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## Toxicity assessment of microplastics within the Loire River in the clam *Corbicula fluminea* exposed to environmentally relevant exposure conditions

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1 Toxicity assessment of microplastics from Loire River in the clam *Corbicula*  
2 *fluminea* exposed to environmentally relevant exposure conditions

3  
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10

11 **Abstract**

12 The diffuse pollution caused by microplastic (MPs) represents a current and global concern. While the  
13 fragmentation of plastic debris into smaller particles occurs in rivers, few MPs research on freshwater  
14 species is available compared to the marine environment. The Loire River, the longest French river, is  
15 subjected to moderate to high anthropic pressure while it represents major societal and economic  
16 issues. However, effects of environmental MPs (EMPs) on aquatic organisms have not been studied yet,  
17 and no policies can take place to monitor the plastic pollution. In this study, freshwater bivalves,  
18 *Corbicula fluminea*, were exposed for 21 days to environmentally relevant concentrations of a mixture  
19 of < 200 µm MPs generated from plastic litter collected directly along the Loire river banks. This mixture  
20 was composed of 40% polyethylene (PE), 40% polypropylene (PP), 10% polyethylene terephthalate  
21 (PET) and 10% polyvinylchloride (PVC) (mass percentage). Ecotoxicological effects were assessed from  
22 the individual to the molecular levels on several endpoints: condition index, filtration efficiency, enzyme  
23 activities, lipid peroxidation, energy reserves and gene expression. The ingestion of EMPs caused  
24 damages at the biochemical level. Indeed, we reported an increase in catalase activity in gills and  
25 digestive mass, a decrease in TBARs in gills, a decrease in acetylcholinesterase activity in the digestive  
26 mass, a decrease of glycogen and lipid contents in the whole organisms and a significant induction of  
27 the expression of *gst*, *cat*, *mp*, *acp* genes. The current results suggest therefore that long-term exposure  
28 to realistic doses of EMPs causes toxicity towards freshwater benthic biota. Biomarkers at the  
29 biochemical and molecular levels are complementary tools allowing to prevent an early plastic  
30 contamination for aquatic organisms.

31 **Keywords:** Microplastics; Bivalves; Biomarkers; Gene expression; Energy reserves

32

33 **Introduction**

34 Since the last decade, plastic pollution has become a major environmental concern due to its  
35 increasing production, estimated at 390.7 million metric tons in 2021 (Statista Research Department,  
36 2023). Among these plastics, many are single-used and immediately discarded, in most cases

37 improperly. Then, they reach the terrestrial environment and ultimately end up in aquatic environments  
38 (Oliveira et al., 2019; Ferreira et al., 2019). At the global level, it is estimated that between 70 and 80%  
39 of plastics in oceans come from land-based sources (Duis and Coors, 2016). While rivers are the main  
40 entryway for plastic litter into the marine compartment, these streams are still poorly studied (Li et al.  
41 2018). The Loire River, the longest French river with a length of 1 006 km, presents some major societal  
42 and economic issues for the country (Managing Rivers Wisely, World Wildlife Fund (WWF)). In some  
43 cities, such as Angers, the Loire water is used as a resource of drinking water and fishing is a widespread  
44 activity in this area. Since anthropic activities of towns may affect the Loire River it seems helpful to  
45 characterise the plastic contamination around metropolises in order to implement adequate policies.  
46 Moreover, once plastics reach the aquatic compartment, they can be fragmented into smaller size  
47 polymers by photodegradation (UV radiation), weathering and physical abrasion (e.g., wind and wave  
48 action), and biotic factors (e.g., microorganisms' action). Nowadays, small plastic particles ( $\leq 5$  mm) so-  
49 called microplastics (MPs) represent a current and a global environmental concern. Indeed, numerous  
50 scientific studies reported that MPs are increasingly being observed in all components of most aquatic  
51 ecosystems worldwide, as suspended particulates in the water column, in sediments and in wastewater  
52 (Burns and Boxall 2018; Frias et al. 2020; Habib et al., 2020; Bayo et al. 2020; Dusaucy et al. 2021; Uddin  
53 et al. 2021; Vidal et al. 2023). In addition to being found in abiotic compartments, it has been  
54 demonstrated that MPs are ingested by a wide variety of aquatic biota, at different levels of food webs,  
55 from microalgae to mammals (Ferreira et al. 2019, Wang et al. 2019; Bringer et al. 2020; Qu et al. 2020;  
56 Latchere et al. 2021; Pires et al. 2023).

57 Among invertebrates, bivalves are considered as good bioindicators to assess plastic pollution levels  
58 (Boening, 1999). They are often used in laboratory to study the ecotoxicological effects of xenobiotics.  
59 Indeed, bivalves are filter-feeders and have the ability to bioaccumulate pollutants from their  
60 surrounding environment. Since years, Asian clam (*Corbicula fluminea*) is considered as a relevant  
61 bioindicator to assess the anthropic pollution in aquatic compartments, and recently for plastic pollution  
62 (Su et al. 2018). The presence of MPs is global in the aquatic compartments, especially in the marine  
63 environment, on which many studies have focused. Despite a major research effort conducted to study  
64 the effects of MPs on freshwater streams in general and on freshwater bivalves in particular, literature  
65 on MPs contamination of freshwater bivalves remains still sparse compared to the publication on  
66 marine bivalves. Indeed, Sendra et al. (2021) reported 56 studies dealing with marine bivalves *versus* 13  
67 studies dealing with freshwater bivalves, of which 6 of them used *Corbicula fluminea* as their model  
68 species, the rest (*Unio pictorum*, *Perna canaliculus*, *Dreissena burgensis*, *Elliptio complanate* and  
69 *Dreissena polymorpha*) counted for either 1 or 2 works. In bivalves, it has been demonstrated that MPs  
70 can be retained in the gut over timescales beyond those expected for other ingested matter (Browne  
71 et al. 2008), and that MPs can be ingested by bivalves under environmental conditions (Ward et al.

72 2019). Once MPs are ingested, they may be retained in the digestive tract, egested through defecation,  
73 or translocated from the gut to the circulatory system (Browne et al. 2008). In addition, diverse  
74 chemicals named 'additives' are integrated to plastic polymers during their manufacture to improve  
75 their properties and extend their lifespan (Hahladakis et al. 2018). Studies focused more and more on  
76 the effects of these additives on aquatic organisms (Ateia et al. 2020; Hermabessiere et al. 2017). The  
77 few studies addressing this topic reported worrying effects on bivalves, but toxicological studies using  
78 EMPs (involving additives and aging of plastics) are still scarce. There is therefore a lack of knowledge  
79 about their effects on aquatic organisms. This was demonstrated in the review of Latchere et al. (2021)  
80 who reported that scientists often used unrealistically high concentrations of MPs in laboratory  
81 exposures. This may lead to misleading conclusions about the impacts of plastic particles on aquatic  
82 ecosystems. Also, it appears necessary to test MPs coming from macro-sized plastics collected in the  
83 environment to be as closed as possible to the environmental conditions. In this context, this study aims  
84 to evaluate the toxicity of environmental MPs (EMPs) on the freshwater bivalve *Corbicula fluminea*  
85 under environmental realistic conditions such as a long-time exposure (21 days), environmental  
86 exposure concentrations and use of plastics coming directly from the field. Ecotoxicological effects of  
87 EMPs on clams were assessed at different biological organisation levels, from molecular to individual  
88 levels on both relevant tissues (gill and digestive gland) or on the whole organism. We wondered in this  
89 study if the ecotoxicological effects are dependent on the exposure concentrations and/or the tested  
90 tissues. Also, we wondered if there is a link between effects at the different biological organisation  
91 levels. Results will help establishing first insights about the links between molecular effects and  
92 individual ones in an Adverse Outcome Pathway concept.

## 93 **Materials and methods**

### 94 **Collection, preparation and characterization of EMPs**

95 Plastic wastes were collected by hand along the Loire River banks in March 2022, near Angers,  
96 a medium city in West of France (47°42'38.34"N, -0°60'30.10"W). There is an accumulation of plastic  
97 specially at this site due to the river currents. In the laboratory, plastic wastes have been dried at room  
98 temperature for few days and cleaned with demineralised water to take off duff, sand and other dirt to  
99 avoid this matter to be transformed into microparticles. Plastic litter (bottles, rope, food packaging,  
100 diverse coloured pieces) was pre-cut and analyzed by Attenuated Total Reflection Fourier Transform  
101 Infrared spectrometer (ATR-FTIR Vertex70v, Bruker) equipped with a diamond crystal ATR accessory and  
102 a denudated triglycine sulfate (DTGS) detector. A few grams of each plastic: polyethylene (PE),  
103 polypropylene (PP), polyethylene terephthalate (PET) and polyvinylchloride (PVC) were put into a  
104 sample container with a magnetic bar, and both sides of the container were closed with two stainless  
105 steel caps. The container was inserted into a cryogenic mechanical miller (SPEX, 6770 Freezer-mill) filled

106 with liquid nitrogen. During the milling process, an oscillating magnetic field was generated, which drove  
107 the magnetic bar forward and backward inside the container and make the impacts with the caps.  
108 Milling consisted in 2 min of pre-cooling followed by 2 cycles of milling. Each cycle lasted 4 min with a  
109 1-min pause between the 2 cycles. The obtained powders of plastic particles were then sieved during  
110 60 min on a stain sieve of 200  $\mu\text{m}$ , to select particles size reflecting that of food particles ingested in the  
111 natural environment by Asian clams (Parra et al. 2021; Li et al. 2019). Fractions of PE, PP, PET and PVC  
112 under 200  $\mu\text{m}$  were then stored in a glass bottle at ambient temperature in the dark until preparation  
113 of the stock solution.

