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1 Assessment of microplastic contamination in the Loire River (France) throughout 2 analysis of different biotic and abiotic freshwater matrices

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8 Abstract

9 The contamination of microplastics (MP) in freshwater environments represent an important way
10 for the MP transport in the environment. The assessment of MP pollution in freshwater compartments is
11 then important to visualize the pressure and the impacts on medium, and to set up necessary measures.
12 In this context, this study focused on the influence of anthropogenic activities of a medium French city
13 (Angers) on MP levels in samples collected from the Loire River, the longest river in France. Abiotic and
14 biotic matrices were collected upstream and downstream Angers. A first analysis was performed based
15 on microscopy to determine the size, colour and shape of suspected MP and a complementary analysis
16 by μ -FTIR (micro-Fourier Transform InfraRed) was conducted to determine the composition of plastic
17 particles. Three organisms belonging to different trophic levels were studied: when the MP level was
18 expressed per individual, the lowest abundance of MP was found in *Tubifex sp.* followed by *Corbicula*
19 *fluminea*, while the highest was measured in *Anguilla anguilla*. To establish the relationship with their
20 habitat, the presence of MP in sediment and water was also analysed. Therefore, this works constitutes
21 a complete overview of the MP levels in freshwater abiotic and biotic matrices. Overall, the presence of
22 MP in analysed samples did not follow a particular pattern, neither in the sites nor matrices: the
23 characteristics depending on a multifactorial outcome (feeding mode, organism size...). However,
24 correlation of MP pattern between clams and sediment was quite evident, while the one between worms
25 and their habitat was not. This demonstrates the relevance of investigating plastic contamination both in
26 biotic and abiotic matrices. Finally, a standardisation of sampling and analytical analysis protocols would
27 be helpful to make comparisons between studies more robust.

28 Keywords

29 Microplastics; Freshwater; Environmental abundance; Bioindication; μ -FTIR; Microscopy

30 Introduction

31 Plastic pollution in aquatic ecosystems is now a well-known issue. For the last decade, plastic
32 pollution has become a major environmental concern due to its increasing production, which has reached
33 367 million tons worldwide (PlasticsEurope, 2021). Among these plastics, many are single-used and
34 immediately discarded, most frequently improperly. Some reach the terrestrial environment and

35 ultimately end up in aquatic environments (Oliveira et al. 2019; Ferreira et al. 2019). Globally, it is
36 estimated that between 70 and 80% of plastics in the oceans come from land-based sources (Duis and
37 Coors, 2016; Alimi et al. 2018). Once they reach the aquatic compartment, plastics can be fragmented
38 into smaller sizes by photodegradation (UV radiation), weathering and physical abrasion (e.g., wind and
39 wave action), and biotic factors (e.g., microorganisms' action). Nowadays, microplastics (MP), plastic
40 particles < 5 mm, represent a current and a global environmental concern. Indeed, numerous scientific
41 studies have reported that MP are increasingly observed in all compartments of most aquatic ecosystems
42 around the world, even in Arctic and Antarctic Oceans (Lusher et al. 2015; Waller et al. 2017; Huntington
43 et al. 2020). MP can be found as suspended particles in the water column, in sediments and in wastewater
44 (Habib et al. 2020; Bayo et al. 2020; Frias et al. 2020; Dusaucy et al. 2021; Uddin et al. 2021). In addition
45 to being found in abiotic compartments, MP have been demonstrated to be ingested by a wide variety of
46 aquatic biota, in both marine and freshwater organisms, from microalgae to large mammals (Farrell et al.
47 2013; Santana et al. 2017; Qu et al. 2020), and consequences are multiple: MP retention in the gut causing
48 blockages, reducing nutrient absorption (Cole et al. 2015; Lu et al. 2016; Welden and Cowie, 2016) or MP
49 translocation to other tissues (Browne et al. 2008; Brennecke et al. 2015).

50 Today, studies having reported data on MP levels in sediments, water and organisms are more
51 numerous in the marine compartment than in freshwater systems, since the investigation on the MP
52 contamination in freshwater ecosystems is more recent. Data on their level and composition, and
53 information on MP interaction with the freshwater biota are then still scarce. However, freshwater
54 environments represent an important pathway for the transport of MP (Li et al. 2018; Wagner et al. 2014).
55 Since they are more susceptible to anthropogenic impacts, they usually contain higher MP amount with
56 a more diverse plastic composition (Scherer et al. 2018). Therefore, the assessment of MP pollution in
57 freshwater compartments is of crucial importance to set up necessary measures. Indeed, some rivers may
58 cross a multitude of cities, some of which using water as a source of drinking water. It is the case for the
59 Loire River, the longest French river, representing an important societal and economic interest for the
60 country. As anthropic activities in cities generate pollution ending up nearby the Loire River, it would be
61 helpful to characterise its contamination to implement adequate policy regulations, and preserve the
62 large number of plant and animal species living on the banks or in the bed of the Loire (Wantzen et al.
63 2023). For the moment, to our knowledge, no study reporting the level of MP in living organisms of the
64 Loire River was published and studies reporting MP levels in sediments and water of the Loire are still
65 limited.

