Environmental Science & Technology

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Stable Isotope Insights into Microplastic Contamination within Freshwater Food Webs

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Cite This: *Environ. Sci. Technol.* 2021, 55, 1024−1035

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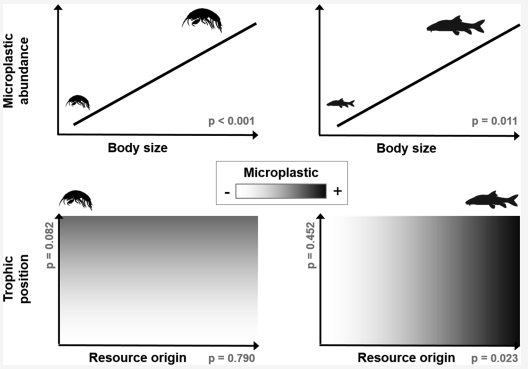
ABSTRACT: Microplastic pollution and ingestion are ubiquitous phenomena in freshwater ecosystems. However, our understanding of the role of trophic niche in microplastic ingestion is still limited. Here, we quantified the level of microplastic (700 μm to 5 mm) contamination for macroinvertebrates and fish within the Garonne river. We then used stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) to quantify trophic niches. We first demonstrated that the abundance of ingested microplastics differed between macroinvertebrates and fish and was not significantly related to microplastic pollution. We then found that microplastic characteristics (shape, color, size, and polymer composition) differ between the abiotic (surface waters and sediments) and biotic (ingested by macroinvertebrates and fish) compartments. The abundance of ingested microplastics increased with the size of organisms in both fish and macroinvertebrates and tended to increase with trophic position in macroinvertebrates only. Finally, the origin of the resources consumed by fish significantly affected the abundance of microplastics ingested. Altogether, these results suggest the absence of microplastic bioaccumulation in freshwater food webs and the dominance of direct consumption, most likely accidentally. The use of stable isotope analyses is therefore crucial to improve our understanding of microplastic ingestion by wild organisms.



1. INTRODUCTION

Freshwater ecosystems provide a myriad of services to humans but are facing growing impacts from human activities[1] with multiple and interacting perturbations altering biodiversity and ecosystem functioning.[2] Microplastic pollution, the presence of small fractions (<5 mm) of plastics[3] in the environment, is a ubiquitous phenomenon that has recently emerged as a growing source of concern. There is, to date, an important lack of knowledge about the contamination pathways and consequences of microplastic pollution on freshwater organisms and ecosystems.[4−6]

Studies on microplastic pollution have typically focused on marine ecosystems,[7,8] but streams and rivers play a crucial role in the global microplastic pollution.[5] Indeed, 70−80% of marine plastics are transported by freshwaters.[9,10] Freshwater microplastic pollution is strongly variable within hydrological networks[11,12] and usually higher in urban and industrialized areas.[13,14] Microplastics are ingested by freshwater organisms and the consequences of these ingestions are variable.[6,7,15] High levels of ingestion generally occur in sites with high microplastic pollution in the water[16,17] or sediment,[18] but this relationship does not hold systematically.[19,20] Microplastic ingestion is also dependent on organism biological traits. This includes, for instance, body size, whereby ingested microplastic size and abundance typically increase with organism body size.[19,21] Microplastic ingestion can differ between functional feeding groups and foraging style,[19,22,23] with microplastic abundance ingested by visual foragers increasing with increased microplastic concentration in water.[20] In addition, microplastic characteristics can also influence their consumption by organisms, with their size being limited by gill raker apparatus in fish,[24] while food-like and sinking particles were reported to be more often ingested by fish.[20] Freshwater organisms might directly ingest microplastics and this is defined as a primary ingestion. Primary ingestion can either be intentional (active) or accidental. Secondary ingestion occurs when microplastics are consumed through the consumption of prey that have consumed microplastics, i.e., indirectly ingested. Secondary ingestion can represent a form of bioaccumulation.[6,24,25] Investigations are therefore needed to better understand the mechanisms of microplastic ingestion by freshwater organisms.

Studies on microplastic ingestion neglected the fact that individuals within species are highly variable ecologically[26] and

Received: September 15, 2020
Revised: December 17, 2020
Accepted: December 17, 2020
Published: January 7, 2021

that trophic niches are shaped by complex and interacting ecological parameters.[27] Intraspecific variability occurs both in terms of functional traits and trophic niches, within and between the life stages of a species.[28−30] Therefore, the use of functional feeding groups might oversimplify individual trophic niches, precluding an integrative understanding of microplastic ingestion. During the past two decades, stable isotope analyses have emerged as an integrative tool used by trophic ecologists to quantify the realized trophic niche.[31] Compared to traditional methods such as stomach content and feces analyses that represent only a snapshot into the diet of organisms, stable isotope analyses provide an integrative quantification, over several weeks to months depending on the tissue analyzed, of the diet of individuals.[31,32] Importantly, trophic niche can be quantified with stable isotope analyses even if the organisms has not consumed any prey recently (e.g., empty stomach contents), maximizing the amount of information obtained from sampled individuals. Specifically, stable isotope analyses of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) provide assessment of the origin of resources consumed and the trophic position in the food chain, respectively, and are commonly used in freshwater ecology, notably to quantify the consequences of global changes.[32−34] The use of stable isotope analyses to understand microplastic contamination in freshwater food webs therefore represents a promising approach.

The general objective of this study is to assess the trophic determinants of microplastic contamination across trophic levels within freshwater food webs using stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$). We first measured microplastic contamination in macroinvertebrates and fish and tested the association between microplastic ingestion and microplastic pollution (surface waters and sediments). We then compared microplastic characteristics (shape, color, size, and polymer composition) between microplastics in the environment and those ingested by organisms. Finally, we quantified the relationship between the trophic ecology of organisms and microplastic ingestion.

2. MATERIAL AND METHODS

2.1. Study Area. The present study was performed in the Garonne river, located in the southwest of France. The Garonne river is the third biggest stream in France, with a length of 525 km and a basin area of 53.536 km^2, flowing from the central part of the Pyrenees, across the main city of Toulouse, and into the Atlantic Ocean near Bordeaux (Figure S1). Six study sites were selected within the watershed of the Garonne river to represent contrasting environmental conditions (Table S1): two sites (LBI and MUG) were located on the Garonne river upstream of Toulouse, two sites (LAU and TOU) were located on tributaries within the Toulouse agglomeration, and two sites (GSG and CAS) were located on the Garonne river, downstream of Toulouse. The presence of dams and subsequent strong flows prevented the sampling of the Garonne river within Toulouse.

