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Spatio-temporal monitoring of MP pollution in the Garonne and potential transfer in the food web

Aline Reis de Carvalho

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THÈSE

**En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE**

Délivré par l'Université Toulouse 3 - Paul Sabatier

**Présentée et soutenue par
Aline REIS DE CARVALHO**

Le 27 octobre 2021

**Suivi spatio-temporel de la pollution de microplastique dans la
Garonne et potentiel transfert dans le réseau trophique**

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Photochimique**

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Spatio-temporal monitoring of microplastic
pollution in the Garonne and potential transfer in
the food web

Unveiling drivers of spatio-temporal variability and of their ingestion
by aquatic organisms

Was she told when she was young
That pain would lead to pleasure?
Did she understand it when they
said
That a man must break his back
To earn his day of leisure?
Will she still believe it when he's
dead?

Girl
The Beatles

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Pai, mãe, florzinha. If I have seen further is by standing on the shoulders of giants. Caio. It would not happen without you. Meu eterno companheiro, amigo e amor.

Abstract

The omnipresence of microplastic (MP) represents a novel environmental pressure acting on freshwater ecosystems and a better understanding of the dynamic of this pollution is needed. Here, we investigated the spatial and temporal changes in MP pollution (size range 700 μm – 5 mm) in the Garonne catchment (Southwestern France) and the consumption of these particles by aquatic organisms. The composition of MP was verified through attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-FTIR). First, a total of 14 sites located in the main river and several tributaries were sampled during four seasons. We found that MP concentration averaged 0.15 particles.m⁻³ (\pm 0.46 SD) and strongly varied both in space and in time, driven by urbanization and hydrological conditions. Higher MP concentrations and smaller particle sizes were observed in warm seasons with low discharge. Second, we analysed the changes in MP pollution caused by flooding. Two sites in the Garonne River, located upstream and downstream of Toulouse, were sampled during two flood episodes. We found a general increase in MP concentration during flood episodes driven by river discharge. However, this increase was greater in the downstream site. Regarding MP characteristics, a predominance of larger particles was observed. Using multivariate analysis of the infrared spectra, we quantified the changes in MP chemical profile during flooding. A higher oxidation profile, represented by an increased carbonyl spectral band, was found in particles collected during the flood. Third, a novel pathway of MP into freshwaters was assessed by quantifying MP contamination in angling baits. We analysed three different categories of industrially-produced baits ('groundbait', 'boilies' and 'pellets'). From 160 bait samples, 28 MP were identified in groundbait and boilies. No MP within the studied size range were found in pellets. We revealed that MP introduced accidentally during bait manufacturing and/or those originating from contaminated raw ingredients might be transferred into freshwaters. Fourth, the consumption of MP by freshwater macroinvertebrates and fish was quantified. This consumption was linked to individual trophic niches, which were measured by stable isotope analyses ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). We demonstrated that the abundance of ingested

MP differed between macroinvertebrates and fish and was not significantly related to MP pollution. We also found that MP characteristics differed between the abiotic (water and sediment) and biotic (macroinvertebrates and fish) compartments. The abundance of ingested MP increased with organism size in both fish and macroinvertebrates and tended to increase with the trophic position of macroinvertebrates. The origin of the resources consumed by fish significantly affected the abundance of MP ingested in fish. Altogether, these results suggested the absence of MP bioaccumulation in the studied size range in freshwater food webs and the dominance of direct consumption, most likely accidentally. In conclusion, we highlighted that MP pollution should be perceived as a multi-stressor due to the particulate behaviour and potential interactions with other environmental contaminants. The consequences of these interactions should be the focus of future research. This work contributes to improve our understanding to elucidate the drivers of the dynamic and consumption of MP, and further studies are needed to quantify the actual risks associated with this pollution in freshwater ecosystems.

Keywords: plastic, polymer, infrared, trophic niche, Garonne river

L'omniprésence des microplastiques (MP) représente une nouvelle pression environnementale agissant sur les écosystèmes d'eau douce et une meilleure compréhension de la dynamique de cette pollution est nécessaire. Ici, nous avons étudié les changements spatiaux et temporels de la pollution en MP (gamme de taille entre 700 μm et 5 mm) dans le bassin versant de la Garonne (Sud-Ouest de la France) et la consommation de ces particules par les organismes aquatiques. La composition des particules a été vérifiée par spectroscopie infrarouge à transformée de Fourier à réflectance totale atténuée (ATR-FTIR). Premièrement, un total de 14 sites situés dans le fleuve principal et plusieurs affluents a été échantillonné pendant quatre saisons. Nous avons constaté que la concentration de MP était, en moyenne, de 0.15 particules.m⁻³ (\pm 0.46 SD) et variait fortement à la fois dans l'espace et dans le temps en fonction de l'urbanisation et des conditions hydrologiques, respectivement. Des concentrations plus élevées de MP avec des tailles de particules plus petites ont été observées pendant les périodes chaudes avec de faibles débits. En deuxième, nous avons analysé les changements dans la pollution des MP causés par les crues. Deux sites dans la Garonne, situés en amont et en aval de Toulouse, ont été échantillonnés lors de deux épisodes de crue. Nous avons constaté une augmentation générale de la concentration de MP pendant la crue. Elle était induite par le débit de la rivière,

mais cette augmentation dans la concentration de MP était plus importante dans le site en aval. En ce qui concerne les caractéristiques des MP, la prédominance de particules plus grosses a été remarquée. À l'aide d'une analyse multivariée des spectres infrarouges, nous avons quantifié les changements du profil chimique des MP lors de la crue. Un profil d'oxydation plus élevé, représenté par une bande spectrale de carbonyle accrue, a été trouvé dans les particules recueillies pendant la crue. En troisième, une nouvelle voie d'entrée MP dans les eaux douces a été évaluée en quantifiant la contamination en MP dans les amorces de pêche. Nous avons analysé trois types d'amorces fabriquées industriellement ("pellets", "bouillettes" et "farines"). A partir de 160 échantillons d'amorces, 28 MP ont été identifiés dans les farines et les bouillettes, tandis qu'aucun MP dans la gamme de taille étudiée n'a été trouvé dans les pellets. Nous avons révélé que les MP introduits accidentellement lors de la fabrication des amorces et/ou ceux provenant de matières premières contaminées pouvaient être transférés dans les eaux douces. En quatrième, la consommation de MP par les macroinvertébrés et les poissons a été quantifiée. Cette consommation a été liée aux niches trophiques individuelles, quantifiées à l'aide d'analyses d'isotopes stables ($\delta^{13}\text{C}$ et $\delta^{15}\text{N}$). Nous avons d'abord démontré que l'abondance de MP ingérée différait entre les macroinvertébrés et les poissons et n'était pas significativement liée à la pollution en MP. Nous avons également constaté que les caractéristiques des MP différaient entre les compartiments abiotique (eau et sédiments) et biotique (macroinvertébrés et poissons). L'abondance de MP ingérée augmentait avec la taille de l'organisme chez les poissons et les macroinvertébrés et avait tendance à augmenter avec la position trophique des macroinvertébrés. L'origine des ressources consommées par les poissons affectait significativement l'abondance des MP ingérés dans les poissons. Dans l'ensemble, ces résultats suggèrent l'absence de bioaccumulation de MP dans la gamme de tailles étudiée dans les réseaux trophiques d'eau douce et la prédominance de la consommation directe, très probablement accidentellement. En conclusion, nous soulignons que la pollution par les MP devrait être perçue comme une forme de stress multiple en raison du comportement particulier et des interactions potentielles avec d'autres contaminants environnementaux. Les conséquences de ces interactions devraient être au centre des recherches futures. Ce travail contribue à améliorer notre compréhension et à élucider les moteurs de la dynamique et de la consommation de MP par les organismes aquatiques. Des études complémentaires sont désormais nécessaires pour quantifier les risques induits par cette pollution dans les écosystèmes d'eau douce.

