant expressing anticholinergic
478 properties, as previously demonstrated in *H. diversicolor* (Urban-Malinga et al. 2022, Abouda
479 et al. 2022). However, several studies showed that neurotoxicity may also be mediated by
480 apoptosis. Xie et al. (2016) attributed the induction of AChE activity to both apoptosis and
481 inhibition of the muscarinic cholinergic receptor in the brain of the crucian carp (*Carassius*
482 *auratus*) exposed to diphenhydramine. Kim and Lee (2018), in their review on invertebrate's
483 acetylcholinesterases, described AChE as a putative stress regulator, exhibiting additional non-
484 neuronal physiological functions. This is in accordance with more ancient studies showing
485 AChE enhancement after exposure of zebrafish to neurotoxic compounds such as aluminium
486 (Senger et al. 2011) or ethanol (Rico et al. 2007). According to Barboza et al. (2020), AChE
487 activity may be induced in response to oxidative stress and damage due to MPs exposure in 3
488 marine fish (*Dicentrarchus labrax*, *Trachurus trachurus* and *Scaber colias*), which inflamed
489 cells and tissues need higher acetylcholine level compared to non-exposed ones. Our conclusion
490 is in accordance with Barboza et al. (2020), the enhancement of AChE, could be a signal of
491 neurologic alterations even if the molecular mechanisms of related to cholinergic signalling are
492 until now not well characterised in worms. Moreover, at T21, the phosphatase acid activity was
493 also enhanced demonstrating alteration in lysosomal membrane and consequently of the

494 immune system. In the study of Revel et al. (2018), no alteration of PA activity was depicted
495 after a 4 days exposure of *H.diversicolor* to MPs (10 and 50 mg.kg⁻¹). Based on our results, it
496 appears that PA activity was enhanced after longer times of exposure (21 days).

497 4.2 DBP effects on worms

498 As dibutylphthalate was the main additive measured in the MPs mixture used in this study, its
499 effect on the worm was studied in parallel at an equivalent concentration. In our study, methanol
500 was used as a solvent control allowing the comparison of exposure with DBP, as methanol was
501 used to dissolve DBP powder. Whatever the duration of the exposure of worms, and compared
502 to control, methanol impacted all enzymatic activities (CAT, GST, AChE, PA, LDH) except
503 AChE and PA after 4 and 21 days of exposure, respectively. This led us to disregard the major
504 enzymatic responses depicted after DBP exposure of worm. To our knowledge, the literature
505 on the toxicity of methanol on invertebrates is relatively scarce. According to Kaviraj et al.
506 (2004), methanol is lowly toxic to aquatic organisms and the estimated concentration of
507 methanol that produces no observed effect (NOEC) to freshwater ecosystem in chronic
508 exposure is 23.75 mg. L⁻¹. However, after 4 days exposure, the activities of CAT, GST and
509 LDH decreased in the worm exposed to methanol and after 21 days of exposure all the
510 enzymatic biomarkers activities and lipid content were impacted. This conduct to consider the
511 conjugate effect of methanol and DBP for the impacted biomarkers and relativize the impact of
512 DBP on most enzyme activities that may be due to the presence of methanol. This reflects the
513 difficulty to study the effect of environmental plastic as they correspond to a mixture of
514 different type of plastic with different additives or adsorbed contaminants.

515 The only clear effects of DBP, except from those of the solvent, was related to an increase in
516 PA activity at both exposure duration and an alteration of the cellular aerobic metabolism: an
517 increase in protein content, ETS and EA, a decrease of CEA after 4 days exposure; a decrease
518 in protein content, EA, CEA and an increase of ETS activity after 21 days of exposure. Those