2.2. Sampling. *2.2.1. Microplastic Pollution in Water and Sediment.* Water and sediment were sampled between 1 and 4 July 2019, with two sites sampled per day. Surface water samples were obtained by filtration using a Manta net (opening, 32 cm × 82 cm) equipped with a 500 μm polyamide mesh size net and a removable cod-end[35] submerged for approximately 10 min. The 500 μm mesh size was selected to maximize a trade-off between the volume of filtered water, net clogging, and particle size and concentration.[14] This mesh size was subsequently applied in all sample types and procedures. Three water samples were collected for each site (18 samples). The net mouth was additionally equipped with a mechanical flowmeter (Hydro-Bios, Germany) to estimate the volume of water filtered. Sediment samples were collected using a Surber net (30 cm × 30 cm, 500 μm mesh size) equipped with a removable cod-end in the riffle areas of each site. Surber nets were used in microhabitats composed of gravels and cobbles as the main substrate and the area delimited by the Surber net (subsequently used to calculated microplastic concentration) was gently washed to remove settled particles, which were subsequently collected in the cod-end of the Surber net. The sampling was repeated three times (approximately 10 min between each replicate) and standardized for approximately 1 min, yielding a total of 18 samples. After each sampling, the contents of the cod-end were filtered in the field through a 500 μm sieve, rinsed with river water (previously filtered at 500 μm), and transferred to plastic sealed bags made of polyethylene. All samples were stored in a cooler in the field and subsequently stored at 4 °C in the laboratory before analyses.

2.2.2. Microplastic Contamination in Macroinvertebrates and Fishes. Macroinvertebrates were collected between 10 and 18 July 2019 (one site sampled per day) using Surber and kick (500 μm mesh size) netting, performed representatively of microhabitats. Specimens were collected as a representative sample for each site, which reflected feeding modes (shredders, collectors, predators, and scrapers)[36] and the macroinvertebrate community present within each site. On average, 65.8 samples (±10.1 SD) were collected in each site. Due to the small size of some macroinvertebrate taxa and the potentially low level of microplastic ingestion, individuals were aggregated to compose a sample for macroinvertebrates. On average, each sample consisted of 5.4 individuals (±2.9 SD), ranging from a single individual for large taxa (e.g., Odonata) to around 15 individuals for the smallest taxa (e.g., Chironomidae). Aggregated samples were made up to have approximately similar masses; for example, the average mass of Gammaridae samples was 0.42 mg (see further details in stable isotope analyses). Within taxa, individuals of similar size (visually estimated to the nearest mm) were grouped within the same sample, euthanized, and stored in glass tubes in a cooler in the field. Additionally, two crayfish species (spiny-cheek crayfish *Faxonius limosus* and red swamp crayfish *Procambarus clarkii*) were collected during electrofishing and processed following the same protocol as for the fish (see details below).

Fish sampling was performed between 23 and 30 July 2019 (one site sampled per day) by electrofishing (model FEG 1500 and 5000, EFKO GmbH, Germany). To limit the potential effect of dial activity in fish foraging behavior, sampling was always performed in the morning (7:00−11:00 a.m.), covering all habitat accessible by wading in each site. Sampled fish were subsequently selected (average: 82 fish (±14 SD) per site) to represent the taxonomic, size-class, and functional (bottom feeders and column feeders) diversity of each sampled community. Selected individuals were euthanized individually in aluminum trays using an overdose of benzocaine (25 mg·L^{-1}) and stored in aluminum foil in a cooler before analysis at the laboratory, which were performed in the same afternoon. In the laboratory, each individual was measured (nearest mm), weighed (nearest 0.01 g), and dissected to extract its entire digestive tract. Crayfish were dissected using the same approach of fish and the entire digestive tract was retrieved

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024−1035

for subsequent analyses. Carapace length was measured with a digital caliper to the nearest mm. All digestive tracts were transferred to glass tubes and stored in a freezer before analyses.

2.3. Sample Treatment. *2.3.1. Water and Sediment.* Water samples were processed following five steps, representing an adaptation of existing protocols,[37,38] namely, (1) sieving and washing, (2) chemical digestion, (3) washing and filtration, (4) wet peroxidation, and (5) washing and final filtration. The samples were first transferred to a sieve (500 μm mesh size) to remove large debris (>1 cm) such as leaves and small tree branches after thoroughly rinsing under running water. The contents of the sieves were transferred to 250 mL glass vials. Screw caps with opening (Schott Duran, DWK LifeSciences, Germany) were equipped with a polyamide fabric with a mesh opening of 500 μm (Nitex, SEFAR, Switzerland) and used to close the bottles. Chemical digestion was performed by incubating each sample with enough potassium hydroxide solution (KOH) (pellets, Sigma-Aldrich, USA) at 10% (w/w) to submerge the sample in a water bath (60 °C) for 8 h under intermittent agitation. The sample was then filtered through the Nitex and rinsed with distilled water. Wet peroxidation was carried out by adding enough solution of hydrogen peroxide (H_2O_2) (Merck KGaA, Germany) at 30% (w/w) to submerge the sample and incubating overnight at room temperature.[39] The samples were finally filtered through a Nitex and washed with distilled water.

Sediment samples were successively filtered through a 5 mm sieve and 500 μm mesh size Nitex. For samples with a high organic matter content, a wet peroxidation step was performed (H_2O_2, 30%) for an overnight period. The final content was filtered through a 500 μm Nitex and transferred to a burette where a density separation step was performed with the addition of zinc chloride solution ($ZnCl_2$) (pellets, Sigma-Aldrich, USA) (d = 1.6 g·cm^{-3}).[40] After a first homogenization, the burette was gently placed in an upright position to settle the denser sediments for 1 h. The denser content was released and saved and the top layer was filtered under the 500 μm Nitex. The burette was rinsed with distilled water. This procedure was repeated three times with the denser fraction. The content retained by the Nitex for the water and sediment samples and the Nitex were then stored in Petri dishes at room temperature for further analyses.

2.3.2. Macroinvertebrates and Fish. Macroinvertebrate (whole specimens except for crayfish) and fish (digestive tracts) samples were digested by wet peroxidation (H_2O_2, 30%) in glass tubes fitted with polytetrafluoroethylene caps. A total of 10 mL of H_2O_2 solution for macroinvertebrates and a volume adapted to the mass of the digestive tract for fish and crayfish was added and samples were incubated in a covered water bath (50 °C) for 48 h. The water bath was turned off overnight and left at room temperature for safety reasons. The samples were filtered through a 500 μm Nitex and then washed with distilled water and absolute ethanol. The contents retained by the Nitex were then stored in Petri dishes at room temperature for further analyses.