Mots clés: plastique, polymère, infrarouge, niche trophique, Garonne

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1 | General introduction

1.1 Freshwater ecosystems and human-induced perturbations

Freshwater ecosystems have a pivotal role in human life: 50% of the world population lives within less than 3 km from a freshwater ecosystem and only 10% further than 10 km (Kummu et al., 2011). The importance of freshwater to Humans encompasses water supply for domestic use, irrigation, livestock, and electric power generation (Suring, 2020). In a broad perspective, freshwaters provide what is called ecosystem services, thus, benefits for human and their society obtained from nature. From social process and flood protection to cultural services, as recreational fishing, some of these services are difficult to measure objectively (Apostolaki et al., 2020; Chan et al., 2012; Villamagna et al., 2014). Moreover, freshwater environments support high species diversity and are more functionally diverse than any other ecosystem. Although they comprise only 2.5% of the water on the planet, from which 0.01% are found in lakes, wetlands, and rivers, they contain more than 10% of described species on Earth, a true oasis of life (Apostolaki et al., 2020; Suring, 2020). Paradoxically, freshwater ecosystems are amongst the most threatened ecosystems on Earth, facing novel and exceptionally large anthropogenic pressures such as climate change, land use, cover alteration, biological invasions and pollution from pesticides, heavy metals and plastics (Blettler et al., 2018; Carpenter et al., 2011). These ecosystems stressors hardly occur alone and measuring their temporal and spatial variability and potential interactive effect are an important challenge of current environmental sciences (Perujo et al., 2021).

In order to determine the risks of environmental pressures and provide robust information for decision-making, designing environment and ecological risk assessments is essential. They are conceived in four steps: (1) hazard identification, (2) dose-response assessment, (3) exposure assessment and, finally, (4) risk characterization (Breitholtz et al., 2006; Commission, 2003). However, risk assessment studies have important limits from an ecological point of view because they typically focuses on individual responses of a small set of species, hampering the extrapolation to higher level of organisations, such as biological communities and ecosystems (Galic et al., 2019). From a chemical point of view, they often fails to consider the diversity of contaminants, their co-occurrence and interaction, and are therefore commonly refuted under the argument of distanced from real environmental conditions (Boberg et al., 2019; Kortenkamp, 2020). Although it is difficulty or even impossible to test all possible iterations, selecting key components and likely concomitant conditions

are essential (Noventa et al., 2021). For instance, location and use patterns drive the variability of some pollutants. Industrialization is a key driver for the increased heavy metals levels in the nearby freshwater, whereas agricultural activities are responsible for increased pesticides levels (Alshemmari et al., 2021; Qiao et al., 2020). In addition, a period of intense anthropogenic activity and / or the occurrence of natural events, such as a flood, can lead to an accumulation phenomena, exceeding perturbation levels (Gong et al., 2021; Huang et al., 2021; Irabien et al., 2019). Determining the fate of contaminants and their bioavailability to organisms are crucial to then assessing their effects on freshwater ecosystems, and this is particularly needed for plastic pollution.

1.2 Plastic production and environmental consequences

The contemporary history of plastic began in the 1950's when the production and daily use of plastics started to grow. The Second World War boosted the material engineering as a whole, motivated by the search for substitutes for materials that were in short supply, such as natural rubber (Crawford and Quinn, 2017; Gilbert, 2017). By 1976, plastics were already deemed as an important material in the world (Crawford and Quinn, 2017). Their unique advantages, such as lower densities and easy processing, reduced the production and transportation costs, as well as energy consumption (Andrady and Neal, 2009). For instance, without plastics, it is estimated that today's cars would be around 200-300 kg heavier, what saves around 0.5 liters of fuel per 100 km (Gilbert, 2017). To date, no material is able to replace plastic, whose demand has constantly grown in the world and Europe (GrandViewResearch, 2021; PlasticsEurope, 2019).

Plastics are the result of an industrial process that combines synthetic polymers with several formulation adjuvants, such as plasticizers (to improve flexibility), flame-retardants, antioxidants, also known as plastic additives (Beiras et al., 2021; Sastri, 2014) (Fig. 1.1). These additives are often not chemically bonded to the polymeric structure. The raw material for synthetic polymer, i.e. the monomers, are mainly obtained from fossil fuels through a cracking procedure, i.e. the breaking down of higher molecular weight petroleum fractions into lower molecular weight products (Fig. 1.1). The plastic industry is heavily integrated within the oil industry and the environmental impacts of their production are no negligible. The carbon impacts of

plastic production might soon reach 15% of the global annual carbon budget, and its growing production is expected to double by 2050 (Gilbert, 2017; MacArthur et al., 2016).

Commodity plastics are the “high volume consumption, relatively low-cost” group of materials, and includes the plastic for packaging and for building (Andrady and Neal, 2009). Indeed, packaging industry uses a large proportion of plastics, most often for a disposable use (PlasticsEurope, 2019). Among the polymers types composing the main plastics are polyethylene (PE) (low density, 17.4% and high density, 12.4%) (Fig. 1.1), polypropylene (PP) (19.4%) (Fig. 1.1), polyvinyl chloride (PVC) (10%), polyurethane (PU) (7.9%), polyethylene terephthalate (7.9%) and polystyrene (PS) (6.2%) (Fig. 1.1), which together represent approximately 81.2% of the total European demand (PlasticsEurope, 2019). Although plastic material was conceived to last, they are more degraded by environmental factors, e.g. temperature, solar radiation, oxygen pressure, than metal and ceramic materials (Xiong et al., 2017). Advances in polymeric formulation aim to overcome this vulnerability and improve its mechanical properties, where its use ranges from technological advances to cultural services (Kean, 2021; Thiam and Fall, 2021).

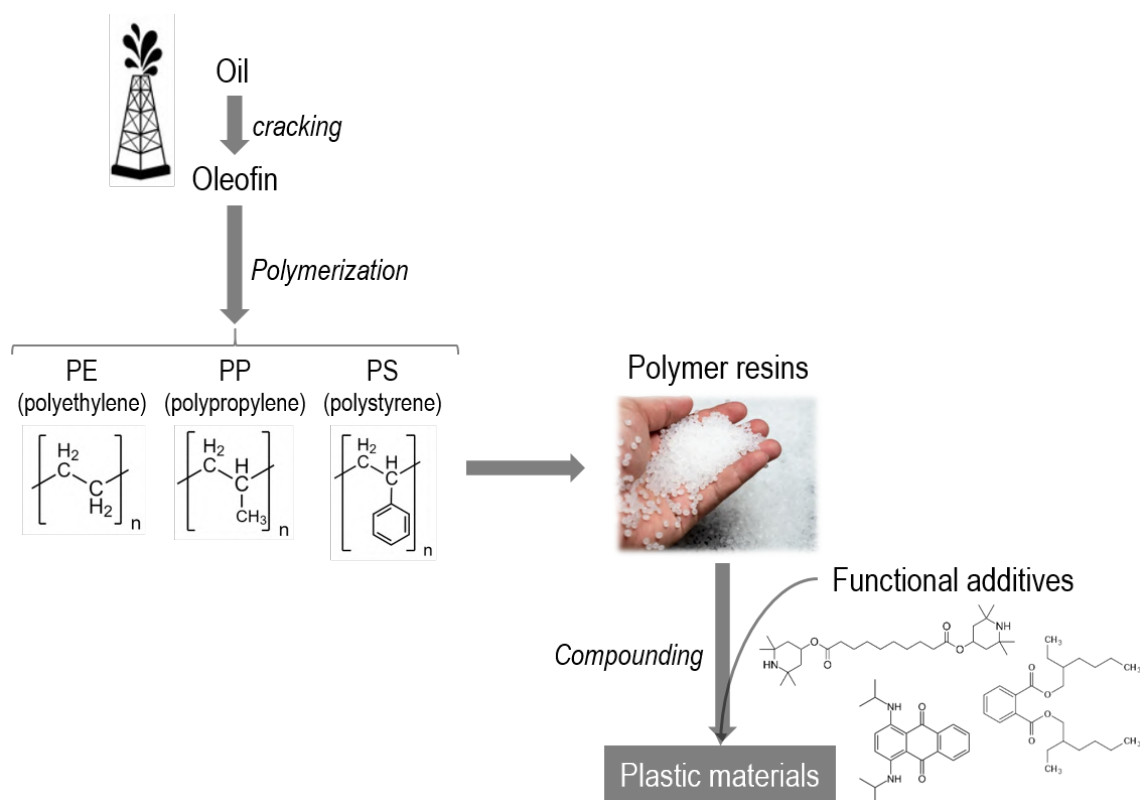


Figure 1.1: Manufacturing of plastic materials combines polymers resins obtained through oil cracking with functional additives. Adapted from Beiras et al. (2021).

Despite the advances that plastic products have brought that revolutionized modern life, e.g. food packaging, construction and medical devices (Agarwal and Gupta, 2017; Sastri, 2010), just like any other synthetic product, the end of its life cycle became a societal concern. In Europe, 32.5 % of the plastic waste collected (29.1 million tonnes out of 51.2 that were demanded in 2018) are recycled, 42.6 % are burned (energy recovery) and 24.9 % are discarded (landfill) (PlasticsEurope, 2020). This proportion is, however, highly variable, with plastic that end up in the landfill reaching values of 0.6 % in countries such as Germany and up to 90% in developing countries (PlasticsEurope, 2020; Thiam and Fall, 2021).

