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

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Abstract

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

Keywords

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

Introduction

Plastic pollution in aquatic ecosystems is now a well-known issue. For the last decade, plastic pollution has become a major environmental concern due to its increasing production, which has reached 367 million tons worldwide (PlasticsEurope, 2021). Among these plastics, many are single-used and immediately discarded, most frequently improperly. Some reach the terrestrial environment and
ultimately end up in aquatic environments (Oliveira et al. 2019; Ferreira et al. 2019). Globally, it is estimated that between 70 and 80% of plastics in the oceans come from land-based sources (Duis and Coors, 2016; Alimi et al. 2018). Once they reach the aquatic compartment, plastics can be fragmented into smaller sizes by photodegradation (UV radiation), weathering and physical abrasion (e.g., wind and wave action), and biotic factors (e.g., microorganisms’ action). Nowadays, microplastics (MP), plastic particles < 5 mm, represent a current and a global environmental concern. Indeed, numerous scientific studies have reported that MP are increasingly observed in all compartments of most aquatic ecosystems around the world, even in Arctic and Antarctic Oceans (Lusher et al. 2015; Waller et al. 2017; Huntington et al. 2020). MP can be found as suspended particles in the water column, in sediments and in wastewater (Habib et al. 2020; Bayo et al. 2020; Frias et al. 2020; Dusaucy et al. 2021; Uddin et al. 2021). In addition to being found in abiotic compartments, MP have been demonstrated to be ingested by a wide variety of aquatic biota, in both marine and freshwater organisms, from microalgae to large mammals (Farrell et al. 2013; Santana et al. 2017; Qu et al. 2020), and consequences are multiple: MP retention in the gut causing blockages, reducing nutrient absorption (Cole et al. 2015; Lu et al. 2016; Welden and Cowie, 2016) or MP translocation to other tissues (Browne et al. 2008; Brennecke et al. 2015).

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

The current study investigated for the first time the level of the MP contamination in three aquatic organisms, around Angers, one of the major cities along the Loire. Tubifex (Tubifex sp. (Müller, 1774)), Corbicula (Corbicula fluminea (Müller, 1774)) and eel (Anguilla anguilla (Linnaeus, 1758)) were used as model organisms due to their dominant stock in the Loire River and to their position at different trophic
levels. Tubifex worms are one of the most abundant invertebrates in freshwater compartments and represent primary consumers in the food chain of their ecosystem. Tubifex tubifex has been widely used to assess the toxicity and the bioaccumulation of some pollutants, especially metals (Lagauzère et al. 2009; Mosleh et al. 2006) making it a suitable species for sediment toxicity tests (Chapman et al. 2001).

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

The first aim of this study was to investigate the MP contamination in abiotic and biotic matrices. In this context, the shape of MP, the colour, the size and the composition were characterised in sediments, water, viscera of A. anguilla, whole body of C. fluminea and Tubifex sp.. In the literature, two methods are generally employed: microscopy and micro-Fourier Transform InfraRed (µ-FTIR) analysis. Imaging µ-FTIR does not distinguish the colours of particles neither the shapes, like fibers, while an observation by microscopy does not determine the polymer composition. In order to perform a global analysis of the plastic particles profile in samples, both complementary methods were used. Thus, we were able to provide points of comparison between these two approaches. The second objective was to compare MP levels in all the samples (sediment, water and organisms) collected upstream and downstream Angers, to evaluate the influence of human activities on the plastic pollution. The final goal was to determine the
correlations between MP ingested by the organisms and MP found in the abiotic matrices, corresponding to the habitats of the analysed species. Most of studies focused on MP levels either on abiotic matrices or biotic matrices, generally on one or two species. Therefore, this study provides less spatial and temporal resolution on *in situ* MP levels than some studies but gives an overview of the MP contamination in freshwater abiotic and biotic matrices at three species belonging to different trophic levels, which constitutes its originality. All of these results will be used to suggest protocols for monitoring microplastic contamination of freshwater streams and provide data to implement public policies for environmental risk management.