2.4. Identification and Characterization of Microplastics. The identification of microplastics was performed using a stereomicroscope (Leica MZ 75 and Nikon SMZ 800). For each sample, two inspections were performed by two independent operators on water, sediment, and fish samples. The order of each operator on a given sample and the order of samples were performed randomly. For macroinvertebrates, a single inspection was performed due to very small amounts of organic matter remaining. All particles ranging from 700 μm (diagonal of the 500 μm mesh) to 5 mm suspected to be microplastics were collected using metal tweezers and stored in small Petri dishes (Figure S2), as previous described.[14] Each item was then photographed using a high-quality optical binocular magnifier (Leica MZ16) equipped with a digital camera (DP20, Olympus, Japan) and classified into predefined color categories: black, blue, green, gray, red, white, and yellow.[12] Each potential microplastic was then individually stored in a styrene multiwell plate. The size of each particle was measured using ImageJ v1.8.0[41] as the length of its longest dimension. The shape of each particle was defined as a fragment (angular and solid or flexible) or fiber (having at least one very small dimension), as adapted from Horton *et al.*[10] (Figure S2). All potential microplastics were then analyzed individually by infrared Fourier spectroscopy with "attenuated total reflectance"[42] (ATR-FTIR, Thermo Nicolet 6700, Thermo Fisher Scientific), equipped with a diamond crystal to determine their chemical composition. The ATR crystal was cleaned with ethanol and the background was performed prior to a batch of analysis (24 particles). The IR spectra were obtained with a resolution of 4 cm^{-1} over the wavenumber range of 400−4000 cm^{-1} by applying eight scans.[43] Each spectrum was compared with the reference spectra of synthetic polymers from commercial libraries using OMNIC software (Thermo Fisher Scientific). The correlation factor of 0.6 was considered as the threshold to assign a recorded spectrum to a database spectrum. If the correlation factor was below this threshold, then the particle was considered as unidentified.[44] Identified particles were classified either as nonplastic or as plastic (polymer or artificial additive[45]). Only plastic particles were used in the subsequent analyses. They were categorized based on the Polymer Properties Database (www.polymerdatabase.com) into six main categories: polyethylene (PE), propylene (PP), polystyrene (PS), polyester, polyacrylate, and artificial additives (considered here as olefin base or alkyd resins, as waxes, oils, and coating lubricants[45−47]).When a polymer type represented less than 2.5% of all polymers, they were grouped in the category "Others", which included tire and bitumen microplastics (TBMP),[48] polyamide, polydiene, polysiloxane, and polyvinylester. The microscopic selection of fibers was based on characteristic visual criteria such as their colors, their textures, their shapes, and their resistance to chemical digestion to keep only the fibers of anthropogenic origins.[49,50] Due to their shape and limited surface area, only one subset of fibers (24%) could have their composition defined by ATR-FTIR. Because the majority of identified fibers were made of synthetic polymers (93% were composed of PE, PP, PS, polyester, artificial additives, polyacrylate, polyamide, and polyvinylchloride (PVC)), all fibers were thus included in the subsequent analyses.

2.5. Stable Isotope Analyses. Samples for stable isotope analyses of macroinvertebrates were collected during regular sampling to replicate the same taxa, microhabitat, number of individuals, and size distribution as invertebrate samples for microplastic analyses. In addition, allochthonous (i.e., tree leaves) and autochthonous (i.e., periphyton and macrophytes when available) were collected (n = 3 per primary producer and per site) and used as stable isotope baselines. In the field, samples were rinsed with distilled water and transported in a cooler to the laboratory where they were oven-dried at 60 °C for 72 h. Periphyton samples were freeze-dried. Crayfish

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024−1035

samples consisted of abdominal muscle collected in the laboratory using pliers and scissors. Fish samples were also collected in the laboratory and consisted of white dorsal muscle collected using a scalpel before individual dissection. The samples were rinsed with distilled water and oven-dried at 60 °C for 72 h. Stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) were performed by the Cornell University Stable Isotope Laboratory (COIL, USA).

2.6. Quality Control and Contamination Assessment. During field sampling, all the equipment was rinsed with river water. In addition, "100% cotton" clothing was used whenever possible to minimize potential contamination. During laboratory analyses, "100% cotton" lab coats and nitrile gloves were used. All procedures were performed under a hood. Metal or glass instruments were used wherever possible and rinsed with ethanol before use. Finally, 81 controls were collected during field sampling by letting tubes open during sampling and placing them next to the experimenters and, during laboratory analyses, by filling tubes with reagents used for the treatment of samples. Solvents were filtered through 8 μm polyethersulfone membranes (Sterlitech, EUA) to avoid contamination. The control samples were subjected to the same protocols as the other samples. Overall, a single fiber was found among the 81 control samples and contamination was therefore considered as null.

2.7. Statistical Analysis. To quantify microplastic contamination in macroinvertebrates and fish, we first calculated microplastic abundance as the number (count) of microplastics ingested per individual. For macroinvertebrates, the number of individuals included in each sample was variable (see details before). However, the maximum number of microplastics measured in each sample was 1 (Table S2), indicating that it could have been ingested only by a single individual. We therefore counted the total microplastics for each sample and then divided it by the number of individuals in the sample to get an average. Due to this methodological difference with fish, macroinvertebrates and fish were analyzed separately. We then used a generalized linear model to test for differences in microplastic abundance between sampled sites for macroinvertebrates and fish. Microplastic pollution in the water was calculated as the number of microplastics divided by the volume of filtered water (microplastic·m^{-3}) and microplastic pollution in the sediment was calculated as the number of microplastics per surface area sampled (microplastic·m^{-2}). Generalized linear mixed-effects models were then used to test the difference of microplastic pollution (log-transformed) in water and sediment between sampled sites using a sample code as a random factor. We then assessed the association between microplastic ingestion by organisms and microplastic pollution in the water and sediment using Spearman correlations.

To compare microplastic characteristics between microplastics from the water and sediment and those ingested by macroinvertebrates and fish, χ^2 tests were used for microplastic shape (fragments and fibers) and color (six categories). χ^2 tests were also used for polymer composition (seven categories), except for the comparisons involving macroinvertebrates where Fisher Exact tests were used due to the limited number of microplastics with known polymer composition in macroinvertebrates. Linear mixed-effects models (lmm) with a sampling site as a random factor were then used to test for differences in microplastic size (log-transformed) between compartments (water, sediment, macroinvertebrates, and fish) and to test the relationship between microplastic size (log-transformed) and body size (log-transformed) of macroinvertebrates and fish.