66 The current study investigated for the first time the level of the MP contamination in three aquatic
67 organisms, around Angers, one of the major cities along the Loire. Tubifex (*Tubifex sp.* (Müller, 1774)),
68 Corbicula (*Corbicula fluminea* (Müller, 1774)) and eel (*Anguilla anguilla* (Linnaeus, 1758)) were used as
69 model organisms due to their dominant stock in the Loire River and to their position at different trophic

70 levels. Tubifex worms are one of the most abundant invertebrates in freshwater compartments and
71 represent primary consumers in the food chain of their ecosystem. *Tubifex tubifex* has been widely used
72 to assess the toxicity and the bioaccumulation of some pollutants, especially metals (Lagauzère et al.
73 2009; Mosleh et al. 2006) making it a suitable species for sediment toxicity tests (Chapman et al. 2001).
74 To date, however, only Hurley et al. (2017) reported data on MP accumulation in *Tubifex sp.*. This is even,
75 to our knowledge, the only *in situ* study that has been published on MP in freshwater worms. Among
76 invertebrates, bivalves are considered, for years, as useful organisms for indicating levels of different
77 pollutants in the environment (Boening, 1999). Literature on MP contamination of freshwater bivalves is
78 more extensive than that on worms, but remains still sparse compared to the publication on marine
79 bivalves. Indeed, Sendra et al. (2021) reported 56 studies dealing with marine bivalves *versus* 13 studies
80 dealing with freshwater bivalves, of which 6 used *Corbicula fluminea* as their model species, the rest (*Unio*
81 *pictorum*, *Perna canaliculus*, *Dreissena burgensis*, *Elliptio complanate* and *Dreissena polymorpha*) counted
82 for either 1 or 2 works. In addition, this species, as an active filter-feeder, is considered as an useful
83 bioindicator to assess the anthropic pollution in aquatic compartments (Su et al. 2018). For now, field
84 studies on the MP ingestion by freshwater invertebrates are limited, even though these organisms are a
85 key entry point into the food chain. So far, studies on freshwater MP contamination have focused on fish
86 (Peters et al. 2016; Jabeen et al. 2017; Biginagwa et al. 2016). The main reason is that fish is commonly
87 and frequently consumed by humans. Even if visceral mass and gills of fish are removed before
88 consumption, small size MP may enter edible tissues (Atamanalp et al. 2021; Guilhermino et al. 2021),
89 which may pose a health risk to consumers. In addition to being consumed by humans, the European eel,
90 *A. anguilla*, is a critically endangered fish according to the International Union for Nature Conservation
91 (IUNC, 2022). Population decline has been demonstrated (Dekker et al. 2007; Andersson et al. 2012; Aalto
92 et al. 2016) and linked to environmental pollution (Jacoby et al. 2015; Drouineau et al. 2018). Regarding
93 the importance of pollution in the decline of the European eel, MP are surely an additional stressor for
94 this species (Menéndez et al. 2022).

95 The first aim of this study was to investigate the MP contamination in abiotic and biotic matrices. In
96 this context, the shape of MP, the colour, the size and the composition were characterised in sediments,
97 water, viscera of *A. anguilla*, whole body of *C. fluminea* and *Tubifex sp.*. In the literature, two methods are
98 generally employed: microscopy and micro-Fourier Transform InfraRed (μ -FTIR) analysis. Imaging μ -FTIR
99 does not distinguish the colours of particles neither the shapes, like fibers, while an observation by
100 microscopy does not determine the polymer composition. In order to perform a global analysis of the
101 plastic particles profile in samples, both complementary methods were used. Thus, we were able to
102 provide points of comparison between these two approaches. The second objective was to compare MP
103 levels in all the samples (sediment, water and organisms) collected upstream and downstream Angers, to
104 evaluate the influence of human activities on the plastic pollution. The final goal was to determine the

105 correlations between MP ingested by the organisms and MP found in the abiotic matrices, corresponding
106 to the habitats of the analysed species. Most of studies focused on MP levels either on abiotic matrices
107 or biotic matrices, generally on one or two species. Therefore, this study provides less spatial and
108 temporal resolution on *in situ* MP levels than some studies but gives an overview of the MP contamination
109 in freshwater abiotic and biotic matrices at three species belonging to different trophic levels, which
110 constitutes its originality. All of these results will be used to suggest protocols for monitoring microplastic
111 contamination of freshwater streams and provide data to implement public policies for environmental
112 risk management.

113 **Materials and method**

114 **Study sites**

115 The Loire River is the longest French river, with a length of 1 006 km. This stream rises in the
116 south-eastern quarter of the French Massif Central at an altitude of 1 350 meters until the Bay of Saint
117 Nazaire, Atlantic Ocean. The watershed covers a total area of 155 000 km², equivalent to 22% of French
118 territory (Managing Rivers Wisely, WWF). The Loire basin has more than 11.5 million inhabitants,
119 markedly rural, with more than a third of communities having fewer than 400 inhabitants (Managing
120 Rivers Wisely, WWF). Moreover, a large number of plant and animal species live on the banks or in the
121 bed of the Loire (Wantzen et al. 2023). The French Biodiversity Agency (OFB) and the National Inventory
122 of Natural Heritage (INPN) listed some of these species, such as the *Schoenoplectus triqueter* plant,
123 various amphibians, birds such as the kingfisher, and mammals such as the beaver, as protected and
124 sometimes threatened species. As human activities in Angers agglomeration (155 850 inhabitants in 2019,
125 INSEE) may generate plastic pollution ending up in nearby freshwater streams, two sites were selected to
126 study MP contamination from anthropic sources near Angers city along the Loire River: one upstream the
127 town, at La Daguinière (47°23'11"NO°50'48"W) and one downstream, at Montjean-sur-Loire
128 (47°23'34"NO°51'58"W) (Fig.1).