114 Based on the composition of polymers found in French Atlantic coastal sediments by Phuong et  
115 al. (2018), the following mixture of plastic particles has been realized (mass percentage): 40% PE, 40%  
116 PP, 10% PET and 10% PVC. This is also consistent with a recent study (Wu et al. 2022), which reported  
117 that the mostly common polymers analyzed in Asian clams sampled in the field (China) were PE (40%),  
118 PET (23%) and PP (18%). A first stock suspension at 1  $\text{mg}\cdot\text{mL}^{-1}$  was prepared in milliQ water at pH 7 and  
119 sonicated for 3 minutes. Then, solutions at 0.1  $\text{mg}\cdot\text{mL}^{-1}$  and 80  $\mu\text{g}\cdot\text{L}^{-1}$  were obtained by successive  
120 dilutions of the stock suspension in milliQ water at pH 7. After sonication (3 minutes), a specific volume  
121 of the stock suspension (1  $\text{mg}\cdot\text{mL}^{-1}$ ) or the working solutions (0.1  $\text{mg}\cdot\text{mL}^{-1}$  and 80  $\mu\text{g}\cdot\text{L}^{-1}$ ) was distributed  
122 every 3 days after a complete water renewal in the aquariums to obtain the following final  
123 concentrations: 0.008, 10, 100 and 500  $\mu\text{g}\cdot\text{L}^{-1}$ . These concentrations were based on Revel et al. (2019):  
124 a low concentration of 0.008  $\mu\text{g}\cdot\text{L}^{-1}$  for the coast regions, a medium concentration of 10  $\mu\text{g}\cdot\text{L}^{-1}$  for coastal  
125 region/gyres, a higher concentration of 100  $\mu\text{g}\cdot\text{L}^{-1}$  for gyres, and the highest concentration of 500  $\mu\text{g}\cdot\text{L}^{-1}$   
126 for freshwater streams. In addition, a Dynamic Light Scattering (DLS) analysis was performed on the  
127 stock solution and the working solutions (Zetasizer Nano-ZS90) to detect the presence of particles  
128 smaller than 1  $\mu\text{m}$ . It allowed to measure the hydrodynamic diameter and the aggregation state of  
129 particles in solution. In parallel, a spectroscopic MPs quantification by  $\mu$ -FTIR imaging (micro-Fourier  
130 Transform InfraRed; Thermo Nicolet iZ10) was performed on the stock solution to characterize the  
131 number, the size and the composition of particles in the used plastic mixture. A pixel with a resolution  
132 of 25 x 25  $\mu\text{m}$  was selected.  $\mu$ -FTIR imaging was performed for the entire sample *i.e.*, the entire filtration  
133 zone of the sample. Acquisition parameters were described in a recent study (Treilles et al. 2021). The  
134  $\mu$ -FTIR maps were treated with siMPle software (v.1.1. $\beta$ ) and the library  
135 MP\_Library\_extended\_grouped\_1\_5.txt. The default matching weight of 0.5 for the first derivative of  
136 the spectra and 0.5 for the second derivative of the spectra was used, and the AAU pipeline was chosen  
137 for data processing. The minimal particle size provided by siMPle is 25  $\mu\text{m}$ . The major dimension of  
138 plastic fragments provided in the output of the siMPle software were considered.

139 ***C. fluminea* sampling and laboratory experimental set up**

140 *C. fluminea* were collected in May 2022 in the Maine Lake located in Angers (France)  
141 (47°27'47.9"N 0°35'21.0"W). Organisms were transported to the laboratory in containers filled with  
142 sediment and water from the sampling site. They were then immediately transferred into aquaria (54  
143 L) containing lake water (0.45 L per individual), in a temperature-controlled room at 13°C, with a  
144 photoperiod maintained at 12:12. A total of 265 clams (mean length = 25.4 ± 0.83 cm and mean weight  
145 = 8.09 ± 0.66 g) were divided among five glass aquaria equipped with bubblers and covered with a glass  
146 plate to avoid plastic contamination by air. During the 10-days acclimation period, the water was  
147 replaced by tap water to progressively decrease the volume of the lake water: 100/0, 75/25, 50/50,  
148 25/75 until 0/100. At the end of the acclimation period, 15 individuals were sampled to provide  
149 biometrical data (mass and length). Organisms were then exposed to MP for 21 days via water spiked  
150 by the four concentrations mentioned above (0.008 µg.L<sup>-1</sup>, 10 µg.L<sup>-1</sup>, 100 µg.L<sup>-1</sup> and 500 µg.L<sup>-1</sup>). A control  
151 tank without added plastic was also put in place (Control). During both acclimation and exposure steps,  
152 the organisms were fed every 3 days with a suspension of unicellular algae *Scenedesmus subspicatus*,  
153 provided by Greensea. Before each feeding, algal concentration was counted on a Malassez cell to  
154 obtain 70,000 cells per individual per liter of water. Individuals were fed one hour before each water  
155 renewal. At the end of the exposure period, individuals were sampled in each aquarium: n=3 for  
156 filtration efficiency; n=10 for condition index and also used for the verification of MP ingestion; n=10 for  
157 biomarker analysis; and n=10 for energy reserves quantification. Whole soft bodies were sampled for  
158 the quantification of energy reserves and immediately frozen at -80°C, while gills and digestive glands  
159 were dissected and immediately frozen at -20°C for further biomarkers analysis.

## 160 **Analysis of MPs in the organisms**

161 A verification of the presence of MP in clams was performed to ensure their ingestion. Since the  
162 microscopy method does not allow to know the plastic composition and the µ-FTIR method does not  
163 give the colour nor the shape of MPs, it seems relevant to use both techniques for a complete  
164 characterization of MPs ingested by the organisms. Therefore, a first analysis was performed by  
165 microscopy to determine the size, the colour and the shape of MPs ingested by individuals for each  
166 condition (n=3 organisms), including the control. Thus, each clam was entirely digested in ethanol  
167 precleaned beakers containing 10% potassium hydroxide (KOH). Beakers, covered with aluminium, were  
168 placed on a heating plate at 60°C with stirring for 24 h to eliminate organic matter. The digesta was then  
169 filtered under a Büchner system using a 1.6 µm filter (GF/A 1820-037). The complementary analysis by  
170 µ-FTIR was conducted to determine the composition and the size of MPs ingested by organisms (pool  
171 of 3 individuals). The digestion protocol was the same as the microscopy analysis and the filtration was  
172 performed on Anodisc (Whatman) membrane filters (0.2 µm, 25 mm).

173 **Ecophysiology measurement**

174 ***Condition index***

175 The total weight and the soft tissue weight were determined for each clam and all conditions at  
176 the beginning (T0) and at the end of the 21 days exposure period (T21). The condition index (CI) was  
177 determined as follows (AFNOR, 1985):

178 
$$CI = \frac{\text{wet weight of soft tissues}}{\text{total wet weight}} \times 100$$

179 ***Filtration efficiency***

180 The filtration efficiency indicates the ability of bivalves to intake microalgae and other food  
181 items from the water column and is a key function for evaluation of the individual fitness. The filtration  
182 efficiency was defined as the volume of water cleared of suspended particles per unit time, and was  
183 estimated for *C. fluminea* previously exposed for 21 days to clean water (Control) or MPs (0.008, 10,  
184 100 and 500  $\mu\text{g.L}^{-1}$ ). Clams were individually placed in a 1 L beaker filled with 400 mL of water from the  
185 aquaria one hour before measurements started. Mixing was promoted by fine bubble aeration to  
186 minimize the re-suspension of faeces and individuals were placed on metal grids to let the faeces fall  
187 into the bottom of the beaker. An initial concentration of 300 000 cells.mL<sup>-1</sup> of *S. subspicatus* was added  
188 in each beaker. The filtration efficiency was then measured by an indirect method assessing the  
189 concentration of the algal solution every 15 min for 45 min according to classical algal cell counting  
190 technique (Utermöhl, 1958). Briefly, algal cells were fixed by adding a drop of Lugol Iodine solution  
191 (Iodine I<sub>2</sub>, 5%; Potassium Iodide KI 10%) in 10 mL of sample. Cells were counted under a light microscope  
192 using a Malassez counting chamber. The filtration efficiency was expressed in cells.mL<sup>-1</sup> of *S. subspicatus*.