To determine the trophic niche using stable isotope analyses, we first transformed stable isotope values using resource baseline values to allow between-site comparisons. $\delta^{13}C$ values were transformed following Jackson *et al.*:[2]

$$RO_{sample} = \frac{\delta^{13}C_{sample} - \delta^{13}C_{Allo}}{\delta^{13}C_{Auto} - \delta^{13}C_{Allo}}$$

where RO_{sample} is the resource origin value for a given consumer sample, $\delta^{13}C_{Allo}$ is the average value of allochthonous primary producers in a given site (i.e., leaf litter), and $\delta^{13}C_{Auto}$ is the average value of autochthonous primary producers in a given sampled site (i.e., periphyton and macrophytes, except for site LBI where only periphyton was used). $\delta^{15}N$ values were then used to calculate the trophic position (TP):[51]

$$TP_{sample} = TP_{base} + \frac{\delta^{15}N_{sample} - \delta^{15}N_{base}}{TEF}$$

Using the primary producers as a baseline, TP_{base} is 1 and TEF is 3.4. The average $\delta^{15}N$ value per sampled site of leaf litter was used as a baseline because some $\delta^{15}N$ values of periphyton and macrophytes were unexpectedly high in the most urbanized sites, likely because of anthropogenic nitrogen inputs, explaining also why the estimated trophic position of consumers was elevated. We specifically compared microplastic ingestion between functional groups and tested the relationship between microplastic ingestion, trophic position estimated using $\delta^{15}N$, and the origin of the resources consumed quantified using $\delta^{13}C$. First, we tested the relationship between the body size (log-transformed) of macroinvertebrates and fish and the abundance of ingested microplastics using generalized linear mixed-effects models with a sampled site as a random factor. Generalized linear mixed-effects models with a sampled sites as a random factor were then used to test the effect of feeding modes, trophic position, and resource origin for macroinvertebrates and fish. Generalized linear mixed-effects models were then used to test the relationship between stable isotope metrics (trophic position and resource origin), microplastic color (abundance of the dominant color for macroinvertebrates and fish, respectively), and microplastic shape (abundance of fragments) using individual identity nested in the sampled site as a random factor. Finally, using the same model structure, linear mixed-effects models were used to test the relationship between stable isotope metrics (trophic position and resource origin) and microplastic size (log-transformed). For macroinvertebrates, all individuals from the same sample were assumed to have the same stable isotope values. All statistical analyzed were performed in R (version 1.3.1056)[52] and generalized linear mixed-effects models and linear mixed-effects models were performed using the package lme4 v.1.1.10.[53] Significant levels of mixed effects models were obtained using the "Anova" function in the car package.[54]

3. RESULTS

3.1. Microplastic Contamination in Organisms, River Water, and Sediment. A total of 50 microplastics were collected in macroinvertebrates samples (n = 396 samples composed of 2010 individuals belonging to 36 taxa, Table S2) and 61 microplastics in fish (n = 492 individuals belonging to 21 species, Table S3), representing occurrences of 2 and 10%,

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024−1035

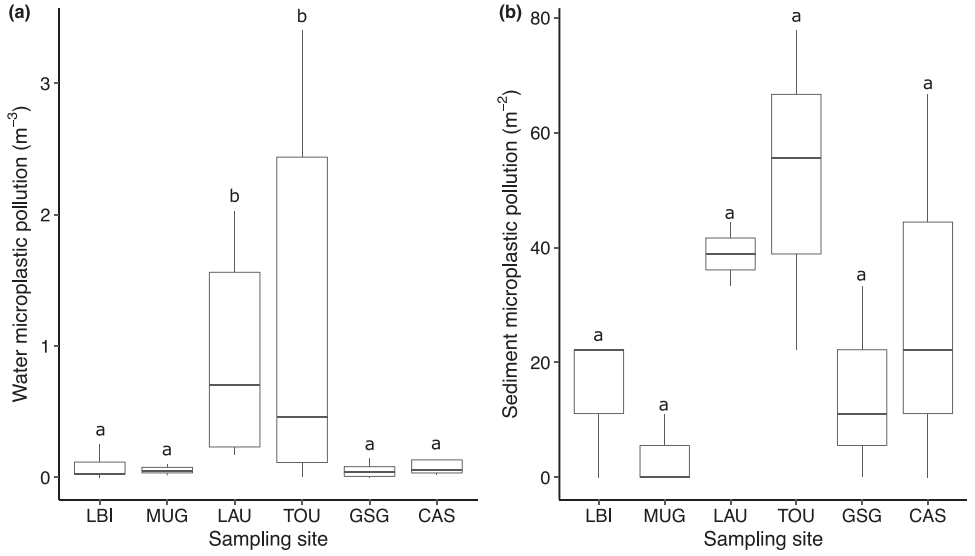


Figure 1. Microplastic pollution in (a) surface waters and (b) sediments in the studied sites. Different letters indicate significant difference (p < 0.05).