129 **Sampling of organisms (biotic matrices)**

130 For this study, three species were analysed: *Tubifex sp.*, *Corbicula fluminea* and *Anguilla anguilla*.
131 *Tubifex sp.* and *C. fluminea* were sampled in May 2022, in both sites. Tubifex worms, deposit feeders,
132 occupy the uppermost layers of sediment and live partially submerged and they were abundant in the
133 banks. They typically burrow to 6–10 cm depth but can be found at a depth of 2 cm in highly contaminated
134 sediments (Karickhoff and Morris, 1985; Lagauzère et al. 2009). For this study, worm species were not
135 identified and only the genus will be considered. The Tubifex organisms were carefully extracted from the
136 sediments using stainless steel tweezers and rinsed with distilled water to remove all organic and
137 inorganic matters. The organisms were then placed into 1.5 mL microtubes and stored at -20°C until MP
138 analysis. In the same way, *Corbicula* were collected in both site using a rake, then rinsed with distilled

139 water and stored at -20°C until analysis. For years, the Asian clam (*C. fluminea*) has colonized diverse
140 freshwater ecosystems (Sousa et al. 2008) and is today an exotic invasive species in Europe and other
141 areas of the world. A total of 300 worms and 50 clams were collected. To characterise MP in an upper
142 and essential link of the aquatic trophic chain, viscera of eels (n=6 females) were also analysed to estimate
143 the MP ingestion (biometric data reported in Table S1). Eel guts provided from a professional fisherman
144 who caught the individuals in December 2021, in the Chalonnes-sur-Loire sector, downstream from
145 Angers (Fig.1).

146 **Sampling of sediment and water (abiotic matrices)**

147 As for the biotic matrices, abiotic (water and sediment) matrices were sampled in May 2022, in
148 both sites. Two types of sediment were sampled: habitat of the collected bivalves and habitat of the
149 collected worms. Visually, the granulometry of sediment where clams were sampled was constituted by
150 coarser grains, while the sediment where worms were sampled was smaller-grained (close to mud). To
151 simplify the understanding of results, sediment samples in which *C. fluminea* were collected were called
152 *sediment*, while sediment samples in which *Tubifex sp.* were collected were referred as *mud*. For each
153 sampling, three replicates of sediment were collected at 1 meter from each other, retrieved near to the
154 banks and stored at 4°C in aluminium boxes to avoid plastic containers. In addition, 1 L of water was
155 collected directly in the water column and stored in a glass bottle at 4°C.

156 **Characterization of MP by microscopy**

157 All biotic and abiotic matrices were analysed under microscope to report the colour (transparent
158 or colored MP), the size (from 25 µm (detection limit size) to 5 mm) and the shape (fragments, fibers,
159 films and beads) of suspected MP.

160 **Biotic matrices (organisms).** The sample treatment procedure for worms and clams was adapted
161 from the protocol published by Revel et al. (2019). Briefly, 6 pools of 3 clams sampled downstream or
162 upstream, as well as 6 pools of 10 worms sampled downstream or upstream were analysed. The clam soft
163 tissues and the worm whole body were placed separately in 250 mL beakers precleaned with ethanol
164 absolute (70%) and filled with 5 volumes of the individual pool mass of hydroxide potassium (KOH) 10%
165 for bivalves and 20% for worms. Then, the beakers were covered with aluminium, placed on a heating
166 plate at 60°C with stirring for 24 h to eliminate organic matter. For the eel viscera MP analysis, preliminary
167 tests for sample digestion were conducted based on Biginagwa et al. (2016) and Thiele et al. (2019).
168 Viscera of 6 eels were analysed separately. To facilitate the digestion step, the viscera were cut into pieces
169 and placed into different beakers due to their important mass (Table S1). A volume of 40% KOH equivalent
170 to 10 times the organism mass (m/v) was added into each beaker. Then, the beakers were covered with
171 aluminium, placed on a heating plate at 60°C with stirring for 7 days to eliminate organic matter. Once
172 the digestion step finished, the solution was transferred to a 50 mL Falcon tube containing a 1:1 ratio of

173 KOH solution and saturated NaCl solution to extract the lipid layer. The tube was then centrifuged at 6
174 500 rpm for 15 min, at 4°C. The solid supernatant was removed and immersed in a new NaCl solution.
175 Another centrifugation (6 500 rpm for 15 min at 4°C) was proceeded before recovering the supernatant,
176 added with the first one for the filtration step. The filtration procedure for the biotic matrices was the
177 same as for the abiotic matrices.

178 ***Abiotic matrices (sediment and water).*** The procedure of treatment of sediment samples was
179 adapted from the protocol published by Blair et al. (2019). Briefly, samples were dried in an oven at 60°C
180 for 48 h and were sieved (1.6 mm) to remove the larger debris. Six replicates of 20 g of sediment were
181 placed in a 50 mL Falcon tube with 20 mL of saturated NaCl solution. After mixing thoroughly, samples
182 were centrifuged (6 500 rpm for 10 min). The supernatant was recovered while the bottom sediment was
183 taken up twice in 20 mL of saturated NaCl to optimize the extraction of all MP. NaCl is often used in the
184 sample treatment for MP microscopic observation. This solution was thus chosen to compare our data
185 with those published in the literature. Then, samples undergo a second centrifugation (6 500 rpm for 10
186 min) and the supernatant was added to the one already recovered, for filtration. Five replicates of 200
187 mL of water were directly filtered without prior digestion step. All samples were filtered under Büchner
188 on a 1.6 µm filter (GF/A 1820-037). Filters were then kept in a Petri dish, precleaned with ethanol absolute
189 (70%), until further analysis.