The mismanaged plastics enter aquatic ecosystems directly in surface run-off or through stormwater and wastewater treatment plants (Dris, H. Imhof, et al., 2015; Skalska et al., 2020). Some studies showed that 10% of all discarded plastic has been actually released into the oceans and that, by the end of 2050, the mass of plastics in the oceans will equal fish biomass (Barnes, 2002; MacArthur et al., 2016; Thompson et al., 2009). Undoubtedly, the accumulation of plastic debris in the environment is an emerging global concern corroborated by the finding that the global mass of

produced plastic is now greater than the overall mass of all terrestrial and marine animals combined (Elhacham et al., 2020; Jambeck et al., 2015).

As famously quoted by Antoine Lavoisier, “*rien ne se perd, rien ne se crée, tout se transforme*”, plastic in the environment does not simply disappear. In fact, they last enough to accumulate, interact with their surroundings and slowly degrade. Plastic degradation, i.e. loss of its original properties, occurs through abiotic and biotic processes. The latter normally comes first, essentially driven by ultraviolet (UV) radiation (photo-initiated), presence of oxygen and mechanical abrasion (Gewert et al., 2015; Song et al., 2017). As a consequence, chemical alterations (e.g. oxidation and polymeric chain scission (cross linking) and physical changes (e.g. colour and crazing on surface) lead to plastic embrittlement, facilitating their fragmentation into smaller pieces (Gewert et al., 2015; Halle et al., 2017; M. Simon et al., 2021) (Fig. 1.2). Although visible environmental consequences of plastic pollution are ubiquitous and well-documented, such as polluted beaches, entanglement and ingestion of plastic debris by animal species (Barnes, 2002; Gregory, 2009; Laist, 1997; Miller, 2008), it was only recently that the threat from smaller fraction, micro-sized, plastic was acknowledged (Thompson, 2004)

1.3 Microplastic and the environment: the smaller the size the higher the risk?

Microplastic, as defined by European Chemicals Agency, are pieces of plastic smaller than 5 mm in size, solid, particulate, insoluble (ECHA, 2020; Thompson, 2004). They may be directly produced and are called as primary microplastics (e.g. resin pellets, personal care products, glitter) representing about 15-31 % of plastic pollution if fibres from synthetic clothing are considered (Cole et al., 2011; Sun et al., 2020; Yu et al., 2021). Additionally, the fragmentation process of larger materials originates secondary microplastics, the main source of microplastic in the environment (Andrady, 2011; Browne et al., 2007; Yang et al., 2021) (Fig. 1.2.Ia). Following this step, the fragmentation of a plastic in the environment or as consequence of wearing during a product service's life may reach the nanometric scale (Fig. 1.2.Ib), therefore denominated nanoplastics, reaching the size of a virus (Fig. 1.2.II) (Gigault et al., 2018). Ultimately, there is the formation of chemical fragments, such as low molecular weight alkanes, alkenes, ketones, aldehydes, carboxylic acids, also called by-products (Fig. 1.2.Ic and II). Amongst these compounds, hydrophilic ones are readily biodegradable

and well dissolved (Fig. 1.2.II) (Bond et al., 2018). Degradation rates of plastics in the environment are highly uncertain and dependent of plastic composition, like polymer type, presence of antioxidants and stabilizers (MacLeod et al., 2021). Environmental conditions significantly influence the degradation of particles. For instance, higher light penetration, consequently higher levels of UV, higher temperatures and mechanical stress can increase the weathering of plastic particles (Free et al., 2014; Law and Thompson, 2014).

The accumulation of small pieces of plastics in aquatic environments was firstly reported in marine ecosystems in the 1970s, but it is only 30 years later that the term microplastic was first used (Thompson, 2004). Then, studies about the impact of microplastics in marine ecosystems have grown exponentially. It was only 8 years ago, with the recognition that microplastics entered the environment upstream of oceans (Faure et al., 2012; Moore et al., 2011), that an active discussion about the prevalence of microplastic in freshwater ecosystems occurred (Eerkes-Medrano and Thompson, 2018). Estimations have revealed that 70 to 80% of plastic on the oceans was, in fact, transported by freshwater ecosystems (Dris, H. Imhof, et al., 2015; Horton, Walton, et al., 2017). Still, considering data until 2018, only 4% of studies on microplastics were related freshwater ecosystems (Lambert and Wagner, 2018). There is an urgency in understanding the prevalence and variability of microplastic pollution in freshwaters ecosystems, a temporary sink and cradle of degradation.

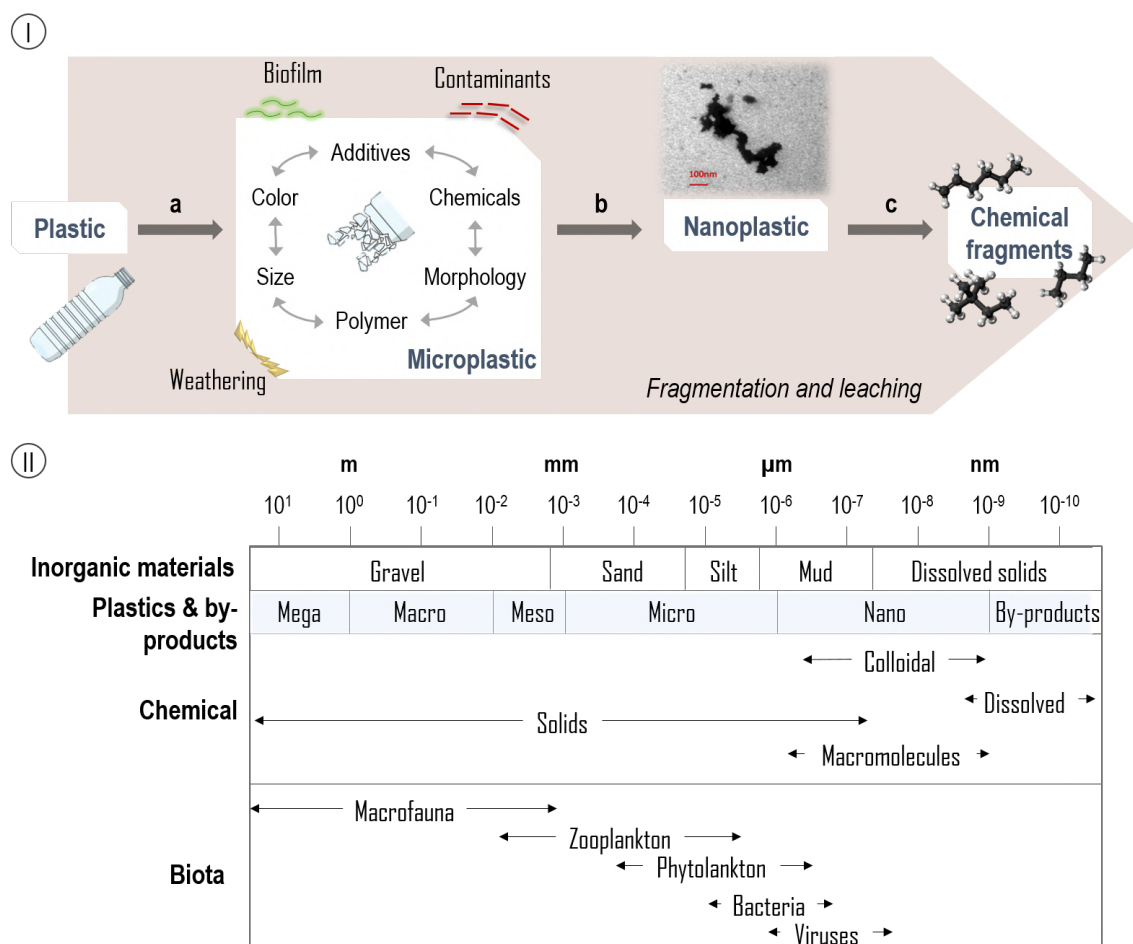


Figure 1.2: Plastic fragmentation process (I) that originates (a) microplastics, (b) nanoplastics and (c) chemical fragments. Microplastic can yet be directly produced. Microplastic features are described as well as the environmental process of weathering, sorption of contaminants and biofilm formation. Nanoplastic image obtained from Gigault et al. (2018). Size description (II) of inorganic and plastic materials, chemical and biota for comparison. Adapted from Stubbins et al. (2021).