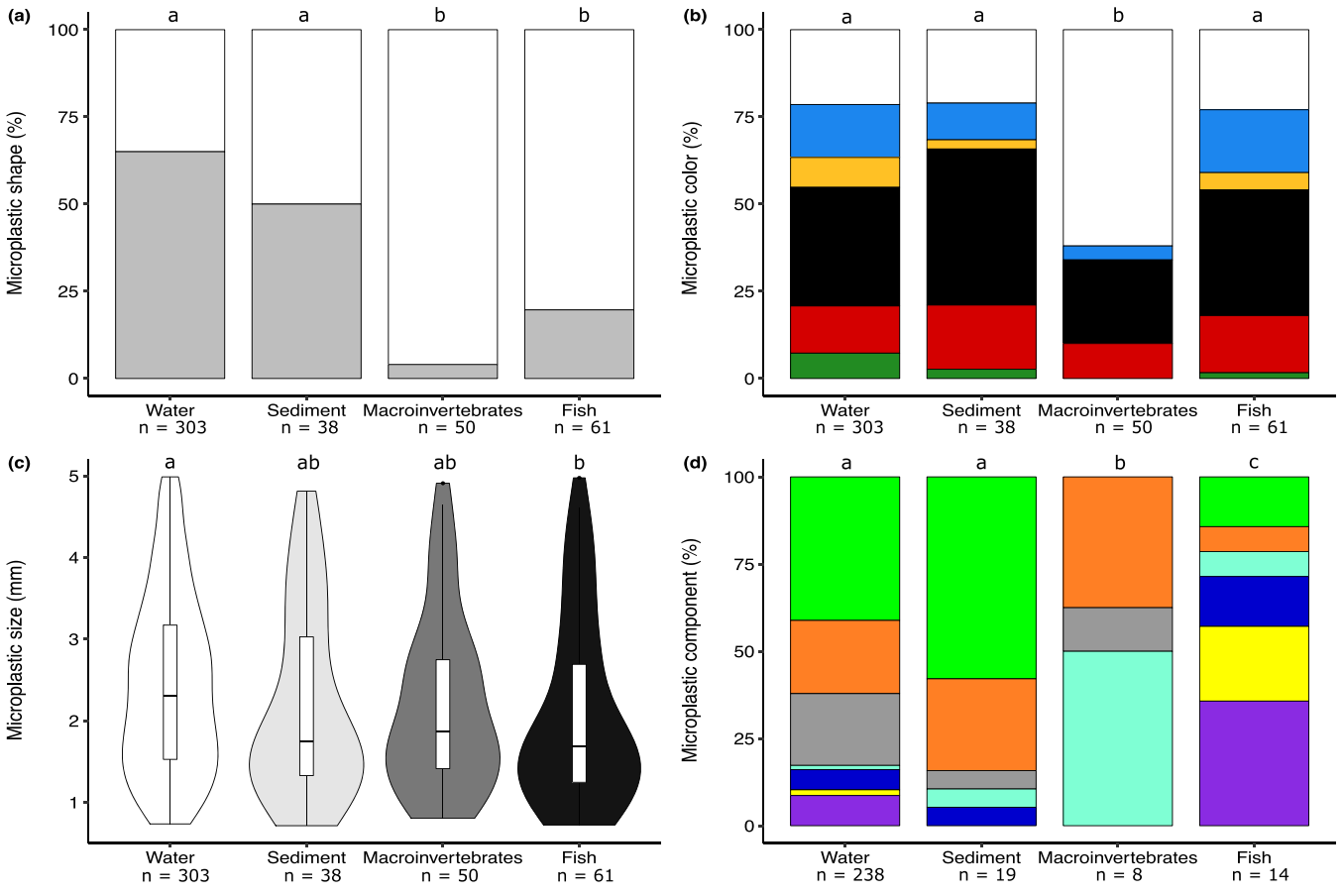


Figure 2. Microplastic characteristics in surface waters, sediments, macroinvertebrates, and fish: (a) shape (fibers in white and fragments in gray), (b) color (white, blue, yellow, black, red, and green), (c) size (mm), and (d) polymer composition (polyethylene in green, polypropylene in orange, polystyrene in gray, artificial additives in cyan, polyacrylate in yellow, polyester in purple, and others in dark blue). Different letters indicate significant difference (p < 0.05).

respectively. Microplastic abundance in macroinvertebrates (mean = 0.02 microplastic·ind^{-1} ± 0.15 SD) was significantly lower than that in fish (mean = 0.13 microplastic·ind^{-1} ± 0.42 SD, glmm: χ^2 = 73.26, p < 0.001). Microplastic abundance in macroinvertebrates and fish did not differ significantly among sampled sites (glm: χ^2 = 7.4467, p = 0.190 and χ^2 = 9.172, p = 0.102, respectively, Figure S3).

Microplastic pollution in the surface water (mean = 0.87 microplastic·m^{-3} ± 1.24 SD) was significantly different among sampled sites (glmm: χ^2 = 77.297, p < 0.001), with a

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Environ. Sci. Technol. 2021, 55, 1024−1035

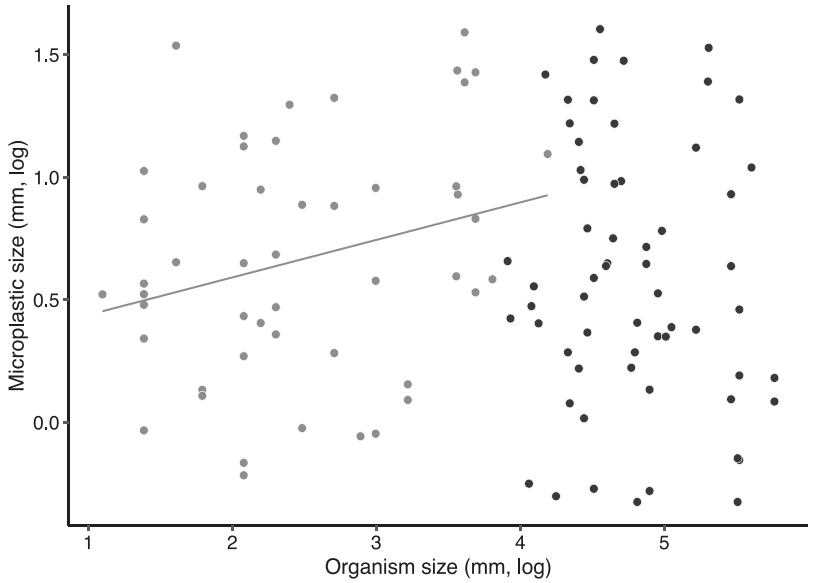

Figure 3. Relationship between organism size (mm) and the size of ingested microplastics (mm). Macroinvertebrates are displayed with gray symbols and fish are displayed with black symbols. The gray line represents the significant relationship between the size of the microplastics ingested by macroinvertebrates and their own size.

significantly higher level of microplastic pollution in the surface water in sites TOU and LAU (Figure 1a). Microplastic pollution in the sediment (mean = 24.84 microplastic·m^{-2} ± 24.38 SD) was not significantly different between sampled sites (glmm: χ^2 = 7.770, p = 0.169, Figure 1b). Microplastic pollution in the sediment was strongly positively correlated with microplastic pollution in the surface water (Spearman correlation, ρ = 0.90, p = 0.015), but there was no significant correlation between environmental microplastic pollution and microplastic contamination of macroinvertebrates and fish, respectively (Spearman correlation, ρ < 0.38, p > 0.462).

3.2. Microplastic Characteristics. Fragments represented 51% of all collected microplastics, while fibers represented 49%. There was no significant difference in the proportion of particles and fibers between surface water and sediments (χ^2 test: χ^2 = 7.359, p = 0.289). Macroinvertebrates and fish ingested a significantly higher proportion of fibers than available in the environment (χ^2 test: χ^2 > 8.653, p < 0.001 and χ^2 > 22.677, p < 0.001, respectively). There was no significant difference in the proportion of particles and fibers between macroinvertebrates and fish (Figure 2a).

Across all collected microplastics, black and white were the most abundance colors, representing 34 and 26%, respectively, followed by red (14%), blue (14%), yellow (7%), and green (5%). The distribution of microplastic color did not differ significantly between the surface water and sediment (χ^2 test: χ^2 = 4.7647, p = 0.445). The distribution of microplastic colors significantly differed between microplastics sampled in the environment (surface water and sediment) and those ingested by macroinvertebrates (χ^2 test: χ^2 > 16.089, p < 0.007) with a higher proportion of white microplastics (post-hoc test: p = 0.002). This difference was not significant for microplastics ingested by fish (χ^2 test: χ^2 < 10.928, p > 0.091). The color of microplastics ingested by macroinvertebrates was significantly different from those ingested by fish (χ^2 test: χ^2 = 20.371, p = 0.001), with a higher proportion of white microplastics (post-hoc test: p < 0.001, Figure 2c).

Microplastic sizes averaged 2.44 mm (±1.09 SD) in the surface water, 2.19 mm (±1.16 SD) in the sediment, 2.19 mm (±1.05 SD) for macroinvertebrates, and 2.07 mm (±1.13 SD) for fish. Microplastic size was significantly different among these compartments (lmm: χ^2 = 10.835, p = 0.013, Figure 2c) with microplastics ingested by fish significantly smaller than microplastics in the water (post-hoc test: p = 0.026). There was a significant relationship between macroinvertebrate size and the size of ingested microplastics (lmm: χ^2 = 5.469, p = 0.019), while this relationship was not significant for fish (lmm: χ^2 = 1.785, p = 0.182, Figure 3).