190 All the filters from the biotic and abiotic matrices were then observed under a binocular
191 microscope at X40 magnification. The suspected MP were counted and their colour, shape and size were
192 reported. MP counted on the blank filters, performed for each matrix, were subtracted from the number
193 of MP counted on associated sample filters. MP found on the blank filters (between 0 and 4 particles per
194 filter) were mainly fibers (92%) measuring from 300 to 2000 µm, either transparent, black, blue or pink.
195 The only fragment was grey and measured 100 µm (major dimension).

196 **Characterization of MP by µ-FTIR analysis**

197 To determine the plastic polymer, sediment, mud, worm and clam samples were analysed using
198 a µ-FTIR. MP in water and in eel viscera were not characterized by µ-FTIR. These matrices considered as
199 moving, a correlation between MP in fish and the ones in water is difficult to establish. 3 pools of 3 clams
200 and 3 pools of 30 worms collected downstream, and 3 pools of 3 clams and 3 pools of 30 worms collected
201 upstream, were analysed.

202 ***Biotic matrices (organisms).*** The MP extraction procedure was based on an adaptation of the
203 protocol published by Phuong et al. (2018). Briefly, whole worms and soft tissue of clams were separately
204 placed in 100 mL beakers with a volume of 10% KOH equivalent to 10 times the organism mass (m/v). The
205 mixture was heated at 45°C under stirring for 24 h for *Corbicula* and for 76 h for *Tubifex*. The solutions
206 were then filtered through a metallic filter (10 µm cutoff) using a Büchner filtration. The metallic filter
207 was immersed in a beaker containing NaI solution and sonicated for 1 minute. As for NaCl used for

208 microscopy, NaI was chosen for the μ -FTIR method. Most studies used it to proceed a pre-treatment step
209 followed by a densimetric separation. The NaI solution, after removing and rinsing the filter, was
210 introduced in a JAMSS display to extract MP for at least 5h. The NaI solution was then filtered on a new
211 metallic filter and rinsed with distilled water. The solution was sonicated for 1 minute before being filtered
212 through an Anodisc membrane filters (0.2 μ m, 25 mm, Whatman). Finally, all the filters were stored in
213 closed glass Petri dishes until analysis.

214 ***Abiotic matrices (sediment)***. 3 pools of 10 g of sediments downstream and upstream, and 3 pools
215 of 1 g of mud (more complex matrix than sediment) downstream and upstream were prepared. A three-
216 time successive density separation using JAMSS display was performed and the organic matter digestion
217 was performed with hydrogen-peroxide 30% (Fluka Germany). The solution was then sonicated for 1
218 minute before being filtered through Anodisc membranes.

219 For all matrices, μ -FTIR imaging (Thermo Nicolet iZ10) using 25 x 25 μ m pixel resolution was
220 performed for the entire sample *i.e.*, the entire filtration zone of the sample. Acquisition parameters were
221 described in Treilles et al. (2021). The μ -FTIR maps were treated with open siMPle software (v.1.1.β,
222 Primpke et al. 2018) and the library MP_Library_extended_grouped_1_5.txt. The default matching weight
223 of 0.5 for the first derivative of the spectra and 0.5 for the second derivative of the spectra was used, and
224 the AAU pipeline was chosen for data processing. The minimal particle size provided by siMPle is 25 μ m.
225 The maximal plastic sizes provided in the output of the siMPle software were considered.

226 **Contamination control**

227 To prevent cross-contamination, all equipment used in the laboratory was previously rinsed with
228 milliQ water and ethanol, dried at room temperature in a hood and kept under aluminum foil to avoid
229 contact with the ambient air. During the experiments, cotton laboratory coats and nitrile gloves were
230 worn. For both methods (microscope and μ -FTIR analysis), during the digestion, beakers were covered
231 with aluminum foil. After filtration, the filters were stored in closed glass Petri dishes until microscopy
232 and μ -FTIR analysis. Due to the remaining risk of cross-contamination of the samples, blanks were
233 performed in KOH 10%, following the same protocol. Each time a series of digestion was performed, a
234 parallel analysis without organism tissues nor abiotic compartments were realized at the digestion and
235 filtration steps, and microscope and μ -FTIR analysis. Finally, the use of plastic material was avoided at
236 best throughout this experimental study.

237 **Statistical analysis**

238 Statistical analysis was carried out using R software (version 4.2.2). Normality of the data
239 distribution and homogeneity of variance were tested using the Shapiro-Wilk test and Levene test,
240 respectively. If the data did not meet the conditions for parametric tests, a Kruskal-Wallis test was used
241 to compare the concentration and the characteristics of MP between the matrices analysed at all sites. If

242 the overall test was significant, a Nemenyi test was performed to determine which means were
243 significantly different. If the data followed the conditions for applying the parametric tests, an ANOVA
244 was used to test for differences between the treatments. If the overall test was significant, a Tukey post
245 hoc test was performed to determine which means were significantly different. In all cases, p values \leq
246 0.05 were considered statistically significant. Also, a principal component analysis (PCA) was carried out
247 on data obtained on clams and worms to evaluate the influence of MP characteristics (size, shape and
248 colour) in the study area and to estimate the relationship between MP profiles in sediments, water and
249 organisms. PCA were thus based on a quantitative variable, the maximum size of MP (<0.1 mm, 0.1– 0.5
250 mm, 0.5-1mm and 1-5mm), and qualitative variables: fragment or fiber, and the colored or non-colored
251 aspect.