By definition, risk means “the possibility of something bad happening at some time in the future” (Dictionary, 2021). Risk is also commonly perceived by toxicologists as the result of exposure *versus* hazard (Backhaus and Wagner, 2020). The smaller size of plastics presents greater environmental and biological barrier permeability, notably increasing the risks of organisms exposure. Microplastics are known to reach from remote lands (Allen et al., 2019; Napper et al., 2020; Woodall et al., 2014) to internal tissues of organisms (Amato-Lourenço et al., 2021; Ragusa et al., 2021; Roch et al., 2019). The increased area-to-volume ratio of microplastics and their likely altered polymeric structure facilitate inter-molecular interactions with their surrounding environment, resulting in a greater sorption capacity for contaminants. Considering also

the hydrophobic nature of microplastics, accumulation of organic contaminants, such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), perfluoro alkyl substances (PFAs), polybrominated diethers (PBCDs), pharmaceuticals, and heavy metals is expected (Atugoda et al., 2021; Hartmann et al., 2019; Naik, 2019) (Fig. 1.2.I - **Microplastic**). In fact, contaminants may sorb more readily to plastics than to some natural sediments and levels of organic contaminants in microplastics might greatly exceed environmental levels (Mato et al., 2001; Teuten et al., 2007). Residues of plastic manufacturing process and formulation additives, as plasticizers (phthalates, bisphenols), colorants, flame retardants, lubricants, can slowly migrate within the plastic to the surface and leach from plastic debris into water (Hahladakis et al., 2018; Horton, Walton, et al., 2017). It is anticipated that these chemicals, most of them recognized as carcinogenic and endocrine disruptor, might accumulate in organisms bodies (Yang et al., 2021). Moreover, while in environment, an “eco-corona” of organic matter and microorganisms are rapidly formed around plastic particles within days (Harrison et al., 2014; MacLeod et al., 2021; Rummel et al., 2017). This complex biofilm, denominated “plastisphere”, have important implications, such as the development of specific microbial communities and the potential surface transport of pathogens and antibiotic resistant’s genes (Bond et al., 2018; Hossain et al., 2019; Kirstein, 2016; Zettler et al., 2013) (Fig. 1.2.I - **Microplastic**). Altogether, these properties lead to the idea of a “Trojan Horse effect” of this “chemicals cocktail” that is a microplastic particle (Bucci et al., 2021; Trevisan et al., 2020; Vethaak and Legler, 2021). Therefore, microplastics are perceived as a vector of residuals monomers (remnants of the manufacturing process), plastic additives, adsorbed contaminants and also pathogens and specified microbiota, representing physical, chemical and microbiological hazards (Noventa et al., 2021).

The complexity of microplastic pollution might also due to the variability of microplastic particles. Contrary to heavy metals and pesticides, microplastic can be perceived as a “multiple stressor” itself, highly variable within a particle. Therefore, assessing microplastic pollution should not only consider quantitative aspects (concentration in count or in mass) but also qualitative ones, including evaluation of their physical and chemical properties (Bucci et al., 2021; Rochman et al., 2019; Waldman and Rillig, 2020). Studies in the field of microplastics consider the identification of particle main chemical composition, i.e. the polymer, as the first and primordial step (Hartmann et al., 2019). Under the umbrella of ‘microplastic’ lie synthetic polymers, anti-fouling paint particles, abrasion particles from synthetic rubber (tyre wear particles) and textile fibres (Dris et al., 2017; Gaylarde et al., 2021; Hartmann et al.,

2019; Järnskog, 2020; Simon et al., 2018). Physical properties (e.g., size, morphology, color), chemical properties (e.g., additives, level of chemical alteration), degradation state (e.g., oxidation, molecular chain size), are some of the parameters to be considered in microplastic research (Fig. 1.2.I - **Microplastic**). Common morphological types of microplastics include pellet/spherule, fragment/sheet, foam, fiber/line and film shape, depending from both original form and degradation process (Yang et al., 2021). Moreover, these properties were found to represent a main driver of microplastic toxicity (Latchere et al., 2021). For instance, the leaching of chemicals might be facilitated by reduced polymer chain size and increased free volume, which in turn is closely related to the degradation process (Hahladakis et al., 2018). Regarding their morphology, fibres and fragments had greater negative effects on functional traits of fishes than spherical microplastic (Salerno et al., 2021). The colour and the polymeric composition of particles seem to be an important driver of their consumption by aquatic organisms, mistaking with their prey while feeding (Collard et al., 2019).

An important aspect of microplastic research is the definition of size boundaries. While the upper size limit of microplastic category has reached a consensus, i.e. 5 mm in its longest dimension (ECHA, 2020), the inferior cut-off is still under debate in the scientific community. It is argued that size boundaries are not scientifically justified but rather based on pragmatic reasons and general consensus (Hartmann et al., 2019). Indeed, to assess a particle composition, instrumental analytical issues arise. Considering available analytical techniques, a particle size is exactly what hampers and influences the unequivocal identification of its composition (Filella, 2015). Therefore, the choice of an analytical procedure, as discussed below, should be aligned with a study objectives and consider limitations related with plastic size.

1.4 The analytical process of microplastic pollution assessment

To extract a (bio)chemical information about an object or a system, an adequate analytical process should be applied, comprising sample collection and laboratorial sample treatment and sample analysis (Valcárcel Cases et al., 2018). Assessment of microplastic pollution in freshwater starts with sample collection, which should be adapted for the studied compartment, such as surface water, sediment, soil, sludge or aquatic organisms. Among interacting factors that influences the sampling, hydrology and particles properties (density, size and morphology) might also influence

a particle's hydrodynamics (Eerkes-Medrano and Thompson, 2018).

Sample treatment aims to extract the microplastic through the removal of interferences (also known as clean-up). Several efforts to develop a standard sample treatment protocol to detect microplastic have emerged, although it is highlighted that there is no "one size fits all" (Caron et al., 2018; Dehaut et al., 2016; Hurley, Amy L. Lusher, et al., 2018; Karami et al., 2017). Protocol adaptations should take into account the study design, matrix components (inorganic and organic contents), and the selected analytical instrument. A reduction in the inorganic content might be achieved through a density separation procedure, where sample is incubated with a high-density solution such as saturated sodium chloride ($d = 1.2 \text{ g.cm}^{-3}$) and zinc chloride ($d = 1.4 - 1.6 \text{ g.cm}^{-3}$) (Carson et al., 2011; Rodrigues, 2020). Organic components, such as plastics, are expected to float due to their density in the range of $0.90 - 1.37 \text{ g.cm}^{-3}$ (from polypropylene to polyethylene terephthalate - PET, for example), and are therefore recovered (Bond et al., 2018; Nguyen et al., 2019). The reduction in the natural organic matter content through a digestion procedure is based on a chemical (e.g. potassium hydroxide, hydrogen peroxide, Fenton reagent, etc.) or an enzymatic (lipases, proteases, etc.) process (Nguyen et al., 2019; Prata, J. da Costa, A. C. Duarte, et al., 2019).

From an analytical point of view, challenges still include the detection and unequivocal identification of (1) polymeric composition to assure the plastic category, (2) chemical alterations and (3) the complex mixture of chemicals (Filella, 2015). Therefore, selecting the sample treatment that precedes sample analysis should consider the aims of the study and ensure that microplastic particles remain unaltered (Dehaut et al., 2016). Since microplastic analysis strongly relies on their chemical identification, infrared spectroscopy is the most widely accepted instrumental analytical technique to characterize plastics (Andrade et al., 2020). This technique is based on the energy absorbed by chemical bonds vibrations, depending on the change of their permanent dipole moment, once an electromagnetic radiation is incident (Siesler, 2016). The absorption bands representing distinct chemical functionalities culminate with the assignment of its composition through an expert or an algorithm based on comparison with a database (commercial, homemade, collaborative - OpenSpecy) (Cowger et al., 2020). The relative low cost, simplicity, non-destructiveness and easy handling of Fourier-transformed infrared (FTIR) spectroscopy made it the main analytical technique used in the field of microplastic research (Jung et al., 2018). Moreover, formation of new functional groups during microplastic degradation, as a result of an oxidation process, and changes in the proportion of existing ones can be monitored

through FTIR analysis (Gardette et al., 2013; Jung et al., 2018; Rouillon et al., 2016). FTIR is an outstanding tool not only for qualitative analyses, but also for quantitative analyses. Also, combination with chemometrics tools, to extract relevant chemical information by analysing chemical data, represents a promising approach (Jamwal et al., 2020; Mazivila and Olivieri, 2018).