Across all particles, polyethylene (PE) represented 41% of the total particles, followed by polypropylene (PP, 21%), polystyrene (PS, 18%), polyester (9%), artificial additives (3%), polyacrylate (2%), and other polymers (6%). There was no significant difference in polymer composition between microplastics from the water and from the sediment (χ^2 test: χ^2 = 7.359, p = 0.289, Figure 2d). Polymer composition significantly differed between microplastics found in the environment (water and sediment) and those ingested by organisms (χ^2 test: χ^2 > 39.665, p < 0.001 and p = 0.005, respectively), with a higher proportion of artificial additives for macroinvertebrates (post-hoc test: p < 0.001, Figure 2d) and a higher proportion of polyacrylate and polyester for fish (post-hoc test: p < 0.018, Figure 2d). Polymer composition of microplastics ingested by macroinvertebrates was significantly different from those ingested by fish (p = 0.007), with a higher proportion of artificial additives and polypropylene for macroinvertebrates and a higher proportion of polyester and polyacrylate for fish (Figure 2d).

3.3. Microplastic Contamination and the Trophic Niche of Organisms. Microplastic abundance significantly increased with increasing body size for both macroinvertebrates and fish (glmm: χ^2 > 6.494, p < 0.011). Microplastic abundance did not significantly differ between feeding groups of macroinvertebrates (glmm: χ^2 = 3.151, p = 0.369, Figure S4a), while the difference was significant in fish (glmm: χ^2 = 4.104, p = 0.043, Figure S4b), with bottom feeders displaying a higher microplastic abundance than column feeders. Stable isotope analyses (Figure 4 and Figure S5) revealed a high level of trophic niche variability within species. In macroinverte-

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024−1035

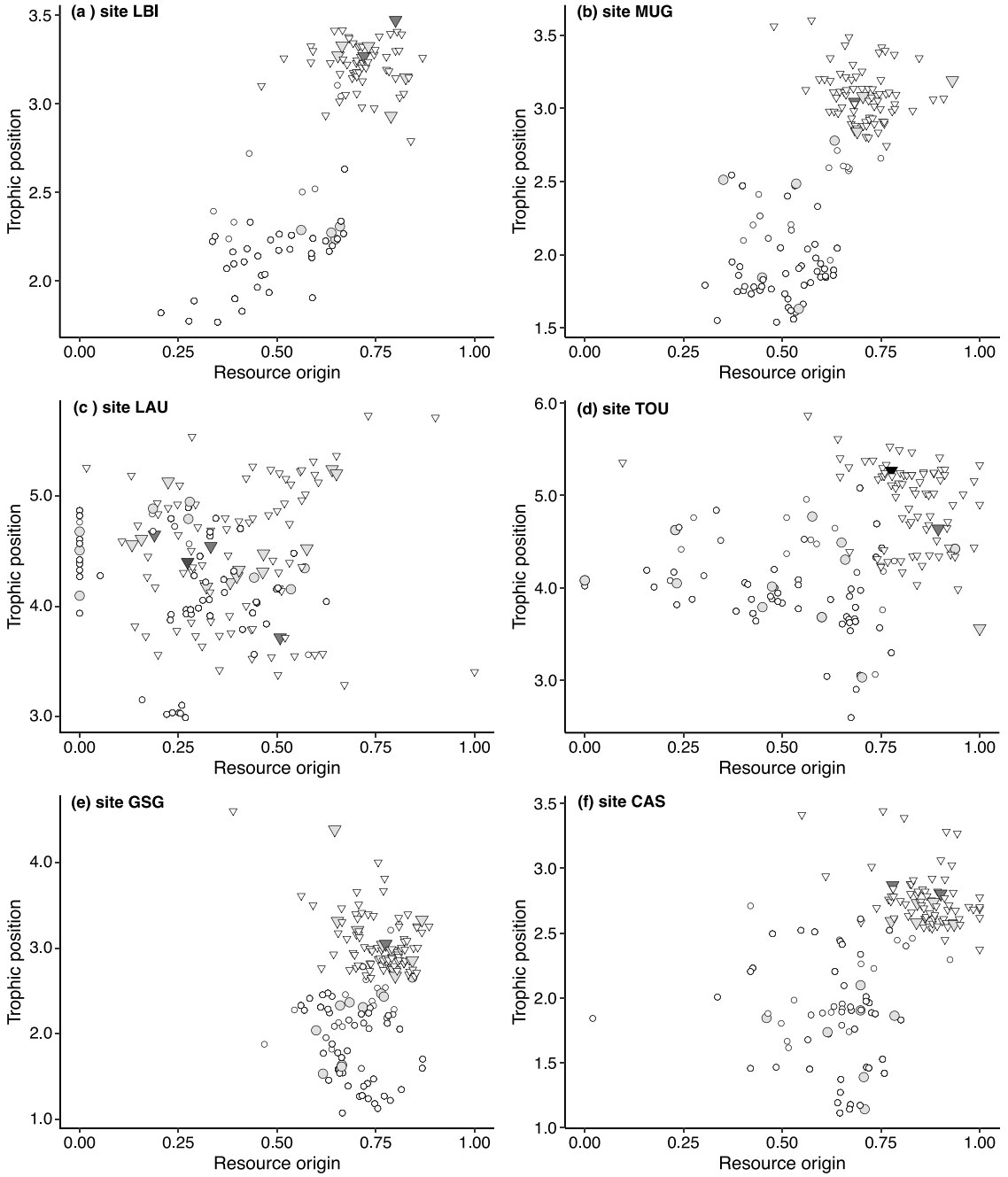

Figure 4. (a–f) Biplot of resource origin and trophic position of each organism measured using stable isotope analyses in each studied site. Macroinvertebrates are displayed with circles and fish are displayed with triangles. Microplastic abundance is displayed using the following colors: white (no microplastic), clear gray (one microplastic), medium gray (two microplastics), dark gray (three microplastics), and black (four microplastics).

brates, there was a nearly significant relationship between microplastic abundance and their trophic position (glmm: χ^2 = 3.029, p = 0.082), and this relationship was not significant with resource origin (glmm: χ^2 = 0.071, p = 0.790, Figure 4). Microplastic abundance in fish was not significantly related to their trophic position (glmm: χ^2 = 0.566, p = 0.452) but decreased significantly when the resource origin increased (glmm: χ^2 = 5.140, p = 0.023, Figure 4); i.e., microplastic abundance was higher in fish consuming resources containing a higher proportion of allochthonous carbon. There was no significant effect of the stable isotope metrics (trophic position and resource origin) on the color and shape of microplastics ingested by macroinvertebrates. Microplastic size was unrelated to the trophic position of macroinvertebrates (glmm: χ^2 = 0.372, p = 0.542); however, microplastic size was significantly higher in macroinvertebrates consuming resources with autochthonous carbon (glmm: χ^2 = 6.644, p = 0.010, Table 1 and Figure 5). There was no significant relationship between stable isotope metrics and microplastic characteristics (color, shape, and size) in fish (Table 1).