**Materials and method**

**Study sites**

The Loire River is the longest French river, with a length of 1 006 km. This stream rises in the south-eastern quarter of the French Massif Central at an altitude of 1 350 meters until the Bay of Saint Nazaire, Atlantic Ocean. The watershed covers a total area of 155 000 km$^2$, equivalent to 22% of French territory (Managing Rivers Wisely, WWF). The Loire basin has more than 11.5 million inhabitants, markedly rural, with more than a third of communities having fewer than 400 inhabitants (Managing Rivers Wisely, WWF). Moreover, a large number of plant and animal species live on the banks or in the bed of the Loire (Wantzen et al. 2023). The French Biodiversity Agency (OFB) and the National Inventory of Natural Heritage (INPN) listed some of these species, such as the *Schoenoplectus triqueter* plant, various amphibians, birds such as the kingfisher, and mammals such as the beaver, as protected and sometimes threatened species. As human activities in Angers agglomeration (155 850 inhabitants in 2019, INSEE) may generate plastic pollution ending up in nearby freshwater streams, two sites were selected to study MP contamination from anthropic sources near Angers city along the Loire River: one upstream the town, at La Daguenière (47°23’11”N 50°48’’W) and one downstream, at Montjean-sur-Loire (47°23’34”N 51’58”W) (Fig.1).

**Sampling of organisms (biotic matrices)**

For this study, three species were analysed: *Tubifex sp.*, *Corbicula fluminea* and *Anguilla anguilla*. *Tubifex sp.* and *C. fluminea* were sampled in May 2022, in both sites. Tubifex worms, deposit feeders, occupy the uppermost layers of sediment and live partially submerged and they were abundant in the banks. They typically burrow to 6–10 cm depth but can be found at a depth of 2 cm in highly contaminated sediments (Karickhoff and Morris, 1985; Lagauzère et al. 2009). For this study, worm species were not identified and only the genus will be considered. The Tubifex organisms were carefully extracted from the sediments using stainless steel tweezers and rinsed with distilled water to remove all organic and inorganic matters. The organisms were then placed into 1.5 mL microtubes and stored at -20°C until MP analysis. In the same way, Corbicula were collected in both site using a rake, then rinsed with distilled
water and stored at -20°C until analysis. For years, the Asian clam (*C. fluminea*) has colonized diverse freshwater ecosystems (Sousa et al. 2008) and is today an exotic invasive species in Europe and other areas of the world. A total of 300 worms and 50 clams were collected. To characterise MP in an upper and essential link of the aquatic trophic chain, viscera of eels (n=6 females) were also analysed to estimate the MP ingestion (biometric data reported in Table S1). Eel guts provided from a professional fisherman who caught the individuals in December 2021, in the Chalonnes-sur-Loire sector, downstream from Angers (Fig.1).

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

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

**Characterization of MP by microscopy**

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

**Biotic matrices (organisms).** The sample treatment procedure for worms and clams was adapted from the protocol published by Revel et al. (2019). Briefly, 6 pools of 3 clams sampled downstream or upstream, as well as 6 pools of 10 worms sampled downstream or upstream were analysed. The clam soft tissues and the worm whole body were placed separately in 250 mL beakers precleaned with ethanol absolute (70%) and filled with 5 volumes of the individual pool mass of hydroxide potassium (KOH) 10% for bivalves and 20% for worms. Then, the beakers were covered with aluminium, placed on a heating plate at 60°C with stirring for 24 h to eliminate organic matter. For the eel viscera MP analysis, preliminary tests for sample digestion were conducted based on Biginagwa et al. (2016) and Thiele et al. (2019). Viscera of 6 eels were analysed separately. To facilitate the digestion step, the viscera were cut into pieces and placed into different beakers due to their important mass (Table S1). A volume of 40% KOH equivalent to 10 times the organism mass (m/v) was added into each beaker. Then, the beakers were covered with aluminium, placed on a heating plate at 60°C with stirring for 7 days to eliminate organic matter. Once the digestion step finished, the solution was transferred to a 50 mL Falcon tube containing a 1:1 ratio of
KOH solution and saturated NaCl solution to extract the lipid layer. The tube was then centrifuged at 6500 rpm for 15 min, at 4°C. The solid supernatant was removed and immersed in a new NaCl solution. Another centrifugation (6500 rpm for 15 min at 4°C) was proceeded before recovering the supernatant, added with the first one for the filtration step. The filtration procedure for the biotic matrices was the same as for the abiotic matrices.