The scientific community acknowledge the compromise between a particle size and their unequivocal identification through a clean infrared spectrum. For instance, visual pre-sorting and analysis through attenuated total reflectance (ATR) – FTIR, a surface technique of passing light through a crystal in contact with the sample, were impaired for particles smaller than 1 mm (Christensen et al., 2020). Therefore, a combination of FTIR with imaging and microscopy techniques are needed to lower the limit of detection for microplastic particles down to 20 μm (Mintenig et al., 2017). However, these improvements substantially increase the time of analyses. Filtered samples are commonly analysed directly on the filter (around 48 hours for a filter), demanding a considerable sample preparation efforts towards a clean matrix (Huppertsberg and Knepper, 2020; K  ppler et al., 2016). It is highlighted that the analytical protocol, comprising identification and quantification techniques (or their combination), should be chosen to suit the most the research and monitoring questions under investigation (Noventa et al., 2021).

The quantification of such a ubiquitous pollutant as microplastic must consider sources of contamination during sample manipulation. Thus, to avoid and mitigate contamination and ensure the accuracy of results, measures of quality assessment and quality control (QA/QC) should be adopted during all steps of microplastics analysis. A non-exhaustive list of QA/QC measures includes implementing blank controls, wearing cotton lab coats, use of metal and glass materials and rinsing all materials before their use (Prata, Reis, et al., 2021).

1.5 Microplastic pollution in freshwater ecosystems

The environmental cycle of microplastics comprises their transport from one pool to another and within pool. The first might be characterized as the transport between biotic and abiotic compartments, while the second is well represented by the microplastic dynamics in streams, where they are continuously deposited and resuspended (Bank and Hansson, 2019; Rochman and Hoellein, 2020). With the recognition of the per-

vasiveness of microplastic pollution in freshwater ecosystems grew the concern about the presence of microplastic in human and animal body and the potential associated ecotoxicological impacts (Noventa et al., 2021; Prata, J. P. da Costa, et al., 2021; Zhang et al., 2019). Therefore, understanding their origin, transport and accumulation, but also the drivers of their consumption and potential trophic transfer in the food chain (C. Li et al., 2020; Provencher et al., 2019), is fundamental (Fig. 1.3).

In general, freshwater hot spots of microplastics often occur in urban areas (Chen et al., 2020; Dris, Gasperi, et al., 2015), where microplastics can directly enter rivers, streams and lakes through drainage, runoff, and wastewater treatment plants (Horton and Dixon, 2018; Simon et al., 2018). Then, microplastics might be transported downstream, as rivers act as an important conveyor belt until the oceans (Lebreton et al., 2017; Weiss et al., 2021), and/or accumulate midway. Their particulate behaviour results in a high susceptibility to hydrodynamics conditions in streams (Fig. 1.3b and c) (Horton and Dixon, 2018; Horton, Walton, et al., 2017), with river beds and river banks perceived as temporal sinks for microplastics and hot spot for aquatic contamination (Fig. 1.3d) (Christensen et al., 2020; Hurley, Woodward, et al., 2018). The temporal response of cumulated microplastics is somehow correlated with natural events. Storm runoff events seem to mobilized this pollution, potentially leading to unthinkable concentrations peaks and profile diversity (Christensen et al., 2020; Gündoğdu et al., 2018; Hitchcock, 2020; Hurley, Woodward, et al., 2018) (Fig. 1.3c). This process might directly affect microplastic bioavailability and consumption by organisms, potentially causing acute toxicity and mortality (Rillig et al., 2021; Tian et al., 2020). The temporal dynamics of microplastic pollution might be dependent of its spatial variability, as subjected to different anthropogenic pressures, but this remains to be further investigated (Wagner et al., 2019). The various facets of anthropogenic activity call the attention to the presence of microplastics in yet imperceptible and unknown sources (Fig. 1.3a), resulting in growing efforts to map and identify cryptic sources of this contamination (Dris et al., 2018; Horton, Svendsen, et al., 2017). Also, because the usage of a plastic material is linked with their properties, assessing the spatial variability in microplastic characteristics should allow to identify local source-apportionment, serving as important resource for waste management strategies (Grbić et al., 2020; T. Wang et al., 2019).

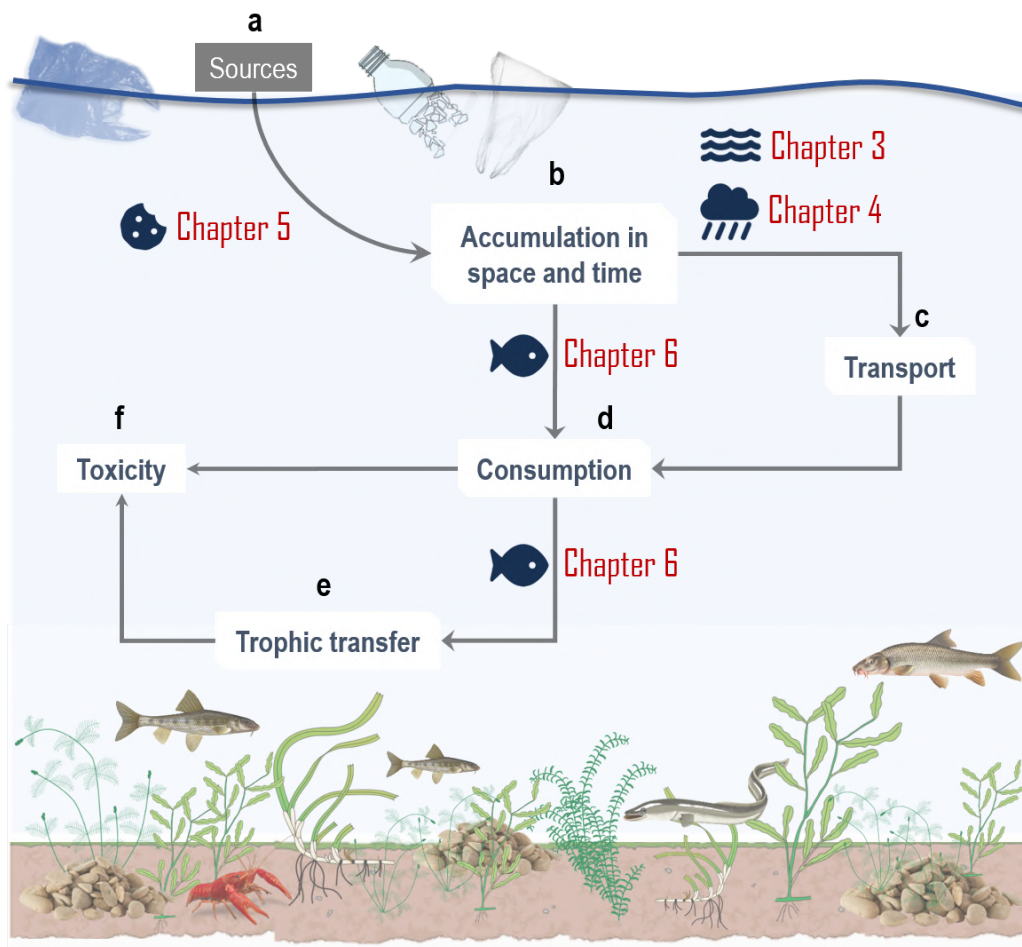


Figure 1.3: Overview of microplastic pollution and contamination process in freshwater ecosystems. The subject of each chapter is displayed.

Freshwater macroinvertebrates and fish consume microplastics (Collard et al., 2019; Prata, J. P. da Costa, et al., 2021; Sloomackers et al., 2019; Windsor, Tilley, et al., 2019) (Fig. 1.3d). They might mistake microplastics with their prey, intentionally consuming it, or consume prey already contaminated with microplastics (Latchere et al., 2021). However, aquatic organisms are not equally exposed to microplastics. Some of them might be at higher risks, due to their feeding strategy and behaviour, and their ability to retain the particle within their bodies (Collard et al., 2017; Salerno et al., 2021) (Fig. 1.3f). For instance, bottom feeding fish might be more exposed to microplastic contamination due to particles accumulation in sediments (Fig. 1.3b) (He et al., 2020). To date, the relationship between microplastic pollution and biotic contamination is not fully understood, with higher pollution levels not necessarily correlating with higher contamination and the key drivers of microplastic consump-

tion by aquatic organisms remain to be investigated (Collard et al., 2017; Salerno et al., 2021).