252 Results

253 Microplastics were found in all biotic and abiotic samples in all sites. Their levels, measured by
254 binocular microscopy and μ -FTIR were presented Table 1. Levels of MP measured by μ -FTIR were higher
255 than those measured under the microscope, but the trend was similar. All suspected MP were determined
256 as fragments or fibers (Fig.2A). Neither beads nor film were found in the different matrices. MP found in
257 the matrices were categorized into the following size ranges: 1-5 mm; 0.5-1 mm; 0.1-0.5 mm and 0.025
258 mm-0.1 mm (Fig.2B). Diverse colours were observed in the biotic and abiotic matrices (Fig.2C). Finally,
259 different types of plastic were analysed in both, abiotic and biotic matrices (Fig.2D).

260 Microplastic pollution in organisms

261 The average levels (\pm standard deviation, sd) of MP in *Tubifex sp* (n=60), *C. fluminea* (n=18) and
262 *A. anguilla* (n=6) were expressed as number of particles per individual, and also expressed as number of
263 particles per gram of tissue wet weight (ww) allowing the comparison of the MP levels measured in
264 sediment and water (Table 1). When the MP level is expressed per individual, the lowest value was found
265 in worms followed by clams, while the highest abundance of MP was measured in eel ($p < 0.05$). When
266 converted into number of particles per gram of tissue ww, MP concentration was higher in worms,
267 followed by clams and finally by eels. The weight of *C. fluminea* sampled upstream were not significantly
268 different from those collected downstream ($p > 0.05$) (Table S1). However, *Tubifex* sampled upstream
269 were bigger than those collected downstream ($p < 0.05$) (Table S1). Eel mass was quite similar between
270 the six individuals (Table S1).

271 In *Tubifex* sampled upstream, 68.4% of analysed MP were fibers, whereas they represented
272 39.5% in worms sampled downstream (Fig.2A). At both sites, *C. fluminea* ingested more fragments (65%)
273 than fibers (Fig.2A). On the contrary, fibers were the most dominant shape in fish (69.3%) (Fig.2A). The
274 difference in fibers found in fish was significant with *Corbicula* sampled downstream. MP measuring
275 between 0.1 and 0.5 mm were dominant in *C. fluminea* collected up- and downstream (60 and 43.2%,

276 respectively), and in eels (42.6%) (Fig.2B). In the upstream Tubifex, the dominant range size of analysed
277 MP was also 0.1 to 0.5 mm (52.6%), while MP below 0.1 mm were dominant in the downstream worms
278 (46.5%) (Fig.2B). Fragment and fiber sizes were detailed in the Fig.S1. Plastic particles up to 5 mm,
279 exclusively fibers, were only found in eel viscera (Fig.2B). Although nonlinear, there was a positive trend
280 between the size of plastic particles ingested by organisms and the mass of individuals (Fig.S3). Larger
281 plastic particles were ingested by fish compared to clams and worms ($p < 0.05$).

282 As shown in Fig.2C, as many transparent (49.9%) as coloured MP were determined in eel viscera.
283 In Corbicula and Tubifex, more coloured (65 – 94.7%) than transparent MP were counted. Percentages of
284 coloured fragments and fibers found in organisms are presented in Fig.S2. Fibers were mainly blue (24%
285 to 64% according to species) followed by pink (between 1% to 15%). Brown, purple, green, grey, black,
286 yellow and red fibers were also found in organisms but in low amount (<8%) (Fig.S2). Colour profiles of
287 the fragments varied according to the organisms and the site (Fig.S2). Considering all the analysed MP,
288 blue was the main colour found in all organisms: 15 and 35% for upstream and downstream Corbicula
289 respectively, 35 and 53% for upstream and downstream worms respectively, and 26% for eels (Fig.2C and
290 Fig.S2). Finally, blue and transparent MP accounted for more than 30% of all plastic particles (Fig.2C).
291 Plastic particles analysed by μ -FTIR revealed that PE was the dominant type in the organisms (> 60%),
292 followed by PA ($\approx 10\%$) and by PP (between 1.4 and 28.6% according to the species) (Fig.2D), except in
293 the downstream Tubifex, in which PP was the main plastic (93.1 %) followed by PE (3.4%) and PA (2.3%).

294 **MP pollution in sediment and water**

295 The highest level of MP was reported in the upstream mud samples, followed by the downstream
296 mud samples, the downstream sediment samples and the upstream sediment samples. While only a few
297 MP were counted per liter of water, more than a hundred MP were observed per kg of sediment (Table
298 1), confirming that the sediment constitutes a sink for MP. However, there was no significant difference
299 between the total number of MP found in the upstream and the downstream abiotic matrices. Generally,
300 as many fragments as fibers were found in the upstream and downstream abiotic matrices ($p > 0.05$)
301 (Fig.2A), except for the sediment samples, in which more fibers (75%) were found downstream than
302 upstream (25%) ($p < 0.05$) (Fig.2A). Size of suspected MP analysed in mud, sediment and water was
303 different according to the matrices and location (upstream and downstream) (Fig.2B). For example, in the
304 upstream water, no particle ranging from 0.5 to 1 mm was measured, while the reported MP size range
305 in the downstream water corresponded to particles measuring between 1 to 5 mm. However, at both
306 sites, MP mostly measured between 0.1 and 0.5 mm (36.4 – 70.6%), except for MP found in the
307 downstream sediment samples, ranging more between 1 – 5 mm (43.8%). Particles below 0.1 mm
308 constituted the minimum size range for all abiotic matrices. Fragment and fiber sizes were detailed in the
309 Fig.S1: 95.2% of fragments measured less than 0.5 mm, while 100% of fibers ranged from 0.1 to 5 mm.
310 Transparent MP accounted for 59.1% in the upstream mud, while it represented less than 40% in other