4. DISCUSSION

Understanding the pathways and mechanisms leading to the consumption of microplastic by freshwater organisms is a

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024–1035

Table 1. Summary Results of the Linear Mixed-Effects Models Testing the Effects of Trophic Position and Resource Origin Obtained from Stable Isotope Analyses on the Characteristics (Color, Shape, and Size) of Microplastics Ingested by Macroinvertebrates and Fish

response variable	predictor	estimate (SE)	z	p
macroinvertebrates				
color (white)	TP	−0.21 (0.25)	0.845	0.398
	intercept	1.17 (0.89)	1.320	0.187
	RO	−1.12 (1.34)	−0.834	0.404
	intercept	1.10 (0.81)	1.354	0.176
shape	TP	−2.24 (10.57)	0.212	0.832
	intercept	−22.91 (48.43)	−0.473	0.636
	RO	−0.93 (15.78)	−0.059	0.953
	intercept	−13.67 (9.87)	−1.385	0.166
size	TP	−0.05 (0.08)	−0.610	0.542
	intercept	0.79 (0.26)	3.064	NA
	RO	0.72 (0.28)	2.578	0.010
	intercept	−0.21 (0.25)	0.845	0.398
fish				
color (black)	TP	−0.16 (1.48)	−0.105	0.916
	intercept	−9.17 (5.70)	−1.608	0.108
	RO	−29.33 (17.47)	−1.679	0.093
	intercept	12.06 (10.36)	1.164	0.244
shape	TP	0.68 (2.66)	0.257	0.797
	intercept	−15.15 (11.19)	−1.354	0.176
	RO	−1.44 (9.61)	−0.150	0.881
	intercept	−11.70 (6.71)	−1.744	0.081
size	TP	−0.01 (0.12)	−0.120	0.904
	intercept	0.62 (0.44)	1.405	NA
	RO	−0.56 (0.46)	−1.215	0.224
	intercept	0.97 (0.35)	2.796	NA

central research question and the present study reveals that stable isotope analyses can provide novel knowledge. Specifically, we first found that the abundance of microplastics (size range, 700 μm to 5 mm) ingested by macroinvertebrates and fish was not related to the level of microplastic pollution in surface waters and sediments. We then demonstrated that microplastic characteristics (shape, color, size, and composition) observed in the environment differ from those ingested by organisms. For both macroinvertebrates and fish, the abundance of ingested microplastics increased with increasing organism size. Finally, feeding groups and trophic niche measured using stable isotope analyses affected the ingestion of microplastics differentially for macroinvertebrates and fish. In macroinvertebrates, there was no difference between feeding groups and the trophic position tended to be positively associated with the abundance of ingested microplastics, while there was no effect of resource origin. In fish, the ingestion of microplastics was higher in bottom feeders than in column feeders and was significantly associated with resource origin, while there was no significant relationship with trophic position.

Our findings support the hypothesis that MP particles are ingested by organisms during feeding[55] and are not passively obtained because microplastic characteristics strongly differed between the environment and organisms. White microplastics were found in a significantly higher proportion in macroinvertebrates than in the environment, while there was no significant difference in the proportion of colors for fish. Fibers were the main microplastic shape consumed by both macroinvertebrates and fish. Microplastic color and shape are important characteristics responsible for their ingestion by organisms and the existence of such preferences has already been reported in freshwater organisms.[56−58] Although the mechanisms leading to these findings remain to be identified, they could represent a preferential ingestion[20] and/or a higher retention time and accumulation in the digestive system,[59] increasing the likelihood of microplastic detection in organisms. Fibers were already shown to be dominant in subsurface water, highlighting the vertical transport of microplastics through the water column,[60] which could potentially affect its availability to aquatic organisms.

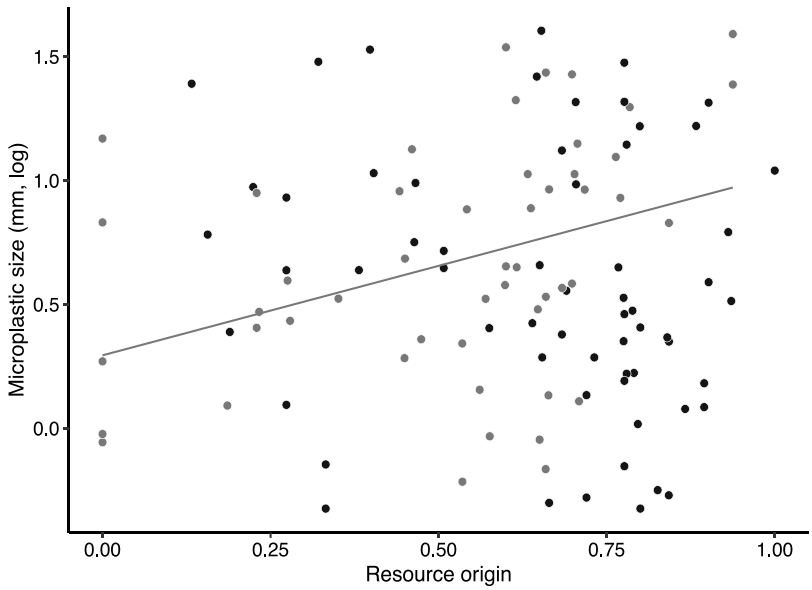

Figure 5. Relationship between the resource origin of organisms and microplastic size (mm). Macroinvertebrates are displayed with gray symbols and fish are displayed with black symbols. The gray line represents the significant relationship between the size of the microplastics ingested by macroinvertebrates and the resource origin.

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024−1035

Microplastics ingested by macroinvertebrates had a similar size than those found in the environment but had a different polymer composition than the abiotic compartment. Specifically, although the number of microplastics with known composition was limited for macroinvertebrates (n = 8), there was a high proportion of polypropylene and artificial additives, which are expected to have a lower density than water.[61] In macroinvertebrates, microplastic contamination tended to increase with trophic position and the size and quantity of ingested microplastics increased with body size. Body length is an important ecological driver of the size of prey and microplastics ingested by aquatic animals.[21] Considering the size of studied macroinvertebrates, further investigations are needed to determine the relationship between the body size and microplastic size for microplastics smaller than our size limit. At the functional feeding group level, there was no significant difference for predators, but predatory taxa such crayfish (*F. limosus* and *P. clarkii*), Odonata (dragonfly and damselfly larvae), and Planariidae had the highest occurrence of microplastics (Table S2), highlighting the importance of measuring the realized trophic position using stable isotopes. Assessing the role of gut structure across species in retention time could help us have a better understanding microplastic contamination.[62] These findings suggest that macroinvertebrates primarily ingest microplastics directly (i.e., primary ingestion) and that the microplastics present higher in the food chain were unlikely the result of a trophic transfer. Because there was no relationship between microplastic ingestion, resource origin (quantified using $\delta^{13}C$), and feeding modes, a deliberate ingestion by organisms was unlikely to be the main pathway of contamination. We hypothesize that microplastic ingestion was mainly accidental and was modulated by microplastic characteristics that influence their availability, such as shape, size, or density.