193 **Biochemical analyses**

194 ***Energy reserves***

195 Protein, lipid and glycogen contents were quantified in the total homogenate using a thawed  
196 subsample for each energy storage fraction. For each condition, 10 clams were individually ground in  
197 liquid nitrogen, then the powder was homogenized with a motorized grinder in a Tris magnesium buffer  
198 (0.1 M Tris-HCl pH 8.5, 0.4 M MgSO<sub>4</sub>, 15% polyvinylpyrrolidone (antioxidant) and 0.2% (w/v) Triton X-  
199 100 (cell membrane permeabilizer)). Lipid and protein fractions were directly stored at -80°C while the  
200 glycogen fraction was boiled during 4 minutes before storage at -80°C. Total lipids were determined by  
201 the sulfo-phospho-vanillin reaction and measured spectrophotometrically at 540nm (TECAN® Sunrise)  
202 (Frings et al. 1972). Glycogen content was estimated using enzymatic digestion by amyloglucosidase and  
203 measured spectrophotometrically (TECAN® Sunrise) at 420 nm (Carr and Neff 1984). For protein  
204 quantification, the homogenates were centrifuged at 3000 g, 10 min at 4°C. Protein concentration was

205 quantified using Bradford method (Bradford 1976). Lipid, glycogen and protein concentrations were  
206 expressed as mg.g<sup>-1</sup> of wet weight (ww).

### 207 ***Oxidative stress, neurotoxicity and lipid peroxidation***

208 Enzyme activities: catalase (CAT), glutathione S-transferases (GST) and acetylcholinesterase  
209 (AChE), and lipid peroxidation were quantified in the gills and digestive gland tissues of clams. Firstly,  
210 the tissues were thawed on ice and homogenized in Tris buffer at pH 7.2 (Tris 0.8 M (Trizma-base Sigma®  
211 T6791); NaCl 2 M (Sigma® S9888); DTT 100 mM (Sigma® 8349-12-3); CIP (Protease Inhibitor Cocktail,  
212 Sigma® P8340)). The homogenates were then centrifuged at 9000 g at 4°C for 30 min. The amount of  
213 total proteins was quantified using the Bradford method (Bradford, 1976). CAT activity was measured  
214 spectrophotometrically at 240 nm ( $\epsilon = 0.04 \text{ mM}^{-1}.\text{cm}^{-1}$ ; UviLine® 9600), following the dismutation of  
215 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Claiborne, 1985). GST activity was measured spectrophotometrically at  
216 340nm ( $\epsilon = 9.6 \text{ mM}^{-1}.\text{cm}^{-1}$  TECAN® Sunrise) at 25°C by observing the conjugation of the 1-chloro-2,4-  
217 dinitrobenzene (CDNB) and the L-glutathione reduced (GSH) (Habig et al. 1974). AChE activity was  
218 determined spectrophotometrically at 412nm (TECAN® Sunrise) at 25°C, monitoring the conversion of  
219 acetylthiocholine into thiocholine in the presence of 5,5-dithiobis (2-nitrobenzoic acid) called DTNB  
220 (Ellman et al. 1961; Galgani and Bocquene 1991). To determine lipid damage, the formation of  
221 thiobarbituric acid reactive substances (TBARs), including malondialdehyde (MDA) was measured  
222 according to the method of Sunderman et al. (1985) with a colorimetric assay (540 nm) that quantifies  
223 lipid degradation products as an index of lipid peroxidation. All specific activities were expressed either  
224 in  $\mu\text{moles}.\text{min}^{-1}.\text{mg protein}^{-1}$  (CAT) or in  $\text{nmoles}.\text{min}^{-1}.\text{mg protein}^{-1}$  (GST, AChE and MDA).

### 225 **Analysis of gene expression by quantitative PCR**

226 *Corbicula fluminea* were pooled by three for each condition and were analyzed by quantitative  
227 RT-PCR for each condition. Total RNA was extracted using TRIzol reagent® (Life Technologies) from the  
228 gills and the digestive mass, according to the manufacturer's recommendations and precipitated with  
229 propan-2-ol. RNA concentration ( $\mu\text{g}.\mu\text{L}^{-1}$ ) was quantified using a NanoDrop 2000 spectrophotometer  
230 (ThermoScientific®). First-strand cDNA was synthesized from 5  $\mu\text{g}$  of total RNA using the Invitrogen™  
231 SuperScript™ III kit (ThermoScientific®; T100™ Thermal Cycler, BIORAD®) according to the  
232 manufacturer's recommendations. The expression levels of seven genes involved in oxidative stress,  
233 detoxication, respiratory chain, immunity, neurotoxicity and apoptosis were analyzed using a set of  
234 forward and reverse primers by quantitative RT-PCR. The ribosomal protein 7 gene (rpl7), previously  
235 tested and compared with other genes in our laboratory, was used as housekeeping gene. Previously,  
236 the quality of each pair of primers was checked: cDNA tests were amplified by PCR (T100™ Thermal  
237 Cycler, BIORAD®. 30 cycles: 30 s à 95°C, 30 s 181 à 60°C, 30 s à 72°C), then the amplification products  
238 were separated on 1.5% agarose electrophoresis gel. After staining with ethidium bromide, the  
239 presence and size of each amplicon were verified. Quantitative PCR (qPCR) amplifications were carried



240 out in triplicate in 96-well microplates (CFX Connect™ Real-Time System, BIORAD®) using SYBR™ Master  
241 Mix PCR Power SYBR™ Green (Invitrogen) containing the SYBR Green dye, DNA Taq Polymerase and  
242 dNTPs. For each reaction, 1 µL of each primer (50 pmoles.µL<sup>-1</sup>), 6.25 µL of SYBR Green mix, 3.75 µL of  
243 water treated with DEPC (DNase-free water) and 0.5µL of cDNA were added in each well. The qPCR  
244 reactions consisted of a first step of 10 min at 95°C (enzyme activation) followed by 45 cycles (95°C for  
245 30 s, 60°C for 30 s and 72°C for 30 s) and 5 min at 72°C. For each primer pair, amplification specificity  
246 was verified revealing the presence of a single product on a melting curve and a no-template control  
247 assay was realised. Expression levels were estimated by evaluating the fluorescence signal emitted by  
248 SYBR-Green®. This fluorescent marker binds to double-stranded DNA (dsDNA) and the fluorescence  
249 emitted is proportional to the dsDNA present in the reaction mix. Calculations are based on cycle  
250 threshold (Ct) values. The relative gene expression ratio of each target gene was calculated following  
251 the delta-delta method normalized with reference genes (Livak and Schmittgen, 2001), which is defined  
252 as:

$$253 \quad \text{ratio} = \frac{2 - \Delta\Delta\text{Ct}(\text{exposed})}{2 - \Delta\Delta\text{Ct}(\text{control})}$$

#### 254 **Quality control**

255 To prevent a cross-contamination, all equipment used in the laboratory was previously rinsed  
256 with milliQ water and ethanol, dried at room temperature in a hood and kept under aluminum foil to  
257 avoid contact with the ambient air. During the experiments, cotton laboratory coats and nitrile gloves  
258 were worn. For both methods (microscopy and µ-FTIR analysis), during the digestion, beakers were  
259 covered with aluminum foil. After filtration, the filters were stored in closed glass Petri dishes until  
260 microscopy and µ-FTIR analysis. Due to the remaining risk of cross-contamination of the samples, blanks  
261 were performed in KOH 10%, following the same protocol. For each series of digestion, a blank sample  
262 (without organisms nor water) was performed in parallel. Blanks were also performed for the filtration  
263 step, and for the microscopy and µ-FTIR analysis. Finally, the use of plastic material was avoided at best  
264 throughout this experimental study.

#### 265 **Statistical analysis**

266 Statistical analysis was carried out using XLSTAT 2019 (version 21.4.63762) and R software  
267 (version 4.0.3). Normality of data distribution and homogeneity of variance were tested using the  
268 Shapiro-Wilk and Bartlett tests, respectively. If data did not meet the conditions for parametric tests,  
269 Kruskal-Wallis test was therefore used to test for differences between the treatments. If the overall test  
270 was significant, a Dunn procedure was performed to determine which means were significantly  
271 different. If the data followed the conditions for application of parametric tests, an ANOVA was used to  
272 test for differences between the treatments. If the overall test was significant, a Tukey post hoc test was  
273 performed to determine which means were significantly different. In all cases, p values ≤ 0.05 were

274 considered statistically significant. Also, Principal Component Analysis (PCA) was realised using all data  
275 of biomarkers obtained for gills and digestive gland (quantitative variables) to evaluate the influence of  
276 MPs concentrations, on control and exposed *C. fluminea*, along the period of 21 days exposure. PCA  
277 was performed with the ade4 package (R software) and all missing values were removed from database  
278 to ensure the PCA realisation. Finally, overlaps were performed with qualitative variables (not used to  
279 build the PCA).

## 280 Results

### 281 Analysis of MPs in the exposure solutions and clams

282 The DLS analysis revealed the presence of particles under 25 µm in the stock solution and in the  
283 two working solutions (Figure S1). This is due to the non-selection of the grinded microplastics (<200  
284 µm). In this study, all MPs ≤ 200 µm were considered but only particles ranging between 25 and 200 µm  
285 (analyzed by both visual and spectrometry methods) were described below. The visual observation of  
286 the stock solution (3 replicates of 100 µL) revealed the presence of 92% of fragments and 8% fibers.  
287 Among the fragments, 73% ranged from 25 to 100 µm and 27% from 100 to 200 µm, and 55% were red,  
288 30% were black, 10% were grey and 5% were transparent. 42% of the fibers measured between 275  
289 and 1 000 µm, and 58% between 1 000 and 1 250 µm, with 50% being grey, 39% black and 11% blue.  
290 The µ-FTIR analysis confirmed the presence of PE, PP, PVC and PET in the stock solution. Indeed, among  
291 the 851 MPs identified in 100 µL of the stock solution, PE particles counted for 71.1%, PP for 19.9%, PET  
292 for 7.9% and PVC for 0.1%. The 1.1%-part remaining was constituted of PA (0.4%) and PS (0.7%). Also, 3  
293 particles of acrylic and 3 of polyamide (PA) were analyzed, probably due to a cross-contamination during  
294 a step of the experiment. The sizes of the MPs analyzed by µ-FTIR were identified as follows: between  
295 31.8 and 1790.9 µm for PE; between 31.8 and 632.9 µm for PP; between 38.2 and 220.3 µm for PVC;  
296 and between 25 and 183.1 µm for PET. The mean size ± SD associated to this µ-FTIR analysis were: 144.8  
297 ± 121.7 µm for PE; 105.4 ± 72.1 µm for PP; 80.2 ± 34.0 µm for PET and 103.6 ± 56.0 µm for PVC.  
298 Therefore, based on the mean values, the exposure was realised using plastic particles with a size less  
299 than 200 µm.