**Abiotic matrices (sediment and water).** The procedure of treatment of sediment samples was adapted from the protocol published by Blair et al. (2019). Briefly, samples were dried in an oven at 60°C for 48 h and were sieved (1.6 mm) to remove the larger debris. Six replicates of 20 g of sediment were placed in a 50 mL Falcon tube with 20 mL of saturated NaCl solution. After mixing thoroughly, samples were centrifuged (6500 rpm for 10 min). The supernatant was recovered while the bottom sediment was taken up twice in 20 mL of saturated NaCl to optimize the extraction of all MP. NaCl is often used in the sample treatment for MP microscopic observation. This solution was thus chosen to compare our data with those published in the literature. Then, samples undergo a second centrifugation (6500 rpm for 10 min) and the supernatant was added to the one already recovered, for filtration. Five replicates of 200 mL of water were directly filtered without prior digestion step. All samples were filtered under Büchner on a 1.6 µm filter (GF/A 1820-037). Filters were then kept in a Petri dish, precleaned with ethanol absolute (70%), until further analysis.

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

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

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

**Biotic matrices (organisms).** The MP extraction procedure was based on an adaptation of the protocol published by Phuong et al. (2018). Briefly, whole worms and soft tissue of clams were separately placed in 100 mL beakers with a volume of 10% KOH equivalent to 10 times the organism mass (m/v). The mixture was heated at 45°C under stirring for 24 h for Corbicula and for 76 h for Tubifex. The solutions were then filtered through a metallic filter (10 µm cutoff) using a Büchner filtration. The metallic filter was immersed in a beaker containing NaI solution and sonicated for 1 minute. As for NaCl used for
microscopy, NaI was chosen for the µ-FTIR method. Most studies used it to proceed a pre-treatment step followed by a densimetric separation. The NaI solution, after removing and rinsing the filter, was introduced in a JAMSS display to extract MP for at least 5h. The NaI solution was then filtered on a new metallic filter and rinsed with distilled water. The solution was sonicated for 1 minute before being filtered through an Anodisc membrane filters (0.2 μm, 25 mm, Whatman). Finally, all the filters were stored in closed glass Petri dishes until analysis.

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

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

**Contamination control**

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

**Statistical analysis**

Statistical analysis was carried out using R software (version 4.2.2). Normality of the data distribution and homogeneity of variance were tested using the Shapiro-Wilk test and Levene test, respectively. If the data did not meet the conditions for parametric tests, a Kruskal-Wallis test was used to compare the concentration and the characteristics of MP between the matrices analysed at all sites. If
the overall test was significant, a Nemenyi test was performed to determine which means were significantly different. If the data followed the conditions for applying the parametric tests, an ANOVA was used to test for differences between the treatments. If the overall test was significant, a Tukey post hoc test was performed to determine which means were significantly different. In all cases, p values ≤ 0.05 were considered statistically significant. Also, a principal component analysis (PCA) was carried out on data obtained on clams and worms to evaluate the influence of MP characteristics (size, shape and colour) in the study area and to estimate the relationship between MP profiles in sediments, water and organisms. PCA were thus based on a quantitative variable, the maximum size of MP (<0.1 mm, 0.1–0.5 mm, 0.5-1mm and 1-5mm), and qualitative variables: fragment or fiber, and the colored or non-colored aspect.