The presence of microplastics across trophic levels, from primary producers to top predators fostered the hypothesis of a potential trophic transfer (Ibañez et al., 2020; Nelms et al., 2018; Prata, J. da Costa, Lopes, et al., 2019; Wenfeng Wang et al., 2019) (Fig. 1.3e). Experimental models in laboratory conditions have demonstrated the transfer of micro and nanoplastics through food chains (Farrell and Nelson, 2013; Hasegawa and Nakaoka, 2021). However, particles used in such experimental approach are far beyond the diversity found in the environment and further investigations are therefore needed to quantify the distribution of microplastic across trophic levels in wild freshwater food webs (Latchere et al., 2021; Phuong et al., 2016).

1.6 Objectives

The main objective of this thesis was to characterise environmental microplastic pollution and contamination of organisms in a freshwater ecosystem. Microplastic pollution and contamination of particles ranging from 700 μm to 5 mm were assessed in the Garonne catchment, located in south-western France. Microplastic pollution and contamination were characterised quantitatively (in count or in mass of microplastic content, whenever possible) and qualitatively (measurement of microplastic properties).

Bearing in mind the challenges of dealing with a high amount of environmental samples, which are rich in natural organic matter content, we firstly developed and validated a highthroughput sample processing protocol focused on water samples (**Chapter 2**). Then, we investigated the spatial and temporal variability in microplastic pollution in surface waters and determined its environmental drivers. In this chapter (**Chapter 3**), investigation were performed in fourteen sites distributed along the Garonne catchment in four seasons to capture a high level of spatial and temporal variability in environmental conditions. The following chapter (**Chapter 4**) focussed on the role of flooding events in modulating microplastic pollution. We then quantified microplastic contamination in angling baits, an underestimated and potentially important source of microplastic to freshwater ecosystem (**Chapter 5**). Finally, the last chapter (**Chapter 6**) aimed to quantify the consumption of microplastic by freshwater macroinvertebrates and fish and to test the existence of a potential trophic transfer using stable isotope analyses.

2 | General material & methods

2.1 Studied area

The Garonne river catchment is located in southwestern France. The Garonne river is the third largest French river with an average annual discharge of $630\text{m}^3\cdot\text{s}^{-1}$. It drains about 53.536 km^2 , equivalent to 3 million people (Adour Garonne, 2021), and the main channel flows northwards over 525 km from its source in central Pyrénées in Spain to the Atlantic Ocean nearby Bordeaux, France (Fig. 2.1). It notably flows through the large cities of Toulouse and Bordeaux. Discharge is strongly dependent on snow melt and is also influenced by precipitations, typically resulting in a flood peak in May–June and a period of low flow from summer to early autumn (Lambs et al., 2009).

2.2 Sampling design

A total of 14 sites were sampled, with different number of sites being used for the analyses of each chapter (Table 2.1), considering prior information about the local accessibility and professional experience. Proximity to wastewater effluents and locations upstream dams might influence microplastic abundance (Mason et al., 2016; Murphy et al., 2016; Zhang et al., 2015) and were therefore avoided while selecting sites within a river.

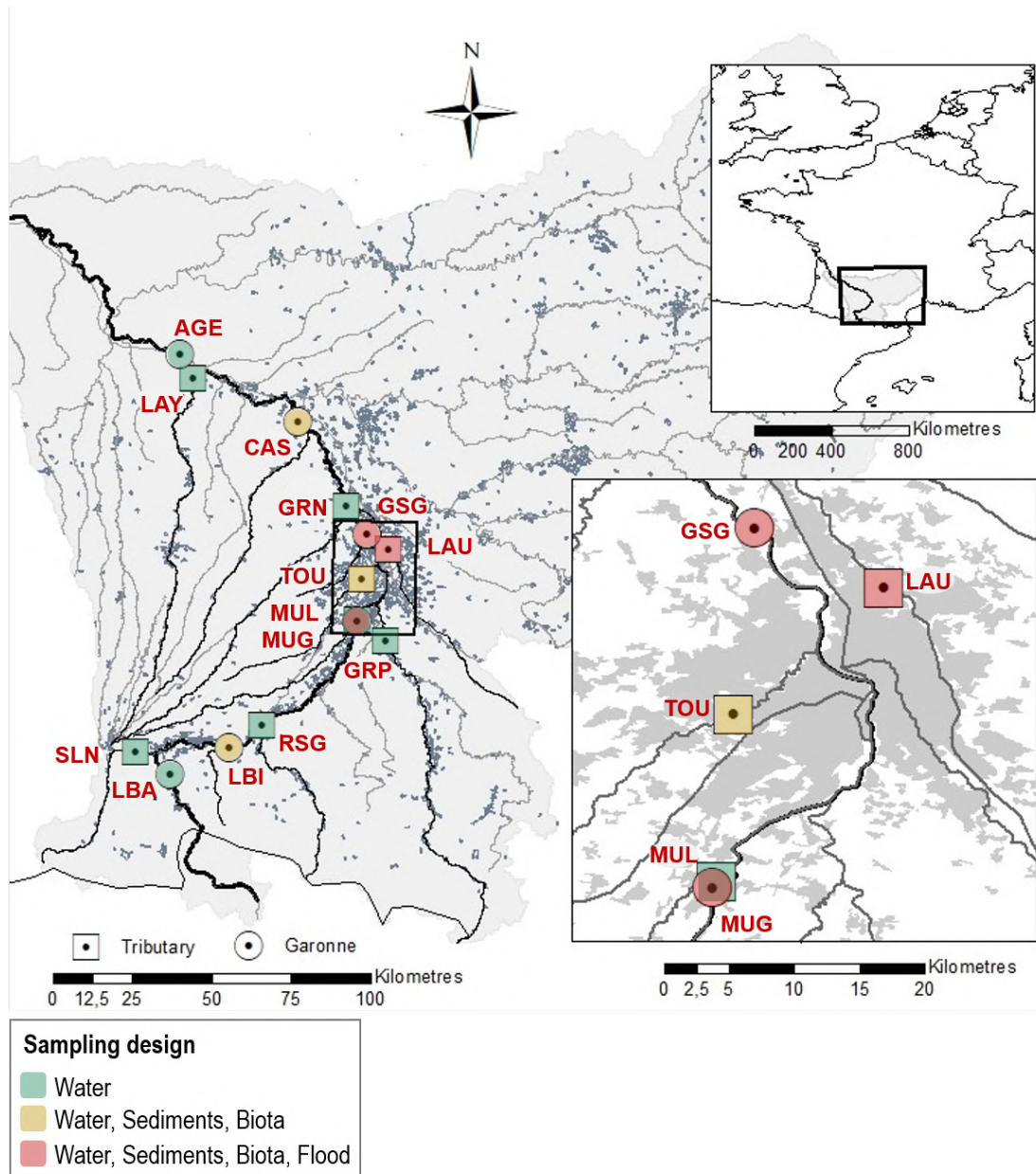


Figure 2.1: Map of the study area and localization of all sampling sites

Table 2.1: Locations of each sampled site and the correspondent chapter they are studied. Sites are ranked from upstream to downstream

Code	River	Latitude (N)	Longitude (E)	Chapter 2	Chapter 3	Chapter 4	Chapter 5
LBA	Garonne	43.01164	0.616240	X	-	-	-
SLN	Neste	43.07222	0.486136	X	-	-	-
LBI	Garonne	43.09458	0.842243	X	-	-	X
RSG	Salat	43.15890	0.967583	X	-	-	-
MUL	Louge	43.46283	1.331124	X	-	-	-
MUG	Garonne	43.45862	1.327305	X	X	-	X
GRP	Ariège	43.40767	1.444286	X	-	-	-
LAU	Hers	43.66534	1.447144	X	X	-	X
TOU	Touch	43.57827	1.344242	X	-	-	X
GSG	Garonne	43.70675	1.361795	X	X	-	X
CAS	Garonne	44.02239	1.078778	X	-	-	X
GRN	Save	43.78390	1.275918	X	-	-	-
LAY	Gers	44.13599	0.663983	X	-	-	-
AGE	Garonne	44.20253	0.606713	X	-	-	-

In Chapter 3, 14 sites distributed across the Garonne basin were selected to capture most of the spatial heterogeneity in terms of environmental conditions (Table 2.1 and Table 2.2). Six sites were located in the Garonne river (from upstream to downstream: LBA, LBI, MUG, GSG, CAS and AGE; Fig. 2.1, displayed with circles). Eight sites were located in the downstream part of some of the main tributaries of the Garonne river (from upstream to downstream: SLN in the river Neste, RSG in the river Salat, MUL in the river Louge, GRP in the river Ariège, LAU in the river Hers, TOU in the river Touch, GRN in the river Save and LAY in the river Gers) (Fig. 2.1, displayed with squares).