300 The verification performed to ensure that this specific size range of EMPs was ingested by the  
301 exposed clams confirmed this hypothesis (Figure S2). Indeed, both the visual count under the binocular  
302 microscope and the µ-FTIR analysis revealed an increasing presence of MPs in the clams, related to the  
303 increase in MPs concentration used in the different treatments (Figure S2). Analysis performed on the  
304 control group also revealed the presence of MPs despite subtracting the MPs found on the control filter,  
305 although fewer than those found in the contaminated conditions. MPs found on the blank filters  
306 (between 2 and 7 particles per filter) were mainly fibers (78%) measuring from 150 to 1 500 µm, either  
307 transparent, blue, grey or pink. The two fragments were blue and pink, and measured 25 and 250 µm

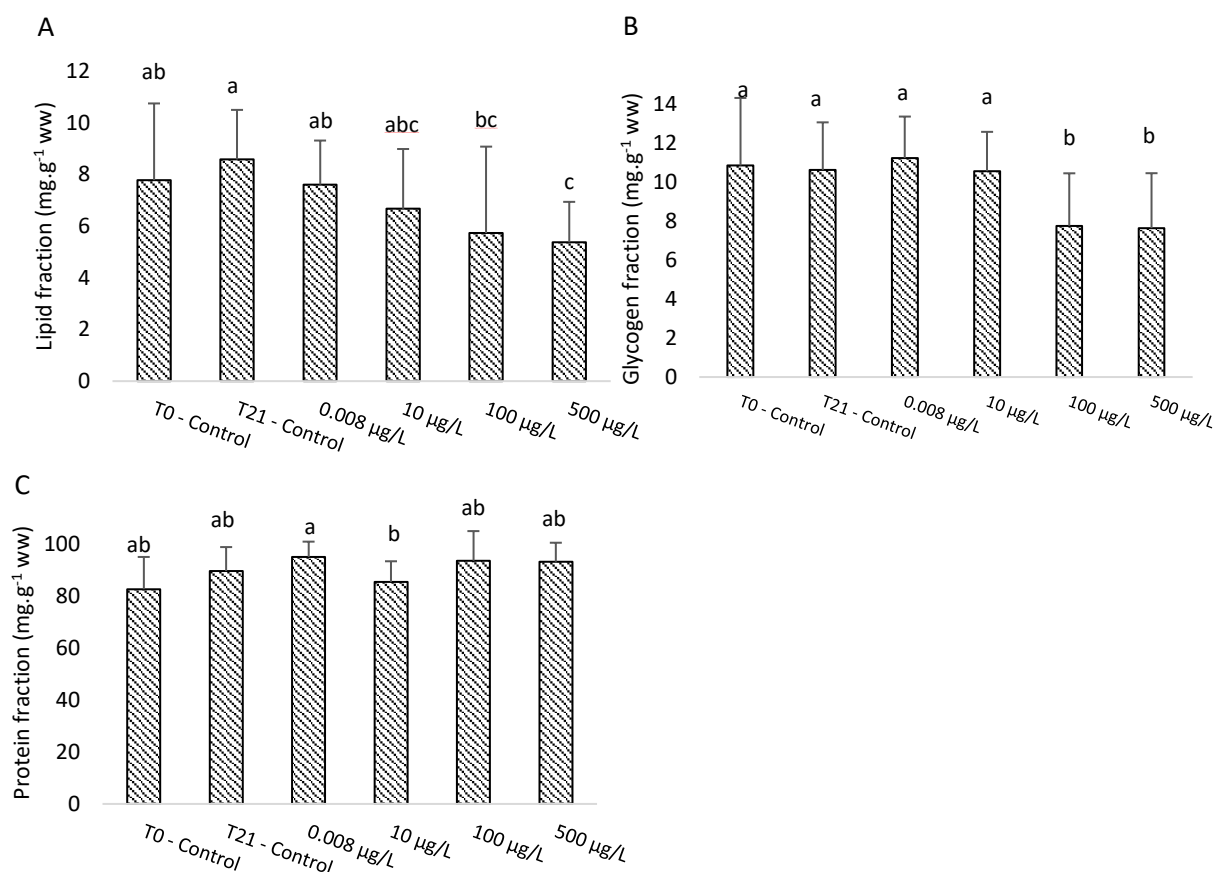
308 (major dimension), respectively. A light cross-contamination by acrylic and PA was also observed,  
309 probably coming from textiles worn during the experiment period, despite the precautions. Fragments  
310 and fibers with different colours (transparent, whitish/opaque, blue, grey and red) have been found in  
311 all organisms (Figure S2). PE was the dominant plastic polymer in the clams followed by PP, reflecting  
312 the MP profile of the stock solution. Neither PVC nor PET particles were found in the clams. The size of  
313 the analyzed MPs in clams measured by  $\mu$ -FTIR confirmed that determined by microscopy (Figure S2)  
314 and was of the same range as the sizes of MPs determined in the stock solution.

### 315 **Condition index and filtration efficiency**

316 No mortality was recorded during the exposition period of 21 days. No significant CI difference  
317 (Figure S3) was observed between the clams sampled at the beginning of the experiment (T0,  $19.30 \pm$   
318  $1.52$ ) and between the clams sampled from the control treatment and from the two lowest  
319 concentrations:  $0.008 \mu\text{g.L}^{-1}$  ( $19.08 \pm 2.28$ ) and  $10 \mu\text{g.L}^{-1}$  ( $17.73 \pm 2.68$ ) after 21 days of exposure (T21,  
320  $17.90 \pm 2.42$ ). However, a significant decrease of the CI was observed between the clams sampled at T0  
321 and those sampled at T21 at the highest concentrations:  $100 \mu\text{g L}^{-1}$  ( $16.20 \pm 2.21$ ) and  $500 \mu\text{g L}^{-1}$  ( $16.82$   
322  $\pm 3.02$ ). In addition, after 21 days exposure, the MPs contamination had no significant impact on the  
323 filtration efficiency of *C. fluminea*, independently of MPs concentration and measurement time (Figure  
324 S4).

### 325 **Energy reserves**

326 The exposure to EMPs during 21 days caused a decrease in glycogen and lipid contents in *C.*  
327 *fluminea*. Results indicated a significant post-exposure decrease in lipid content for individuals exposed  
328 to  $500 \mu\text{g MP.L}^{-1}$ , and in glycogen content for individuals exposed to  $100$  and  $500 \mu\text{g MP.L}^{-1}$  (Figure 1, A  
329 and B). No significant change in protein contents was observed between T0 organisms and control  
330 organisms at 21 days, and the treated organisms.