519 effects of DBP on worms are relatively similar to those due to MP exposure. However, no other
520 effect, particularly concerning enzymatic activities (CAT, GST, AChE, LDH) can be attributed
521 directly to DBP due to the presence of the solvent. DBP has been previously shown to be the
522 most toxic phthalate among PAE (Li et al 2020) causing oxidative damages on the swimming
523 crab *Portunus trituberculatus* (He et al. 2022), both oxidative and energy metabolism
524 disturbances on the marine mussel *Mytilus coruscus* (Gu et al. 2021), oxidative stress, lipid
525 peroxidation and DNA damage in zebrafish (*Danio rerio*) liver (Song et al. 2022) and to disrupt
526 embryonic development in a sessile marine invertebrate *Galeolaria caespitosa* (Lu et al. 2017).
527 Berenstein et al. (2022) demonstrated the potential impact of DBP, issued from the degradation
528 of horticultural PE covers and migrating to the soil, on the earthworm (*Eisenia andrei*)
529 reproduction. Comparing the patterns of response between MP and DBP exposure of *H.*
530 *diversicolor*, significant difference was only shown for AChE activity after 21 days of exposure.
531 This could be linked to an effect of the type of polymer plastic on, principally, neurotoxicity
532 for the long-term exposure, mainly to the other additives linked to the MP and their
533 bioavailability. In our study, direct exposure of worms to bioavailable DBP, not bound to
534 materials like organic matter, could lead to higher toxicity. Upon exposure to MP, DBP could
535 also be released by MP, which could have been embrittled during the cryo-grinding. Chi et al.
536 (2021) demonstrated that the bioavailability of DBP was dependant of the type and the
537 concentration of MPs in sediments. The environmental MP mixture used in this study was
538 composed by four types of plastic polymers (PE 40%, PP 40%, PET 10%, PVC 10%), whose
539 neurotoxicity has already been demonstrated, such as PE/PP (m/m) mixture (0.06 g.kg⁻¹ of sand)
540 on *Donax trunculus* (Tlili et al. 2020) and reviewed on different organism by Prokić et al.
541 (2019). Further study on *H. diversicolor* reproduction and behaviour is needed to better
542 characterize the impact of DBP and MPs on this species and the potential consequences at the
543 population level and ecosystem levels. Zimmerman et al. (2020) demonstrated that the plastic

544 chemical contribution to toxicity on *Daphnia magna* was plastic type-specific associated to
545 chemicals for PVC, and to the mere particle for polyurethane (PUR) and polyactic acid (PLA)
546 microplastics. Gomiero et al. (2018) compared the adverse effect on *H. diversicolor* exposed to
547 sediment spiked with PVC MPs, with or without adsorbed benzo(a)pyrene B[a]P, or with
548 benzo(a)pyrene alone, demonstrating the effective role of MPs as vectors of contaminant and
549 the efficient transfer of this organic compound from sediment to the worm's coelomocytes
550 leading to a greater adverse response when MPs were previously combined with B[a]P.

551

552 **5 Conclusion**

553 In conclusion, our study showed an impact of environmental microplastics on the worm *H.*
554 *diversicolor* after 4 days and 21 days exposure. Can we link directly the effect of environmental
555 MPs to the presence of DBP? DBP has been chosen in the present study as it represented the
556 most abundant chemical found on plastics collected in the Seine River, but the presence of the
557 methanol as a solvent control during exposure interfered with the response of worms to DBP.
558 In general, similar pattern were observed in worms exposed to MP and DBP principally on
559 energy-related biomarkers indicating that DBP seems to be responsible of MP toxicity in tested
560 organisms. However, AChE, GST and LDH activities showed different induction when animals
561 were exposed to DBP vs MP, indicating that for these responses, type of plastic itself caused
562 lipid peroxidation leading to neurotoxic responses. Moreover, the environmental MPs used in
563 this study are associated with many other contaminants as metals or hydrophobic organic
564 contaminants such as PAHs, whose concentrations are much lower than DBP one. This
565 highlight the fact that studying the effects of additives are complex and additional studies must
566 be conducted in order to understand how all these contaminants are released in the sediment
567 depending on the concentration, environmental and physical parameters. All these factors could

568 also influence MP ingestion and the release of the adsorb contaminant in the sediment or in the
569 worms and must be not neglected.

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571

572

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580 Ethical Approval: Not applicable. This article does not contain any studies with human
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587 Amélie Châtel, Isabelle Métais, Messika Revel and Coraline Roman conducted the
588 conceptualization and the methodology of the experiment. Coraline Roman, Hanane Perrein-
589 Ettajani, Mohammed Mouloud, Isabelle Métais, Amélie Châtel sampled the worm and
590 conducted the lab MP exposure. Enzymatic biomarker analysis was performed by Coraline
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