**Results**

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

**Microplastic pollution in organisms**

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

In Tubifex sampled upstream, 68.4% of analysed MP were fibers, whereas they represented 39.5% in worms sampled downstream (Fig.2A). At both sites, *C. fluminea* ingested more fragments (65%) than fibers (Fig.2A). On the contrary, fibers were the most dominant shape in fish (69.3%) (Fig.2A). The difference in fibers found in fish was significant with Corbicula sampled downstream. MP measuring between 0.1 and 0.5 mm were dominant in *C. fluminea* collected up- and downstream (60 and 43.2%,
respectively), and in eels (42.6%) (Fig.2B). In the upstream Tubifex, the dominant range size of analysed MP was also 0.1 to 0.5 mm (52.6%), while MP below 0.1 mm were dominant in the downstream worms (46.5%) (Fig.2B). Fragment and fiber sizes were detailed in the Fig.S1. Plastic particles up to 5 mm, exclusively fibers, were only found in eel viscera (Fig.2B). Although nonlinear, there was a positive trend between the size of plastic particles ingested by organisms and the mass of individuals (Fig.S3). Larger plastic particles were ingested by fish compared to clams and worms (p < 0.05).

As shown in Fig.2C, as many transparent (49.9%) as coloured MP were determined in eel viscera. In Corbicula and Tubifex, more coloured (65 – 94.7%) than transparent MP were counted. Percentages of coloured fragments and fibers found in organisms are presented in Fig.S2. Fibers were mainly blue (24% to 64% according to species) followed by pink (between 1% to 15%). Brown, purple, green, grey, black, yellow and red fibers were also found in organisms but in low amount (<8%) (Fig.S2). Colour profiles of the fragments varied according to the organisms and the site (Fig.S2). Considering all the analysed MP, blue was the main colour found in all organisms: 15 and 35% for upstream and downstream Corbicula respectively, 35 and 53% for upstream and downstream worms respectively, and 26% for eels (Fig.2C and Fig.S2). Finally, blue and transparent MP accounted for more than 30% of all plastic particles (Fig.2C).

Plastic particles analysed by µ-FTIR revealed that PE was the dominant type in the organisms (> 60%), followed by PA (≈ 10%) and by PP (between 1.4 and 28.6% according to the species) (Fig.2D), except in the downstream Tubifex, in which PP was the main plastic (93.1%) followed by PE (3.4%) and PA (2.3%).

**MP pollution in sediment and water**

The highest level of MP was reported in the upstream mud samples, followed by the downstream mud samples, the downstream sediment samples and the upstream sediment samples. While only a few MP were counted per liter of water, more than a hundred MP were observed per kg of sediment (Table 1), confirming that the sediment constitutes a sink for MP. However, there was no significant difference between the total number of MP found in the upstream and the downstream abiotic matrices. Generally, as many fragments as fibers were found in the upstream and downstream abiotic matrices (p > 0.05) (Fig.2A), except for the sediment samples, in which more fibers (75%) were found downstream than upstream (25%) (p < 0.05) (Fig.2A). Size of suspected MP analysed in mud, sediment and water was different according to the matrices and location (upstream and downstream) (Fig.2B). For example, in the upstream water, no particle ranging from 0.5 to 1 mm was measured, while the reported MP size range in the downstream water corresponded to particles measuring between 1 to 5 mm. However, at both sites, MP mostly measured between 0.1 and 0.5 mm (36.4 – 70.6%), except for MP found in the downstream sediment samples, ranging more between 1 – 5 mm (43.8%). Particles below 0.1 mm constituted the minimum size range for all abiotic matrices. Fragment and fiber sizes were detailed in the Fig.S1: 95.2% of fragments measured less than 0.5 mm, while 100% of fibers ranged from 0.1 to 5 mm. Transparent MP accounted for 59.1% in the upstream mud, while it represented less than 40% in other
abiotic matrices (Fig.2C). Blue was the dominant colour (14.3 – 37.5%) in the abiotic matrices but various
coloured fragments and filaments were also found (0.9 – 6.1% depending to the colour and the matrix)
(Fig.2C; Fig.S2). Samples analysed by µ-FTIR revealed that PE was the dominant type in the abiotic
matrices (> 50%), followed by PP (between 22.2 and 37.5% depending to the matrix) (Fig.2D).