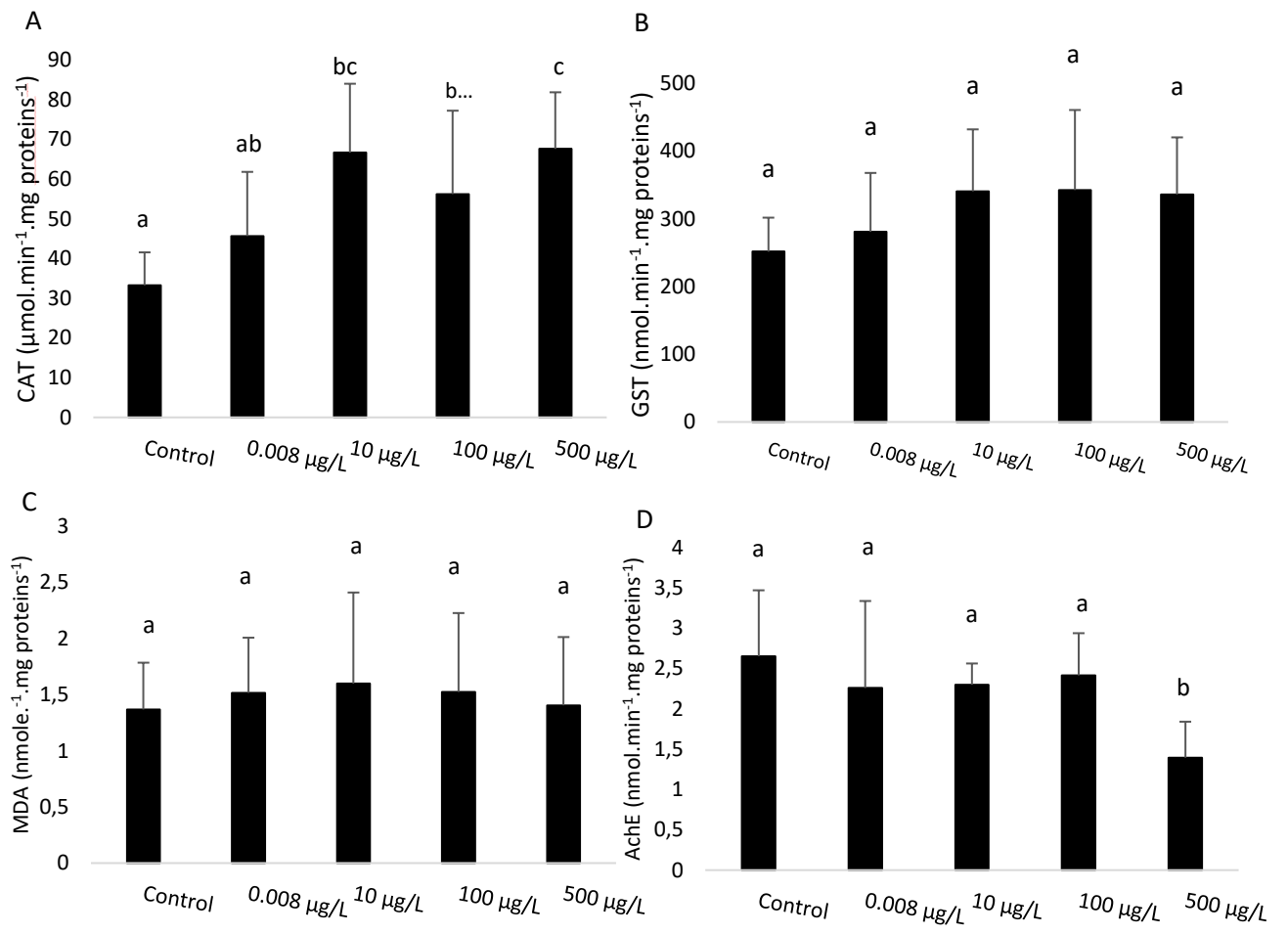
331  
 332 **Figure 1.** Energy reserve contents (mg/g wet weight  $\pm$  standard deviation) in *C. fluminea* (control and exposed to  
 333 EMP). A) Lipid fraction; B) Glycogen fraction; C) Protein fraction. Different lower-case letters indicate significant  
 334 differences between all groups ( $p < 0.05$ ).

### 335 Biomarkers: oxidative stress, lipid peroxidation and neurotoxicity

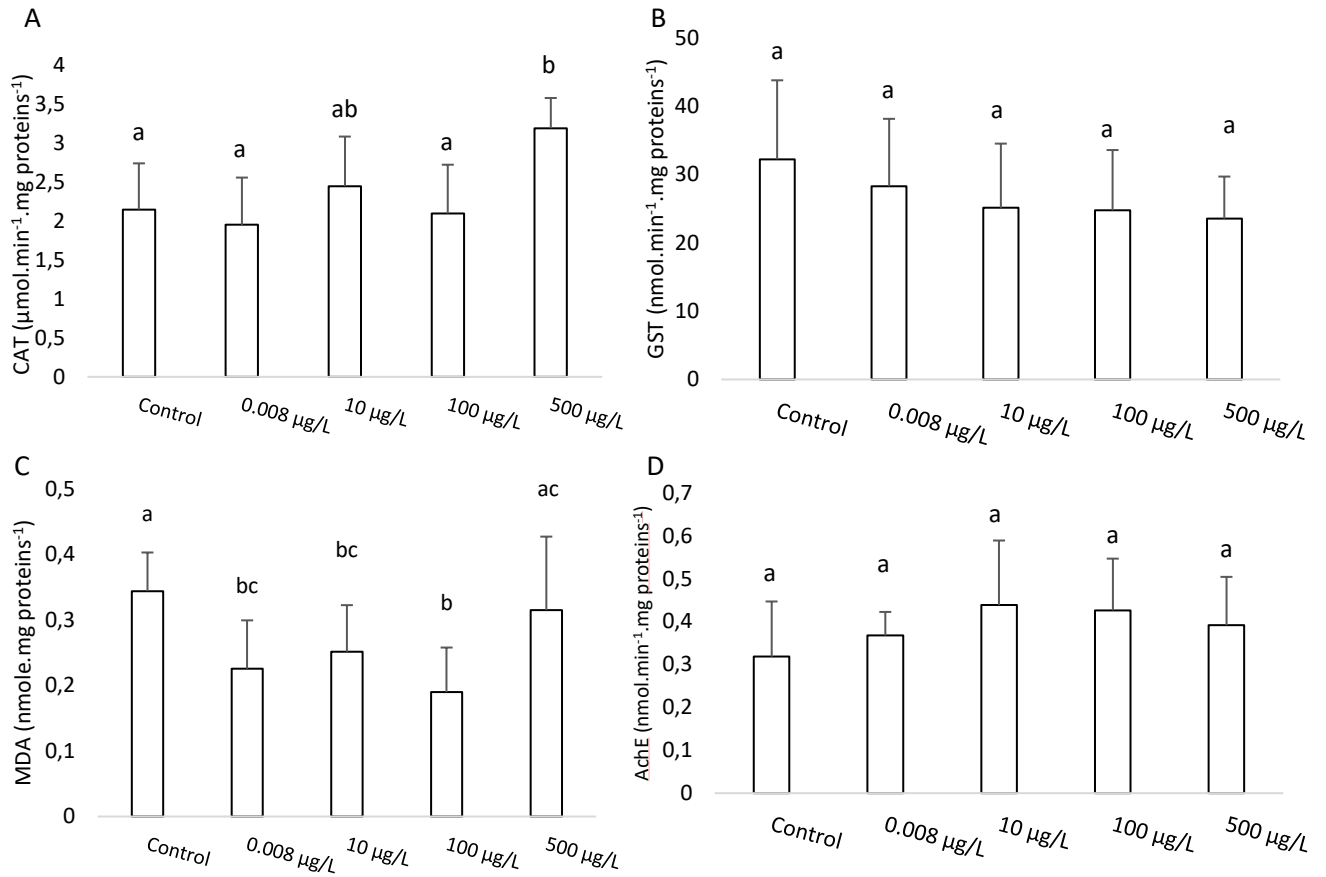
336 For all biomarkers measured after 21 days of exposure, the levels of enzymatic activities were  
 337 higher in the digestive mass than in gills (Figure 2). The biomarker responses to the MP contamination  
 338 differed according to the tissues (Figure 3). CAT activity was significantly increased in digestive mass of  
 339 organisms exposed to 10, 100 and 500  $\mu\text{g.L}^{-1}$  compared to the control organisms, while in gills, CAT  
 340 activity was increased significantly only at the highest concentration (500  $\mu\text{g.L}^{-1}$ ) compared to control.  
 341 In both digestive mass and gills, no significant difference in GST activity was observed for the treated  
 342 organisms compared to the control ones. Concerning MDA levels, significant differences were measured  
 343 only in the gills. Indeed, MDA levels were significantly lower in the clams exposed to 0.008, 10 and 100  
 344  $\mu\text{g.L}^{-1}$ , whereas no significant difference was measured at the highest concentration (500  $\mu\text{g.L}^{-1}$ )  
 345 compared to the control. On the contrary, significant differences in AChE activity were depicted only in  
 346 the digestive mass. Clams exposed to 500  $\mu\text{g MP.L}^{-1}$  showed a significant decrease in AChE activity  
 347 relative to the controls.

348 The first PCA (Figure S5) was based on all biochemical data: CAT, GST and AChE activities, and  
 349 MDA content measured on both organs (gills and digestive mass) of *C. fluminea* exposed to the 5

350 treatments (Control, 0.008, 10, 100 and 500  $\mu\text{g.L}^{-1}$ ). This PCA showed that the studied variables related  
351 to biochemical biomarkers were organ-dependant. Therefore, we chose to represent two distinct  
352 components for a better visual interpretation knowing that PCA representations on a single axis are  
353 rarely presented. Two PCA were then performed separately, one using data obtained on gills and the  
354 other on digestive mass. These two PCA showed the relationships between biochemical biomarkers and  
355 exposure of clams to EMPs (Figure 3). Regarding the PCA performed on digestive mass (Figure 3A), the  
356 two principal components represented 73.76% of total variance, the first axe PC1 and the second axe  
357 PC2 representing 46.30% and 27.46% respectively. Exposure conditions (control, 0.008, 10, 100 and 500  
358  $\mu\text{g.L}^{-1}$ ) were separated and distributed proportionally according to both axes. PC1 is mostly explained  
359 by CAT and GST variables (34.79 and 40.31 % respectively). The exposure condition at the highest  
360 concentration (500  $\mu\text{g.L}^{-1}$ ) was also separated from the others. Relative to PC1, a positive correlation  
361 was established between CAT and GST; likewise, a positive correlation was depicted between MDA and  
362 AChE related to PC2. In the gills (Figure 3B), the two principal components represent 59.73% of total  
363 variance, with PC1 representing 32.26% and PC2 27.46%. PC1 was mostly explained by CAT and AChE  
364 activities (35.35 and 38.25 % respectively), while PC2 was mostly explained by GST activity and MDA  
365 (50.45 and 37.48 % respectively). No evident separation between the conditions was established except  
366 for the highest concentration (500  $\mu\text{g.L}^{-1}$ ) separated from the control. PCA showed that exposure to the  
367 highest concentration of EMPs (500  $\mu\text{g.L}^{-1}$ ) was positively correlated to an increase in the activity of  
368 oxidative stress enzymes CAT and GST in the digestive mass, and only to CAT in gills. In digestive mass,  
369 exposure to high concentration of EMPs was negatively correlated to the activity of AChE (inhibition).