Table 2.2: Environmental characteristics of each sampled site. Sites are ranked from upstream to downstream

Code	River width (m)	Drainage area (km²)	Distance to Garonne source (km)	Mean yearly discharge (2019)	Human population (habitants – 5 km)	Urban land cover (%) – 5 km	Agricultural land cover (%) – 5 km
LBA	34	1082.92	75.83	26.87	227	1.50	55.25
SLN	29	734.73	88.16	26.87	249	3.60	89.33
LBI	65	2561.40	120.59	46.42	353	0.00	97.91
RSG	49	1578.11	136.23	37.72	383	10.15	72.63
MUL	15	465.91	193.78	4.51	1989	42.02	40.99
MUG	87	5335.99	193.41	140.39	1994	38.75	40.89
GRP	55	3521.65	203.92	46.37	344	8.94	76.10
LAU	13	918.70	240.25	2.71	15378	74.26	19.32
TOU	14	425.50	218.58	1.76	1882	70.29	29.71
GSG	133	10644.85	230.77	162.24	1180	50.11	29.18
CAS	123	14971.32	285.61	162.24	497	0.61	74.83
GRN	17	1154.64	245.36	4.09	1080	22.08	77.92
LAY	22	1214.29	333.39	4.86	394	7.45	76.63
AGE	183	34489.90	341.03	315.66	6246	46.46	46.13

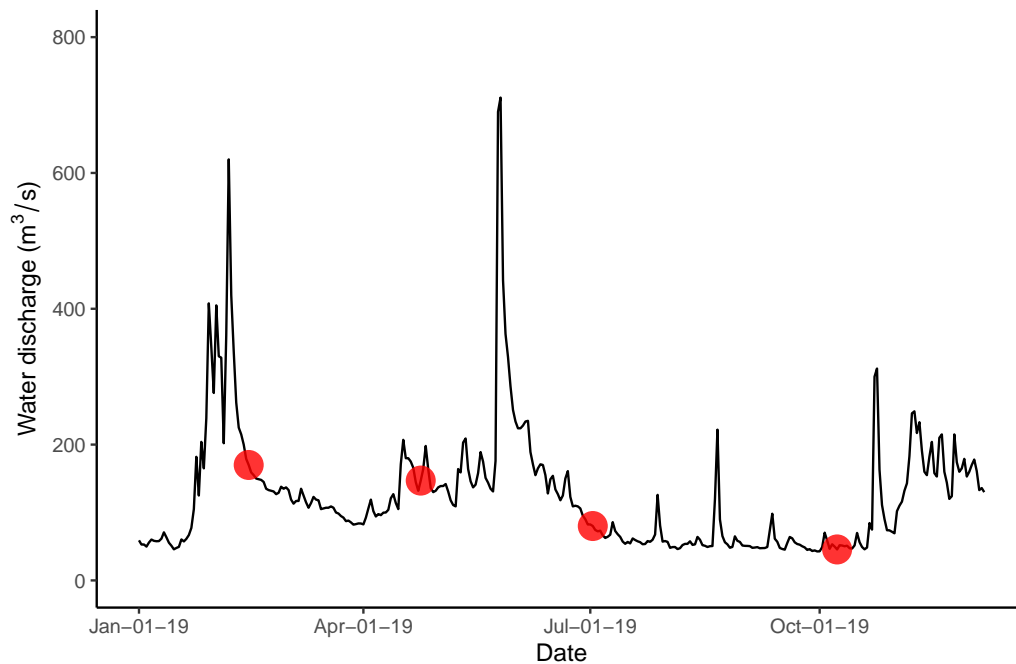


Figure 2.2: River discharge (daily average, $\text{m}^3 \cdot \text{s}^{-1}$) in 2019 in the Garonne river in the middle of the study area (Portet-sur-Garonne, Muret). Sampling events are displayed by a red circle. Data obtained from HydroFrance.

On average, 3 to 5 sites were sampled per day, with a duration of approximately 2 h per site. All sites were sampled at four occasions (Fig. 2.1, all colors): February 13 to 15, April 23 to 26, July 01 to 04 and October 07 to 09, 2019 (water discharge displayed in Fig. 2.2).

Sampling of each site was replicated three times at each sampling event with all replicates being performed successively, leading to a total of 168 samples (4 events \times 14 sites \times 3 replicates). The average volume of filtered water was 99.6 m^3 (± 53.7 SD) and ranged from 4.25 m^3 to 259.10 m^3 depending on the sampling site and the sampling event (Table 2.3). The annual variability in water parameters of pH, temperature and natural organic matter (smaller than 5 mm) were (mean \pm SD) 8.20 ± 0.48 , $14.7^\circ\text{C} \pm 5.34$, $45.1 \text{ g} \pm 76.4$, respectively.

Table 2.3: Volume of filtered water (average and SD, m³) of each sampled site at the four sampling events.

Code	February 2019 (average volume \pm SD)	April 2019 (average volume \pm SD)	July 2019 (average volume \pm SD)	October 2019 (average volume \pm SD)
LBA	150.24 \pm 0.89	256.89 \pm 1.99	229.57 \pm 23.49	171.61 \pm 5.41
SLN	138.53 \pm 5.99	91.35 \pm 3.36	114.98 \pm 2.01	57.23 \pm 1.09
LBI	88.66 \pm 3.75	150.66 \pm 9.70	124.06 \pm 0.55	63.03 \pm 8.09
RSG	126.63 \pm 4.02	72.82 \pm 9.40	71.00 \pm 3.57	27.32 \pm 3.07
MUL	129.31 \pm 13.57	77.46 \pm 3.33	32.88 \pm 4.03	51.04 \pm 3.93
MUG	120.23 \pm 5.02	170.74 \pm 3.53	67.23 \pm 2.10	95.72 \pm 0.75
GRP	160.41 \pm 33.10	163.58 \pm 3.41	86.38 \pm 3.17	81.95 \pm 9.29
LAU	148.86 \pm 11.11	114.83 \pm 5.95	25.74 \pm 1.99	25.27 \pm 1.29
TOU	106.40 \pm 12.21	70.45 \pm 1.93	4.93 \pm 1.05	18.79 \pm 6.85
GSG	155.68 \pm 3.42	58.25 \pm 13.98	56.28 \pm 19.75	40.57 \pm 0.80
CAS	139.57 \pm 5.45	107.37 \pm 6.14	50.77 \pm 0.83	5.51 \pm 0.99
GRN	143.93 \pm 6.31	99.11 \pm 12.24	63.45 \pm 12.46	37.56 \pm 6.71
LAY	161.86 \pm 9.12	129.73 \pm 1.70	75.60 \pm 45.58	118.33 \pm 22.62
AGE	120.23 \pm 21.68	105.96 \pm 15.14	90.92 \pm 3.02	127.76 \pm 10.01

In Chapter 4, two sites located in the main stream were selected during two flood events: upstream (MUG) and downstream (GSG) of Toulouse (Fig. 2.1). Four tributaries flow into the Garonne between the two sampled sites, i.e. the Louge, Ariège, Touch and Hers rivers. The tributary within Toulouse (Hers) was also sampled (site LAU) at the second sampling event, although no flood occurred in this tributary during the monitoring (Fig. 2.1, in red, and Fig. 2.5).

Samples were collected during two different flood events (Fig. 2.3 and 2.4). First, a short-scale sampling was performed on October 15, 16, 18 and 22 2018 (sampling events A1 to A4) (Fig. 2.3 and Table 2.4), with a discharge peak of 336 m³.s⁻¹ occurring in the MUG site at October 15. The second sampling period consisted of six sampling events (B1 to B6) on April 24, May 6, May 22, June 05, June 18 and July 3 2019 (Fig. 2.4 and Table 2.4). At this larger-scale temporal monitoring, sampling events were grouped as presence of flood, i.e. increased water discharge (event B4, Fig. 2.4), or absence of flood (events B1, B2, B3, B5 and B6, Fig. 2.4). A discharge peak of 11 90 m³.s⁻¹ occurred in the GSG site at May 26.