**Microplastic patterns in organisms and their habitat**

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

**Discussion**

Freshwater systems are susceptible to anthropogenic impacts, and usually contain higher amount of
MP particles than marine environments (Scherer et al. 2018). The investigation of MP pollution in
freshwaters is therefore crucial to set up necessary preventive measures and regulations. Yet, studies
investigating, in combined approaches, the abundance of MP in freshwater biota, sediments and water
together are still few. Also, data provided by field campaigns helps to better understand the MP state of
contamination in a particular site and their bioaccumulation by organisms. Indeed, studies carried out to
estimate MP concentration in wild organisms and in their habitats (sediment or water) are essential as
they provide valuable information for further laboratory experiments on biological and ecological effects
at realistic exposure conditions. To the best of our knowledge, this *in situ* work is the first to study levels
and characteristics of MP in organisms, water and sediments from the Loire River, the longest French River, subject to a significant anthropogenic pressure (Wantzen et al. 2023). Although the sampling areas did not cover a large geographic area, the species selected in this study are representative of freshwater ecosystems and the inventory of the MP contamination conducted here allows to know the potential differences between upstream and downstream sites of a city.

Influence of anthropogenic activities in microplastic characteristics

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

In addition, in this study, significantly more fibers were reported in sediments sampled downstream than upstream, whereas no significant differences related to the size of MP or to the colours were reported according to the sites. Browne et al. (2011) showed the presence of fibers at the disposal of wastewater treatment plants (WWTPs). As a consequence, a predominance of fibers is expected in urban areas with large WWTPs (Dris et al. 2018). A substantial wastewater treatment plant is located in Angers (capacity: 257 056 population equivalents (pe), French government website). The discharged water flows into the Maine, then into the Loire. Although smaller, other WWTPs are located along the Loire, near the sampling downstream sites of this study (Chalonnes-sur-Loire: 8 000 pe and Montjean-sur-Loire: 9 000 pe) while there are less WWTPs upstream Angers and these ones are smaller (less than 500 pe).
Moreover, in both sites, blue and transparent MP were mainly found in organisms and abiotic samples. This is in accordance with other studies focusing on bivalves (Wang et al. 2021; Truchet et al. 2021), aquatic worms (Pagter et al. 2021; Bour et al. 2018) and fish (Sarijan et al., 2019; Turhan et al. 2022). In this study, we reported PE and PP as the two main polymer types, which is the general trend, in the aquatic ecotoxicological studies. Therefore, the colour of particles and the plastic type polymer did not depend on the location of the sampling site but to the general anthropic activities. Truchet et al. (2021) also reported that MP found in surface water, sediments, and organisms did not present significant differences between the 3 sampling sites exposed to touristic, agricultural and industrial activities, although presenting different criteria and dynamics (estuary, middle zone of the beaches, and lighthouse). All these results suggest that plastic pollution depends on a multitude of processes, not especially the location up or downstream a town.