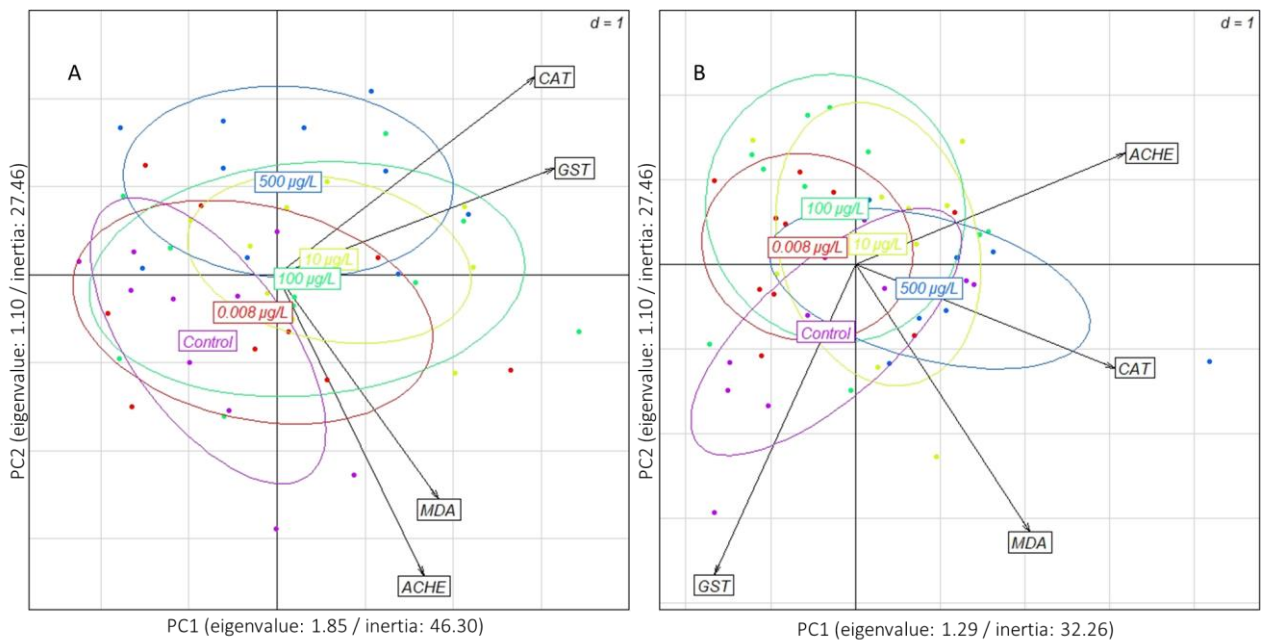


370



371

372 **Figure 2.** Activities of CAT (A), GST (B), AChE (C) and MDA (D) levels in digestive mass (black) and gills (white) of *C.*  
 373 *fluminea* after 21 days of EMPs exposure at 0 (Control), 0.008, 10, 100 and 500 µg MP.L<sup>-1</sup> (mean + sd, N = 10).  
 374 Different lower-case letters indicate significant differences between control and MPs exposed treatments (p <  
 375 0.05).



376

377

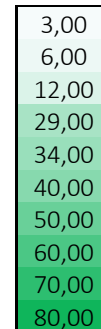
Variables	A: Digestive mass				B: Gills			
	CAT	GST	MDA	AChE	CAT	GST	MDA	AChE
Contribution to PC1 (%)	34.79	40.31	13.63	11.27	35.35	10.40	16.00	38.25
Contribution to PC2 (%)	20.41	5.90	26.38	47.32	5.63	50.45	37.48	6.43

378 **Figure 3.** Principal component analysis (PCA) based on biochemical variables analyzed in *C. fluminea* exposed to  
379 EMPs at 0 (Control), 0.008, 10, 100 and 500  $\mu\text{g MP.L}^{-1}$  in different organs, (A) digestive mass and (B) gills. The table  
380 showed the contributions of the variables to the axis (in %).

### 381 Gene expression

382 As shown in Table 1, at 21 days post-exposure, analyzed genes of *C. fluminea* were only up-  
383 regulated in both gills and digestive mass. These genes are involved in detoxication (*gst* and *mt*),  
384 oxidative stress (*cat*), respiratory chain (*cox1*), immunity (*acp*) and neurotoxicity (*ache*). In gills, the  
385 induction of *gst*, *mt*, *cat* and *acp* was detected only for the lowest EMPs concentration (0.008  $\mu\text{g.L}^{-1}$ ),  
386 except *acp* gene induced also at 10  $\mu\text{g.L}^{-1}$ . In the digestive mass, *cat*, *acp* and *ache* genes were induced  
387 only for the second lowest EMPs concentration (10  $\mu\text{g.L}^{-1}$ ), except *cat* gene induced from 0.008  $\mu\text{g.L}^{-1}$ .  
388 Therefore, significant gene inductions appeared only for the two lowest concentrations (0.008 and 10  
389  $\mu\text{g.L}^{-1}$ ), while there was no significant up or downregulation of genes for *C. fluminea* exposed at 100 and  
390 500  $\mu\text{g.L}^{-1}$ . Overall, there was no evidence of dose-dependent effect for EMPs treatments.

Gills					
		0.008 $\mu\text{g.L}^{-1}$	10 $\mu\text{g.L}^{-1}$	100 $\mu\text{g.L}^{-1}$	500 $\mu\text{g.L}^{-1}$
Detoxication	<i>gst</i>	6.08			
	<i>mt</i>	11.8			
Oxidative stress	<i>cat</i>	5.7			
Respiratory chain	<i>cox1</i>				
Immunity	<i>acp</i>	62.86	38.35		
Neurotoxicity	<i>ache</i>				
Digestive mass					
		0.008 $\mu\text{g.L}^{-1}$	10 $\mu\text{g.L}^{-1}$	100 $\mu\text{g.L}^{-1}$	500 $\mu\text{g.L}^{-1}$
Detoxication	<i>gst</i>				
	<i>mt</i>				
Oxidative stress	<i>cat</i>	16.22	76.2		
Respiratory chain	<i>cox1</i>				
Immunity	<i>acp</i>		32.99		
Neurotoxicity	<i>ache</i>		7.21		



Fold change  
scale bar

391 **Table 1.** Differential gene expression observed in the gills and the visceral mass of *Corbicula fluminea* after 21 days of exposure  
392 to the different treatments. Results are presented as fold-change factors between gene expression in controls and gene  
393 expression in exposed organisms (> 1: induction, in green). Only significant differences are represented (p values  $\leq 0.05$ ) and  
394 only factors <0.5 and >1.5 are considered as significant.

### 396 Discussion

397 The Loire River, the longest French river, represents major societal and economic issues for the  
398 country. Indeed, this stream crosses a multitude of cities of varying sizes, some of them using the Loire  
399 as a resource for drinking water. In the region, scientist federations have even been created to inform  
400 local inhabitants and industrialists of the potential risks of the Loire pollution, and the potential sanitary



401 and environmental impacts. Indeed, since the anthropic activities of cities, such as Angers, may affect  
402 the Loire River, it would be helpful to characterise the plastic contamination in order to implement  
403 adequate policies. In this context, the study of the effects of MPs derived from plastic litter coming  
404 directly from the Loire banks, on the ubiquitous freshwater organism (*C. fluminea*), appeared crucial to  
405 collect data that will help to implement public policies for environmental risk management and  
406 contamination monitoring in the Loire. This is the first study reporting the toxicity of EMPs from the  
407 Loire on *C. fluminea* using a multi-biomarker approach at different levels of biological organization. This  
408 approach allowed 1) to characterize the effects of these plastics by quantifying the biological responses,  
409 aiming to link molecular to individual effects and 2) to propose tools for monitoring plastic  
410 contamination of the Loire by measuring early biological responses in order to predict potential  
411 consequences at higher levels.