311 abiotic matrices (Fig.2C). Blue was the dominant colour (14.3 – 37.5%) in the abiotic matrices but various
312 coloured fragments and filaments were also found (0.9 – 6.1% depending to the colour and the matrix)
313 (Fig.2C; Fig.S2). Samples analysed by μ -FTIR revealed that PE was the dominant type in the abiotic
314 matrices (> 50%), followed by PP (between 22.2 and 37.5% depending to the matrix) (Fig.2D).

315 **Microplastic patterns in organisms and their habitat**

316 Relationship between MP in *Tubifex sp.* and *Corbicula fluminea*, and MP in their habitat (mud or
317 sediment) and water was investigated by PCA (Fig. 3). Since the water samples and eels were not collected
318 at the same time, nor from the same site, MP data related to *Anguilla anguilla* were discarded. Regarding
319 the PCA performed on worms' data (Fig.3A), the two principal components represented 69.10% of total
320 variance: PC1 and PC2 representing 44.41% and 24.69% respectively. PC1 was mostly explained by
321 coloured MP (21.88%) and PC2 by fibers (19.72%). Regarding the PCA performed on clams (Fig.3B), the
322 two principal components represented 72.54% of total variance: PC1 and PC2 representing 50.46% and
323 22.08% respectively. PC1 was mostly explained by the coloured MP (21.00%) and PC2 by the 1-5 mm sized
324 MP (42.19%). In both case, data from the downstream site was more dispersed than those from the
325 upstream site, meaning a more varied profile of MP for the downstream site. Both PCA showed that
326 coloured MP correlated more to fragments, and transparent MP characterised more fibers. They also
327 demonstrated larger size ranges (0.5 to 5 mm) for fibers, while fragments were associated with smaller
328 particles (<0.1 mm). However, the size range of 0.1 to 0.5 mm was not related to fragments nor fibers in
329 *Tubifex* and only to fragments in *Corbicula*. Based on the PCA performed with clam data, the
330 characteristics of MP in the upstream clams had a close relationship with those in the upstream sediment
331 and the upstream water. Although widely scattered, characteristics of MP found in downstream clams
332 were more related to water than sediment. MP relationship between worms and mud was not as
333 correlated as that shown for *Corbicula* with sediment. However, MP characteristics analysed in water
334 were related to those in *Tubifex*, for both sites.

335 **Discussion**

336 Freshwater systems are susceptible to anthropogenic impacts, and usually contain higher amount of
337 MP particles than marine environments (Scherer et al. 2018). The investigation of MP pollution in
338 freshwaters is therefore crucial to set up necessary preventive measures and regulations. Yet, studies
339 investigating, in combined approaches, the abundance of MP in freshwater biota, sediments and water
340 together are still few. Also, data provided by field campaigns helps to better understand the MP state of
341 contamination in a particular site and their bioaccumulation by organisms. Indeed, studies carried out to
342 estimate MP concentration in wild organisms and in their habitats (sediment or water) are essential as
343 they provide valuable information for further laboratory experiments on biological and ecological effects
344 at realistic exposure conditions. To the best of our knowledge, this *in situ* work is the first to study levels

345 and characteristics of MP in organisms, water and sediments from the Loire River, the longest French
346 River, subject to a significant anthropogenic pressure (Wantzen et al. 2023). Although the sampling areas
347 did not cover a large geographic area, the species selected in this study are representative of freshwater
348 ecosystems and the inventory of the MP contamination conducted here allows to know the potential
349 differences between upstream and downstream sites of a city.

350 **Influence of anthropogenic activities in microplastic characteristics**

351 A variation in MP concentrations in organisms, sediment and water was found between both sites.
352 Overall, even if not significant, the level of MP in the samples was slightly higher in all abiotic and biotic
353 matrices collected downstream than upstream, except the mud. In their study, Vermeiren et al. (2021)
354 reported that the average MP abundance was significantly higher at the high anthropogenic site
355 compared to the low impact site due to the human populations considered as a source of plastic debris
356 to the environment and to the proximity with rivers. The city of Angers in our study is crossed by the
357 Maine, a freshwater stream throwing into the Loire River, downstream the town. Since it has been shown
358 that proximity to rivers increases concentrations of MP (Frère et al., 2017; Bancin et al., 2019), this could
359 explain why concentrations of MP are higher downstream than upstream. The higher concentration of
360 MP in the upstream mud compared to the downstream mud in our study may be explained by the
361 granulometry. Although not having measured the grain size of the sampled mud, it was obvious that the
362 upstream mud was composed of finer-grained sediments than the downstream one. Dhivert et al. (2022)
363 demonstrated strong MP levels (on an order of 10^4 items/kg dry weight) over the section characterized
364 by fine-sized sediments. Vermeiren et al. (2021) also showed results in this sense. The MP abundance,
365 measured in the top sediment layer, decreased exponentially with increasing grain size in both sites, one
366 with low anthropogenic impact and one with high impact (Vermeiren et al. 2021). This was corroborated
367 by experimental observations of $>100 \mu\text{m}$ MP retention near the surface in fine sediments compared to
368 coarser sediments (Waldschläger and Schüttrumpf, 2020). Therefore, based on these results, the higher
369 MP levels in the upstream mud compared to the downstream one in this study may be more related to
370 the granulometry than of the location upstream/downstream.