Microplastics in organisms and relationship with MP in their habitat

In natural environment, the ingestion of MP by organisms depends on a combination of parameters (i.e. size, shape and density of the MP) determining the position of particles in the water column, and hence the availability to animals (Van Cauwenberghe 2015). In this study, Tubifex sp, C. fluminea and A. Anguilla were selected to study in situ MP levels since they are able to ingest particles of various size ranges (Hurley et al. 2017, Su et al. 2018, Kılıç et al. 2022). To note that the level of bioaccumulated MP may be under-estimated as the detection limit is 25 µm for both methods, not allowing to characterize the smaller MP. Biology of organisms can also be a key factor for the ingestion of MP. For example, the profile of MP found in A. mactroides was different than B. rodriguezi, another bivalve species sampled in same locations (Truchet et al. 2021). While A. mactroides ingested more MP from 1 to 5 mm, B. rodriguezi ingested more MP lower than 0.5 mm. However, as many 1 to 5 mm MP as MP measuring less than 0.5 mm were found in the sediment and in the water. In addition, this study demonstrated that number of suspected fragments and fibers found in individuals increased with organism mass (Fig.S3). This trend might be explained by the fact that the organism ingests MP in a size-range related to its mass, but also by the ability of MP to transfer through the food chain (Su et al. 2018). Indeed, ecotoxicological studies reported lower MP concentrations in Tubifex tubifex (Hurley et al. 2017), than Corbicula fluminea (Su et al. 2018) or Anguilla anguilla (Kılıç et al. 2022). Hurley et al. (2017) averaged 0.8 ± 1.01 MP ingested by Tubifex tubifex, while 0.4-5.0 items were reported per Corbicula fluminea by Su et al. (2018) and 3.8 ± 1.8 per Anguilla anguilla were reported by Kılıç et al. (2022). However, expressed by gram of tissues, Hurley et al. (2017) measured a mean concentration of ingested MP at 129 ± 65.4 particles g⁻¹ tissue against MP concentrations ranging from 56 to 2543 particles kg⁻¹ in the host sediments. In our study, similar concentrations were measured for worms and mud sampled. As the best of our knowledge, the study of Hurley et al. (2017) and our study are the two only in situ works to have used
Tubifex worms as a bioindicator to assess a plastic pollution. An improved understanding of the uptake of MP by freshwater polychaetes, a key entry point into the food chain, will be essential to better understand MP trophic transfer (Hurley et al. 2017). In our study, downstream Tubifex accumulated ten times more than upstream organisms, suggesting that anthropogenic activities of Angers may influence the level of MP pollution in organisms. Although not significant, measured MP concentrations in downstream Corbicula and in downstream sediment were twice higher than MP concentrations in the upstream matrices, and those in water were thousand times higher suggesting again an influence of the locations. ACP revealed similarities of MP patterns between sediment, water and Corbicula. From a qualitative point of view, this demonstrated that MP contamination in clams follows the trend of MP contamination in sediment and water. Su et al. (2018) found a significant positive correlation between the abundance of MP in C. fluminea and in the surrounding water and sediment. Abundance and size of particles in organisms, however, were more similar to those in the sediment (Su et al. 2018). Therefore, the authors concluded that C. fluminea is an appropriate bioindicator of MP pollution at a large scale. However, in their study the authors also reported that despite MP in sediments are more similar to those in clams, differences in size, colour and types were reported. As previously mentioned, Tubifex only served once as bioindicator for plastic pollution. It is well known that these freshwater worms are highly tolerant of grossly polluted settings (Wiederholm and Dave, 1989) and are one of the last species present under deteriorating environmental conditions (Milbrink, 1987). Since the entire life cycle of Tubifex take place within sediments, this species could be used as indicator of MP pollution in freshwater streams. A replacer dans le texte : Indeed, this study demonstrated that MP profile in Corbicula was similar to those found in their habitat but MP profile in Tubifex was not related to the sediment where they live in. This result may be explained however by the granulometry of the sampled upstream mud compared to the downstream one, as explained in the previous part.

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

**Complementary methods for plastic pollution monitoring**

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

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

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

**Conclusion**

This investigation work provided a global insight into MP contamination of the Loire River. All the organisms sampled in this study bioaccumulated MP. The related abiotic matrices, the sediment and the
water where they live, revealed also an occurrence of MP. The profile of MP found in Corbicula was similar
to the one found in its habitat while the MP profiles in Tubifex and in their habitat were not statistically
correlated. Overall, the characteristics of the analysed plastic particles were different according to the
matrices and the sites, and no generality can be made. This study contributed to the knowledge of the
MP pollution levels in freshwater compartments, and data might be helpful to conduct laboratory
experiment carried out under realistic ecological conditions.

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