### 412 **Environmental realism of the experiment**

413 One of the current questions that arise from MPs exposures conducted in laboratory is whether the  
414 conditions of experiments are realistic from an environmental point of view. In nature, MPs is an  
415 umbrella term that covers particles with various physical and chemical properties, and organisms are  
416 not exposed to uniform sizes and shapes of MPs (Rochman et al. 2017). Laboratory studies often  
417 conduct bivalve exposure at higher concentrations of MPs than the ones found in the environment.  
418 Also, they generally use MPs with a consistent shape and with a calibrated size, particles composed of  
419 only one polymer type (no plastic mixture) (Li et al. 2019; Latchere et al. 2021), and without additives,  
420 which may be responsible for the observed effects on aquatic organisms (Ateia et al. 2020;  
421 Hermabessiere et al. 2017). More precisely, one type of spherical plastic microbeads, calibrated at 1-  
422 100 µm in size, was often used for MPs exposure to clams (Li et al. 2019; Ribeiro et al. 2017; Oliveira et  
423 al. 2018; Moreschi et al. 2020; Fu et al. 2022). However, it has been demonstrated that uptake of MPs  
424 by *C. fluminea* varies based upon physicochemical properties, such as polymer composition and particle  
425 size (Li et al. 2019). Accounting for this, in this study, *C. fluminea* were exposed to a mixture of different  
426 types of EMPs (PP, PE, PVC and PET) generated from separated plastic waste collected directly from the  
427 field. Since these plastics have been more or less aged, they still contain additives integrated during  
428 their manufacture or adsorbed during their release in the environment. Lower exposure concentrations  
429 of EMPs than those used in most bivalve laboratory experiments have been selected for this study. Each  
430 grinded powder presented similar size range of MPs, but not perfectly uniform due to differences in  
431 how the polymers micronized. These size ranges fall within the range of particles ingested by *C. fluminea*  
432 sampled in the field (Wu et al. 2022; Su et al. 2016). Also, laboratory experiments have been conducted  
433 to confirm preferential MPs sizes ingested by Asian clams. For example, Li et al. (2019) reported that  
434 uptake of PET fibers in exposed clams was greater in the size range 100-250 µm than in other size classes  
435 (5-100 µm, 100-250 µm, 250-500 µm, 500-1000 µm and 1000-5000 µm). In our study, size particle

436 ranges of MPs determined by  $\mu$ -FTIR in organisms were similar for all plastic types and are also similar  
437 to the ones measured in the stock solution. The  $\mu$ -FTIR analysis of the clams after the 21-days exposure  
438 revealed the presence of PE and PP but neither PVC nor PET particles were found. This is probably due  
439 to the small amount detected in the stock solution or these particles may aggregate in the solution and  
440 being less bioavailable for organisms (Revel et al. 2019).

#### 441 **Effects of EMPs at individual level**

442 After confirming the ingestion of MPs by exposed organisms, biological changes at individual level  
443 were analyzed. Indeed, statistically significant alteration in the CI was observed between the clams at  
444 the beginning of the exposure (T0) and the ones after 21-days of exposure, exposed to the two highest  
445 MP concentrations. The organisms exposed to these two concentrations did not grow as much as the  
446 control clams and the ones exposed to lower MP concentrations. Naidu et al. (2022) calculated the CI  
447 of yellow clams sampled at three different locations of the northwest coast of India. The lesser CI could  
448 be due to the occurrence of a high number of < 100  $\mu$ m size MPs, as smaller size MPs can result in  
449 oxidative damage, altered feeding, depressed reproductive output in bivalves (Naidu et al. 2022). This  
450 reduced general condition may therefore be explained by disorders at lower biological levels such an  
451 allocation of energy reserves to face a stress rather than for growth, and might cause negative impacts  
452 on a long-term period. Therefore, the effects of EMPs on the whole organisms or on particular tissues  
453 (gills and digestive mass) of the bivalves were evaluated by assessing their ecotoxicological impacts using  
454 a battery of biomarkers and by comparing the expression of some genes involved in the stress  
455 responses.

#### 456 **Effects of EMPs at molecular and biochemical levels: relationship between gene expression 457 and enzymatic activities**

458 The comparison of gene expression levels between exposed and control organisms is useful for  
459 identifying the mechanisms involved in the toxicant-specific responses. These molecular markers can  
460 also provide early-detection of a stress. The biochemical markers were chosen to evaluate the possible  
461 effects related to damage caused by MPs, including the oxidative stress, lipid oxidative damage and  
462 neurotoxicity (AChE activity). Relationship between both, molecular and biochemical markers, is useful  
463 to know if gene expression induces the activity of the associated protein. The results first highlighted  
464 that an exposure to EMPs led to changes in gene expression patterns and biochemical activities at  
465 environmentally relevant concentrations, in the two tissues, with a differential response of biomarkers  
466 between gills and digestive mass (Figure 1 and Figure 2). For all biomarkers, the specific activity levels  
467 were higher in digestive mass compared to gills (Figure 1), and ecotoxicological effects were sometimes  
468 significant in gills and not in digestive mass, or inversely, reflecting the distinct physiological and  
469 metabolic pathways present in the two tissues.

470 Firstly, *cat* and *gst* gene expressions were quantified, both involved in the detoxication pathways:  
471 *cat* gene encodes catalase, an enzyme that acts as a defence mechanism against reactive oxygen  
472 species, allowing the disproportionation of hydrogen peroxide into water and dioxygen; *gst* gene  
473 encodes GST enzymes protecting cells against toxicants by conjugating the glutathione as substrate to  
474 xenobiotics. The induction of both *cat* and *gst* genes observed in the gills and in the digestive mass of *C.*  
475 *fluminea* is a sign of response to an oxidative stress. However, the GST activities was not significantly  
476 modified in both organs of clams in this study and the CAT activity was significantly higher only at the  
477 highest concentrations. The non-significant change of GST activity might be due to a compensatory and  
478 earlier response of the antioxidant enzyme, such as CAT or SOD (not analyzed in this study). However,  
479 some studies have reported a response of the GST in bivalves exposed to MPs. For example, Parra et al.  
480 (2021) found a significant increase of GST activity in gill, digestive gland and gonad of Asian clams  
481 exposed to the combination MPs with Cd, but not with MPs alone (PS commercial particles). The  
482 individuals were exposed to higher concentration (2 mg MP.L<sup>-1</sup>) than those used for our study. Métais  
483 et al. (2023) reported a significant decrease of GST activity, after 21-days exposure, in gills and digestive  
484 glands of *S. plana* exposed to low concentration of MPs (0.008 µg.L<sup>-1</sup>) compared to the control but not  
485 for the highest concentrations (10 and 100 µg.L<sup>-1</sup>). Therefore, GST activity is probably not induced at  
486 low environmental concentrations, and expression of *gst* appeared only at low MP exposure  
487 concentrations, while the *cat* gene takes over for high MP concentrations.

488 The PCA realised on the data obtained from the digestive mass analysis, showing that exposure to  
489 EMPs was positively correlated to an increase in the activity of CAT and GST, and negatively correlated  
490 to the activity of AChE, suggested the clam response to an oxidative stress, the establishment of a  
491 detoxication process and a neurotoxic effect. This response was not observed in gills except for the CAT  
492 activity. This has been previously reported by Revel et al. (2019), who showed an increase in CAT activity  
493 in gills of mussel (*Mytilus spp.*) at the highest tested concentration of MPs (100 µg.L<sup>-1</sup>), while there was  
494 a significant increase in digestive gland at the lowest and medium concentrations of MPs (0.008 and 10  
495 µg.L<sup>-1</sup>). A similar result was reported by Métais et al. (2023): after 21 days of exposure, significant higher  
496 levels of CAT activity were observed in the gills of *S. plana* exposed to 100 µg.L<sup>-1</sup> of MPs compared to  
497 the control ones, but there was no significant change for CAT activity in the digestive glands. This  
498 difference may be explained by the differential exposure level between tissues due to particle  
499 aggregation in stock suspensions (Revel et al. 2019). Another reason of the difference of the antioxidant  
500 enzyme response in gills and digestive glands would be the natural differences in physiology of the  
501 different organs. Gill is the first organ exposed to environmental contaminants, while the digestive gland  
502 is the major organ for the accumulation of metals and other contaminants (Wirnkor et al. 2019). In our  
503 study, CAT activity significantly increased already at lower concentration in digestive mass (at 100 µg.L<sup>-1</sup>)  
504 compared to the gills (at 500 µg.L<sup>-1</sup>). Parra et al. (2021) also reported a higher induction of CAT activity

505 in the digestive gland of *C. fluminea* compared to gills, after an exposure to the mixture MPs and Cd,  
506 while there is no difference between these two organs when organisms were exposed only to MPs (PS  
507 commercial particles). In the green-lipped mussel *Perna canaliculus*, Chandurvelan et al. (2013)  
508 observed an increase of CAT activity in digestive gland but not in gills from organisms exposed to  
509 cadmium ( $200 \mu\text{g.L}^{-1}$ ) for 21 and 28 days. Borković-Mitić et al. (2013) also observed higher CAT activity  
510 in the digestive gland compared to the gills, after exposure of the freshwater bivalve *Unio tumidus* to  
511 metals. Metals and some other contaminants are considered as additives integrated or adsorbed to the  
512 plastics found in the environment. More and more studies focus on the toxicity of additives on bivalves,  
513 and some of them highlighted an oxidative stress (Wu et al. 2018; Capolupo et al. 2021). Since the  
514 plastics used in this study were directly collected from the field to evaluate the toxicity of  
515 environmentally relevant plastic particles, the oxidative stress could therefore be induced by additives  
516 like metals. In this context, it would be interesting to characterize additives associated to the EMPs used  
517 in this exposure experiment, while the number of scientific studies on the effects of additives on aquatic  
518 organisms is still too scarce.

519 The induction of the *MT* gene expression in gills at the lowest concentration suggests a detoxication  
520 mechanism involvement. This can be induced by the presence of metal or other organic compounds  
521 probably integrated or adsorbed as additives on plastics. In both tissues, the overexpression of *acp* gene  
522 indicated an immune system response at low EMP concentration exposures. A similar result in the  
523 immune response has been reported for bivalves exposed to MPs (Mkuye et al. 2022). Métais et al.  
524 (2023) reported a significant higher level of AcP activity from the seventh day of exposure in *S. plana*  
525 after exposure to the lowest concentration of MPs ( $0.008 \mu\text{g.L}^{-1}$ ) compared to  $10 \mu\text{g.L}^{-1}$ , corroborating  
526 the overexpression of *acp* found in this study.