371 In addition, in this study, significantly more fibers were reported in sediments sampled downstream
372 than upstream, whereas no significant differences related to the size of MP or to the colours were
373 reported according to the sites. Browne et al. (2011) showed the presence of fibers at the disposal of
374 wastewater treatment plants (WWTPs). As a consequence, a predominance of fibers is expected in urban
375 areas with large WWTPs (Dris et al. 2018). A substantial wastewater treatment plant is located in Angers
376 (capacity: 257 056 population equivalents (pe), French government website). The discharged water flows
377 into the Maine, then into the Loire. Although smaller, other WWTPs are located along the Loire, near the
378 sampling downstream sites of this study (Chalonnnes-sur-Loire: 8 000 pe and Montjean-sur-Loire: 9 000
379 pe) while there are less WWTPs upstream Angers and these ones are smaller (less than 500 pe).

380 Moreover, in both sites, blue and transparent MP were mainly found in organisms and abiotic samples.
381 This is in accordance with other studies focusing on bivalves (Wang et al. 2021; Truchet et al. 2021),
382 aquatic worms (Pagter et al. 2021; Bour et al. 2018) and fish (Sarijan et al., 2019; Turhan et al. 2022). In
383 this study, we reported PE and PP as the two main polymer types, which is the general trend, in the
384 aquatic ecotoxicological studies. Therefore, the colour of particles and the plastic type polymer did not
385 depend on the location of the sampling site but to the general anthropic activities. Truchet et al. (2021)
386 also reported that MP found in surface water, sediments, and organisms did not present significant
387 differences between the 3 sampling sites exposed to touristic, agricultural and industrial activities,
388 although presenting different criteria and dynamics (estuary, middle zone of the beaches, and
389 lighthouse). All these results suggest that plastic pollution depends on a multitude of processes, not
390 especially the location up or downstream a town.

391 **Microplastics in organisms and relationship with MP in their habitat**

392 In natural environment, the ingestion of MP by organisms depends on a combination of
393 parameters (i.e. size, shape and density of the MP) determining the position of particles in the water
394 column, and hence the availability to animals (Van Cauwenberghe 2015). In this study, *Tubifex sp.*, *C.*
395 *fluminea* and *A. Anguilla* were selected to study *in situ* MP levels since they are able to ingest particles of
396 various size ranges (Hurley et al. 2017, Su et al. 2018, Kılıç et al. 2022). To note that the level of
397 bioaccumulated MP may be under-estimated as the detection limit is 25 µm for both methods, not
398 allowing to characterize the smaller MP. Biology of organisms can also be a key factor for the ingestion of
399 MP. For example, the profile of MP found in *A. mactroides* was different than *B. rodriguezii*, another
400 bivalve species sampled in same locations (Truchet et al. 2021). While *A. mactroides* ingested more MP
401 from 1 to 5 mm, *B. rodriguezii* ingested more MP lower than 0.5 mm. However, as many 1 to 5 mm MP
402 as MP measuring less than 0.5 mm were found in the sediment and in the water. In addition, this study
403 demonstrated that number of suspected fragments and fibers found in individuals increased with
404 organism mass (Fig.S3). This trend might be explained by the fact that the organism ingests MP in a size-
405 range related to its mass, but also by the ability of MP to transfer through the food chain (Su et al. 2018).
406 Indeed, ecotoxicological studies reported lower MP concentrations in *Tubifex tubifex* (Hurley et al. 2017),
407 than *Corbicula fluminea* (Su et al. 2018) or *Anguilla anguilla* (Kılıç et al. 2022). Hurley et al. (2017) averaged
408 0.8 ± 1.01 MP ingested by *Tubifex tubifex*, while 0.4-5.0 items were reported per *Corbicula fluminea* by
409 Su et al. (2018) and 3.8 ± 1.8 per *Anguilla anguilla* were reported by Kılıç et al. (2022). However, expressed
410 by gram of tissues, Hurley et al. (2017) measured a mean concentration of ingested MP at 129 ± 65.4
411 particles g^{-1} tissue against MP concentrations ranging from 56 to 2543 particles kg^{-1} in the host sediments.
412 In our study, similar concentrations were measured for worms and mud sampled. As the best of our
413 knowledge, the study of Hurley et al. (2017) and our study are the two only *in situ* works to have used