Microplastics ingested by fish were smaller than those in the water surface and fish contained a higher proportion of polyacrylate and polyester, two polymers types that have an overall higher density than water[61] and may likely be found in the water column and sediments. Although the proportion of adults and large-bodied piscivorous fish in the sampled communities was limited (e.g., *Esox lucius* and *Silurus glanis*, Table S3), we found no relationship between individual trophic position and abundance of ingested microplastics. Contrary to observations reported elsewhere,[6,63] predatory fish were not more contaminated (at least in terms of abundance) than other trophic levels,[64−66] suggesting that bioaccumulation and biomagnification were overall unlikely to occur in the studied food webs. Direct consumption by fish was more likely, as several studies have already shown.[25,67,68]

Interestingly, $\delta^{13}C$ analyses reported that the resource origin affected microplastic ingestion that was higher in individuals consuming a higher proportion of allochthonous carbon. This can occur directly through the consumption of allochthonous inputs such as falling terrestrial insects or indirectly through the consumption of invertebrates at the base of the detritus food chain such as shredders.[69,70] Because bottom feeders (e.g., *Gobio occitaniae* and *Barbus barbus*) ingested a higher quantity of microplastics than column feeders (e.g., *Squalius cephalus* and *Alburnus alburnus*) (Table S3), accidental ingestion of small microplastics in the sediment when consuming prey on the basis of the detritus food chain (e.g., Gammaridae and Asellidae, Table S2) most likely occurred. Interestingly, the consumption of polymers with an overall higher density (as polyethylene terephthalate (PET), included in the polyester category, and polyacrylates) and the occurrence of sand and small gravels (0.5−3 mm) in the stomach contents of bottom feeders observed here during stomach digestion reinforce the hypothesis of accidental consumption. The quantification of microplastic ingestion through gut contents is likely, as observed in trophic ecology studies, to provide only a snapshot of microplastic contamination that does not include temporal variability, while stable isotope analyses could reveal longer-term trophic patterns. Measurements accounting for the temporal dynamic of microplastic ingestion are needed to improve our knowledge of its mechanisms and pathways into and within freshwater food webs.

The relationship between environmental pollution and microplastic contamination in freshwater organisms is highly context-dependent. Here, we found that, while microplastic pollution differed between sites, microplastic ingestion was not correlated to environmental microplastic pollution. While the relatively low number of studied sites might limit the statistical power, a higher microplastic concentration in water does not necessarily induce a higher ingestion of microplastics.[6,71] This might be caused, for instance, by three mutually nonexclusive mechanisms. First is the spatial changes in microplastic characteristics across sites[38,72] that could modulate their ingestion by organisms. Second is the variability in environmental conditions across sites. Several abiotic parameters such as water turbidity, substrate characteristics, and temperature are known to modulate the ability of freshwater organisms to detect and/or handle their prey, and they are likely to affect the ingestion of microplastics by organisms. Biotic conditions such as population density, predation, and intraspecific conditions, by modulating individual trophic niche,[73] are also likely to affect microplastic ingestion. Third is the structure of macroinvertebrate and fish communities, which vary across sites. Because individual and species traits influence microplastic ingestion,[17,74,75] changes in community structure can strongly modulate the overall ingestion of microplastics at the food web level. Experimental approaches that manipulate microplastic characteristics (e.g., composition, color, and shape), environmental conditions (e.g., turbidity, substrate, and temperature), and community composition are therefore needed to fully assess the relationship between microplastic pollution in the environment and the contamination of freshwater organisms.

The levels of microplastic occurrence in macroinvertebrates and fish observed in the present study, i.e., 2 and 10%, respectively, fell within the range of the values observed in European streams.[6,76] When only contaminated individuals were considered, the number of microplastics was always 1 for macroinvertebrates and ranged between 1 and 4 for fish, as observed elsewhere.[6] The level of microplastic pollution in the surface waters of the Garonne river was similar to the level observed in other French rivers such as the Seine river (0.28−0.47 microplastic·m^{-3}).[49] The two most urbanized sites (LAU and TOU) had the highest level of microplastic pollution in surface waters and sediments and also the highest microplastic loads in macroinvertebrates and fish (Tables S2 and S3), confirming that urbanization is a crucial driver of microplastic pollution[77] and biotic contamination. Urbanization can have profound and multiple effects on freshwater organisms and ecosystems[69,78,79] and is a ubiquitous driver of microplastic contamination.[14,77] It is therefore crucial to decipher the

https://dx.doi.org/10.1021/acs.est.0c06221
Environ. Sci. Technol. 2021, 55, 1024−1035

relative importance of microplastic ingestion compared to other environmental stressors on freshwater organisms and to determine whether they act synergistically, additively, or antagonistically.[2]

In conclusion, this study highlights the importance of quantifying the realized trophic niche when assessing microplastic ingestion by wild organisms and the fact that intraspecific variability in microplastic ingestion within species could be high. Determining how the ecological traits of individuals (e.g., behavior, metabolism, morphology, and trophic specialization) are driving intraspecific variability in microplastic ingestion represents an important and challenging area of research. Large microplastics, as those studied here (700 μm to 5 mm), represent only a small fraction of the microplastics ingested by freshwater fish[65] and stable isotope analyses appear as a robust and insightful method to quantify the distribution and pathways of smaller microplastics in freshwater food webs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c06221.

Map of the studied area and localization of the six sampled sites (Figure S1); examples of microplastics ingested by organisms and collected in the environment (Figure S2); microplastic abundance in macroinvertebrates and fish in the six studied sites (Figure S3); microplastic abundance in the different feeding groups of macroinvertebrates and fish (Figure S4); stable isotope values (δ^{13}C and δ^{15}N) of the organisms collected in the sites (Figure S5); environmental characteristics of the six sampled sites (Table S1); number of individuals analyzed per macroinvertebrate taxa and per site in the study (Table S2); number of individuals analyzed per fish taxa and per site in the study (Table S3) (PDF)

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[§]F.G. and A.R.d.C. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to our colleagues for their precious help during field work and sample analyses. Ben Parker and three anonymous reviewers. We thank Ben Parker for comments on a previous version of this manuscript. Electrofishing and sample collection were performed with the following authorizations: Arrêtés Préfectoraux from the Prefecture de Haute-Garonne (20190506) and from the Prefecture de Tarn et Garonne (2019-1049). This study was funded by the Agence de l'Eau Adour-Garonne (PLASTIGAR project) and by the Region Midi-Pyrenees.

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