527 Moreover, AChE, that splits acetylcholine into choline and acetate, is essential in transmitting nerve  
528 impulses among synapses (Modesto and Martinez, 2010). In gills, neither a change of AChE activity nor  
529 of the *ache* gene expression was depicted. However, in digestive mass, a significant inhibition of the  
530 AChE activity of clams exposed at  $500 \mu\text{g.L}^{-1}$  and a significant increase of the *ache* gene expression at  
531  $10 \mu\text{g.L}^{-1}$  have been reported. This may be related to the inflammation of the visceral mass since it has  
532 been reported that inflammatory conditions can trigger the up-regulation of *ache* gene expression  
533 (Oliveira et al., 2012). Consistent with some previous studies (Guilhermino et al. 2018; Parra et al. 2021;  
534 Fu et al. 2022), AChE activity inhibition induced by MPs was observed in the current study. As explained  
535 by Fu et al. (2022), a possible reason contributing to the inhibition of AChE is that the enzyme structure  
536 was changed through adsorption (Tan et al. 2020). Thus, the MPs could act as AChE inhibitors, that may  
537 affect bivalve behaviour, including the feeding ability and the ability to recognize and avoid predators  
538 (Pan and Dutta, 1998). Fu et al. (2022) highlighted that disturbance to AChE activity may lead to  
539 weakened ability to uptake food from water. These changes were confirmed by a decrease in the

540 filtration rate in the exposed *Corbicula fluminea*. However, in our study, the filtration efficiency was not  
541 disturbed when comparing control and exposed clams. This is in accordance with the results of Revel et  
542 al. (2019). The authors did not report a significant difference in the clearance rate between control and  
543 exposed mussels. Moreover, the expression of the *cox* gene did not change, neither in gills nor in  
544 digestive gland in the exposed clams. This suggests that the respiratory chain was not impacted by an  
545 exposure to EMPs at the chosen concentrations.

546 In addition, MDA levels are an important biomarker of oxidative damage in aquatic organisms, which  
547 contain high amounts of lipids with polyunsaturated fatty acid, a substrate for oxidation (Lushchak,  
548 2011). In our study, no significant increase of MDA level was reported in the digestive mass, which is  
549 consistent with the studies of Parra et al. (2021) and Guilhermino et al. (2018). Indeed, Parra et al. (2021)  
550 did not report significant lipid peroxidation in the digestive gland of Asian clams exposed to 2 mg MP.L<sup>-1</sup>  
551 and Guilhermino et al. (2018) did not report significant lipid peroxidation in the digestive gland of Asian  
552 clams exposed at both 0.2 and 0.7 mg MP.L<sup>-1</sup>. These results indicate that lipid peroxidation does not  
553 occur in this organ, even at high concentrations of MPs, because it might be compensated by the  
554 antioxidant enzymatic activities. However, in our study, a significant decrease of MDA content was  
555 reported in gills of exposed clams to 0.008, 10, 100 µg MP.L<sup>-1</sup> compared to the control group but not  
556 for the highest concentration (500 µg MP.L<sup>-1</sup>). Ribeiro et al. (2017) also reported a decrease of oxidative  
557 damage to lipids in the gills in PS MPs exposed clams (*Scrobicularia plana*) compared to the control  
558 group. The authors explained that this could be linked to antioxidant defences, which consequently limit  
559 the attack of reactive oxygen species to membrane lipids (Ribeiro et al. 2017). Parra et al. (2021) showed  
560 that exposure to Cd alone, MPs alone and the mixture of both induced significant increase lipid  
561 peroxidation levels in gills. Oliveira et al. (2018) also observed a significant oxidative lipid damage in *C.*  
562 *fluminea* exposed to a mixture of MPs (0.13 mg.L<sup>-1</sup> of fluorescent red polymer microspheres of unknown  
563 composition between 1 and 5 µm diameter) with mercury, but not in clams exposed to MPs only. This  
564 suggests that mercury is responsible for this damage.

565 Finally, results of the current study showed the interest of using different complementary  
566 ecotoxicological tools. Indeed, biomarker activities and energy reserves level changes were significant  
567 for the highest concentrations of the EMPs exposure, while changes of gene expressions occurred at  
568 the lowest EMP concentrations. This means that the transcriptomic tools targeting expression of genes  
569 involved in the main pathways of stress responses would help to predict microplastic effects in bivalves  
570 at high biological levels (biochemical and individual) but also at high concentrations of MP exposure.

#### 571 **Effects of EMPs at biochemical level: energy reserves**

572 Exposure to EMPs caused a decrease of the energy reserves in *C. fluminea*. To our knowledge,  
573 this is the first study reporting data on energy reserves on *C. fluminea* exposed to MPs. Although no  
574 difference was observed in protein levels, a decrease in lipid and glycogen contents was depicted in

575 clams exposed to the two higher concentrations of MPs (100 and 500  $\mu\text{g}\cdot\text{L}^{-1}$ ), after 21 days of exposure.  
576 A protein breakdown is considered as a last resort, only occurring during prolonged starvation or periods  
577 of extreme energy demands (Haider et al. 2018). A decrease in the protein content in MPs-exposed  
578 bivalves indicate therefore severe energy deficiency, which is not the case in the current study. A change  
579 in energy reserves in bivalves exposed to MPs was supported by other studies. For example, Bour et al.  
580 (2018) concluded that larger PE particles (125–500  $\mu\text{m}$ ) had more severe effects on energy reserves of  
581 *Ennucula tenuis* and *Abra nitida* than smaller ones (4–6 $\mu\text{m}$  and 20–25 $\mu\text{m}$ ), possibly due to a longer  
582 residence time in the gut that can cause physical damage. Also, Shang et al. (2021) reported a decrease  
583 of energy reserves of mussels *Mytilus coruscus* exposed to 2  $\mu\text{m}$  polystyrene microspheres. MPs have  
584 been demonstrated to significantly suppress the energy assimilation efficiency of mussels (Gardon et al.  
585 2018; Pedersen et al. 2020). In our study, both glycogen and lipid reserves undergo depletion at 21 days  
586 of exposure at 100 and 500  $\mu\text{g}\cdot\text{L}^{-1}$  indicating an energy cost for *C. fluminea* at high MP concentrations.  
587 However, it is possible that these different energy reserves have already decreased before 21 days of  
588 exposure. In their study, Shang et al. (2021) demonstrated an early depletion of glycogen (day-7) but  
589 also a rapid resynthesis after the end of MP exposure of the mussels reaching the baseline levels after  
590 7 days of recovery. In contrast, depletion of lipids and proteins occurs later during the MP exposure and  
591 takes longer to recover after the exposure end. Lipid metabolism is the primary pathway through which  
592 energy is supplied in organisms, playing an important role in the response to environmental stress in  
593 aquatic invertebrates (Lee et al. 2018). However, to deep knowledge of the biological mechanisms,  
594 energy reserves might be analyzed in separate tissues.

## 595 Conclusion

596 This study aimed to evaluate the toxicity of environmental MPs on the freshwater bivalve  
597 *Corbicula fluminea* under environmental realistic conditions. Therefore, for this laboratory experiment  
598 exposure microplastics were made up from plastic litter collected *in situ* and the exposure  
599 concentrations were considered as environmental. This paper is the first that reports ecotoxicological  
600 effects on aquatic organisms exposed to EMPs from the Loire, the longest French river. We  
601 demonstrated that the effects were dependant on the tested concentrations: biochemical responses  
602 have been generally reported at the two highest exposure concentrations while molecular responses  
603 were reported at the two lowest concentrations in both tissues. Also, we showed that effects were  
604 dependant on the tissues. Finally, from these results, it appears that a transcriptomic approach targeting  
605 genes involved in stress response helps evaluating the slight differences in toxicity of the different tested  
606 plastic conditions on the Asian clam. Also, it suggests that this approach could be relevant in order to  
607 predict effects at higher levels (biochemical and individual) of the biological organization in the concept

608 of Adverse Outcome Pathway (AOP). This is particularly important with regard to plastic pollution whose  
609 effects on humans are difficult to investigate.

## 610 **Declarations**

### 611 **Ethical Approval**

612 Not applicable. This article does not contain any studies with human participants. The animal used in  
613 this article is an aquatic invertebrate.

### 614 **Competing interests**

615 The authors declare no competing interests.

### 616 **Authors' contributions**

617 Alice Vidal, Oihana Latchere, Isabelle Métais and Amélie Châtel conceived and designed the  
618 experimental set up. Alice Vidal performed the field campaign and the laboratory analyses, assisted by  
619 Isabelle Métais and Amélie Châtel. Alice Vidal and Johnny Gasperi realised the spectroscopy analyses.  
620 Antoine Le Guernic did all the statistics on the results. Alice Vidal collected all the data, interpreted it  
621 and wrote the first draft of the manuscript. All authors commented on previous versions of the  
622 manuscript. All authors read and approved the final manuscript.

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### 626 **Availability of data and materials**

627 The authors confirm that the data supporting the findings of this study are available within the article  
628 and its supplementary materials.

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