414 Tubifex worms as a bioindicator to assess a plastic pollution. An improved understanding of the uptake
415 of MP by freshwater polychaetes, a key entry point into the food chain, will be essential to better
416 understand MP trophic transfer (Hurley et al. 2017). In our study, downstream Tubifex accumulated ten
417 times more than upstream organisms, suggesting that anthropogenic activities of Angers may influence
418 the level of MP pollution in organisms. Although not significant, measured MP concentrations in
419 downstream Corbicula and in downstream sediment were twice higher than MP concentrations in the
420 upstream matrices, and those in water were thousand times higher suggesting again an influence of the
421 locations. ACP revealed similarities of MP patterns between sediment, water and Corbicula. From a
422 qualitative point of view, this demonstrated that MP contamination in clams follows the trend of MP
423 contamination in sediment and water. Su et al. (2018) found a significant positive correlation between
424 the abundance of MP in *C. fluminea* and in the surrounding water and sediment. Abundance and size of
425 particles in organisms, however, were more similar to those in the sediment (Su et al. 2018). Therefore,
426 the authors concluded that *C. fluminea* is an appropriate bioindicator of MP pollution at a large scale.
427 However, in their study the authors also reported that despite MP in sediments are more similar to those
428 in clams, differences in size, colour and types were reported. As previously mentioned, Tubifex only
429 served once as bioindicator for plastic pollution. It is well known that these freshwater worms are highly
430 tolerant of grossly polluted settings (Wiederholm and Dave, 1989) and are one of the last species present
431 under deteriorating environmental conditions (Milbrink, 1987). Since the entire life cycle of Tubifex take
432 place within sediments, this species could be used as indicator of MP pollution in freshwater streams. A
433 replacer dans le texte : Indeed, this study demonstrated that MP profile in Corbicula was similar to those
434 found in their habitat but MP profile in Tubifex was not related to the sediment where they live in. This
435 result may be explained however by the granulometry of the sampled upstream mud compared to the
436 downstream one, as explained in the previous part.

437 Finally, a comparison between the MP profile in eels in the two sampling sites was not possible as fish
438 were only collected downstream. Since fish are not sedentary organisms, they are not representative of
439 a specific site, as compared to the other bioindicator species analyzed in the present study. However,
440 they are useful indicators of plastic pollution levels of large freshwater streams such as the Loire River.
441 Based on literature, it seems that gut is one of the most relevant organ to assess MP pollution. Lv et al.
442 (2020) showed that MP were mostly found in gut of eel, in lower concentrations in edible parts (head,
443 muscle and bones) and none in internal organs other organs (heart, liver, kidney, bile and gonad). Kılıç et
444 al. (2022) showed a statistically different MP abundance in the examined organs of the few fish species
445 and reported a general higher MP abundance in the gastrointestinal tract, coherent to the results of
446 Guilhermino et al. (2021). Moreover, Lv et al. (2020) reported MP size ranges in eel guts similar to the
447 ones measured in the current study (mainly between 0.1 and 1 mm). Therefore, eels fished in the Loire

448 River might represent a sanitary risk due to a MP contamination, and an assessment of MP abundance in
449 consumable fish may be considered.

450 **Complementary methods for plastic pollution monitoring**

451 Visual assessment allows to quantify and sort the suspected MP based on their characteristics.
452 Even if a bias in their identification under microscope cannot be excluded due to human subjective
453 perception, shape and colour are two fundamental properties to determinate the environmental dynamic
454 of plastics. Composition of polymers is also important to determine MP profile. Use of analytical analysis
455 by μ -FTIR is therefore a relevant complementary method, allowing to verify the accuracy of visual
456 identification of suspected MP. Since the visual method does not allow to know the plastic composition
457 and the μ -FTIR imaging method does not give the colour of particles, it seems therefore essential to use
458 both techniques for a complete assessment of an *in situ* MP pollution.

459 In addition, even if methods are in place, there is still no standardised protocols in the literature.
460 This lack may create uncertainty for interpretation of results of published articles. For example, units of
461 MP concentrations are not the same according to published papers, making comparison of plastic
462 pollution levels difficult. Differences in analytical protocols can be also highlighted, such as the quantity
463 of abiotic matrix digested which is author-dependant. Some of them used aliquots of 30 g (Li et al. 2021),
464 50 g (Truchet et al. 2021) or 100 g (Bonifacio et al. 2022) of sediment. We also reported some differences
465 in digestion method of biotic samples. Indeed, for MP extraction in fish, some studies used hydrogen
466 peroxide (Atamanalp et al., 2022; Turhan, 2022); while, some others used potassium hydroxide (Merga et
467 al., 2020). Also, studies focusing on bivalves are not homogenous on the number of analysed individuals.
468 For example, Suet al. (2018) analysed three replicates of 2 - 4 *Corbicula*, while Truchet et al. (2021)
469 analysed three replicates composed each of 10 *A. mactroides* organisms and three replicates of *B.*
470 *rodriguezii* composed each of 30 individuals. Moreover, the sediment size or additional processes leading
471 to a plastic contamination are not automatically characterized in studies, which might be accounted when
472 comparing among sites, and for relevantly characterising the plastic pollution in abiotic and biotic
473 matrices.

474 Therefore, the lack of standardised protocols for the sampling, the analytical analysis and the way
475 of highlight data makes comparison between *in situ* studies difficult. A well-defined theoretical framework
476 is so really needed to summarise and compare global results regarding abundance of MP and understand
477 their spatial and temporal dynamics. This would help to define the sources, to put in place an adequate
478 policy and to compare existing or future data.

479 **Conclusion**

480 This investigation work provided a global insight into MP contamination of the Loire River. All the
481 organisms sampled in this study bioaccumulated MP. The related abiotic matrices, the sediment and the

482 water where they live, revealed also an occurrence of MP. The profile of MP found in *Corbicula* was similar
483 to the one found in its habitat while the MP profiles in *Tubifex* and in their habitat were not statistically
484 correlated. Overall, the characteristics of the analysed plastic particles were different according to the
485 matrices and the sites, and no generality can be made. This study contributed to the knowledge of the
486 MP pollution levels in freshwater compartments, and data might be helpful to conduct laboratory
487 experiment carried out under realistic ecological conditions.

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