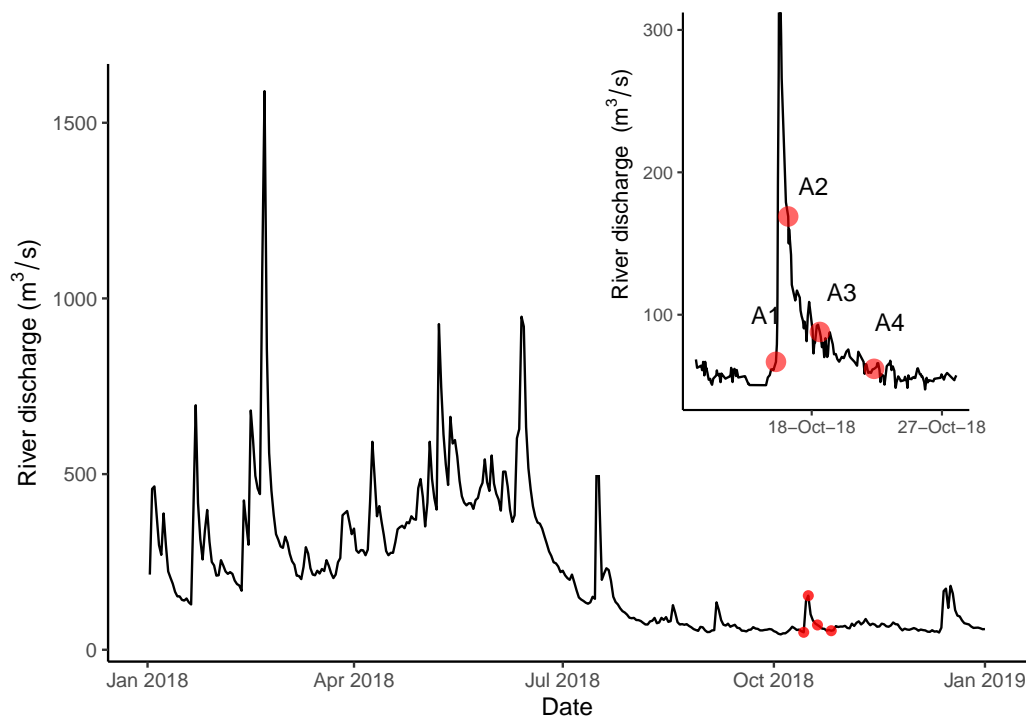


Figure 2.3: River discharge (daily average, $\text{m}^3 \cdot \text{s}^{-1}$) in 2018 in the Garonne river (Portet-sur-Garonne, Muret) zoomed at the four sampling events, A1 to A4, at the top right. Data obtained from HydroFrance.

In Chapter 6, six sites were selected to represent contrasting environmental conditions (Table 2.1): 2 sites (LBI and MUG) were located on the Garonne river upstream of Toulouse, 2 sites (LAU and TOU) were located on tributaries within the Toulouse agglomeration, and 2 sites (GSG and CAS) were located on the Garonne river, downstream of Toulouse (Fig. 2.1, displayed in yellow and red). Water and sediment were sampled between July 1 to 4 2019, with two sites sampled per day. Sampling was repeated three times, totalizing 18 samples for each matrix. Macroinvertebrates were collected between July 10 to 18 2019 (one site sampled per day, Table 2.4) and fish sampling was performed between July 23 to 30 2019 (one site sampled per day, Table 2.4).

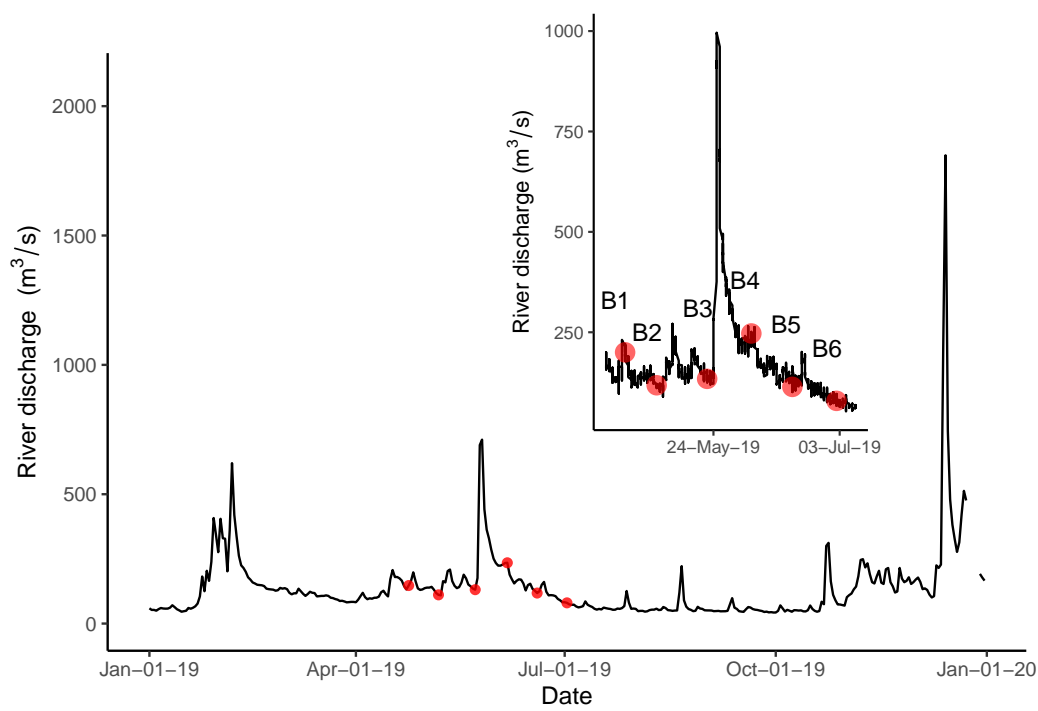


Figure 2.4: River discharge (daily average, $\text{m}^3 \cdot \text{s}^{-1}$) in 2019 in the Garonne river (Portet-sur-Garonne, Muret) zoomed at the six sampling events, B1 to B6, at the top right. Data obtained from HydroFrance.

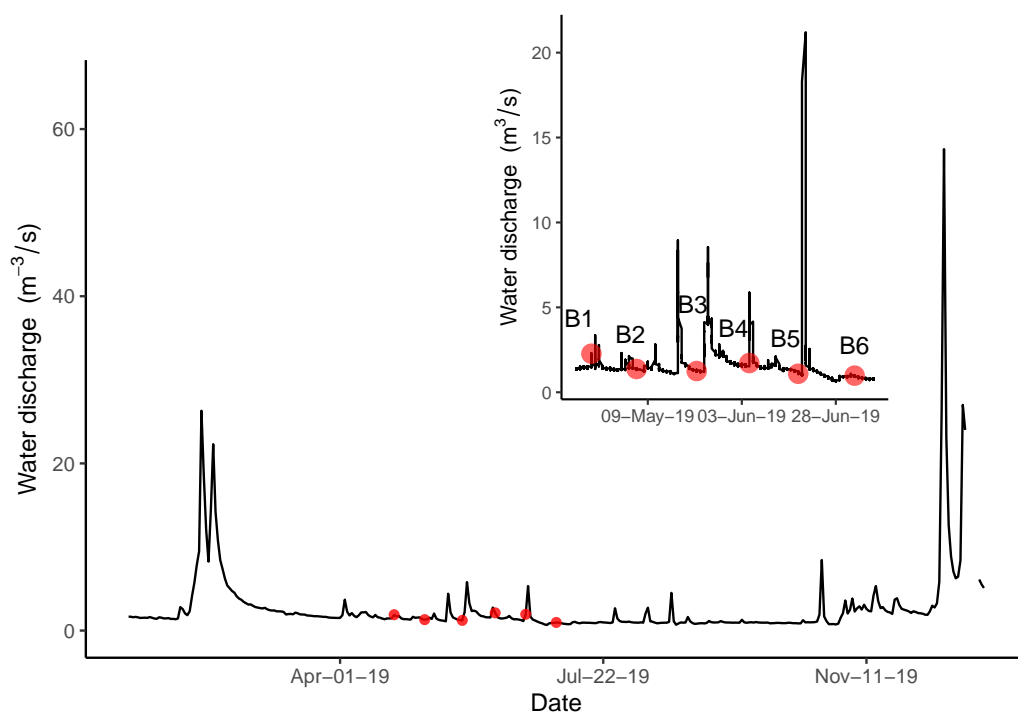


Figure 2.5: River discharge (daily average, $\text{m}^3 \cdot \text{s}^{-1}$) in 2019 in the tributary Hers (LAU site) zoomed at the six sampling events, B1 to B6, at the top right. Data obtained from HydroFrance.

Table 2.4: Sampling events and matrices studied at each chapter

Date	Matrices	Chapter 2	Chapter 3	Chapter 4	Chapter 5
Oct 15-22, 2018	Water	-	24	-	-
Feb 13-15, 2019	Water	42	-	-	-
Apr 23-26, 2019	Water	42	12	-	-
May 06 - Jun 18, 2019	Water	-	36	-	-
Jul 01-04, 2019	Water / Sediments	42 / -	12	-	- / 18
Jul 10-18, 2019	Macroinvertebrates	-	-	-	2010 individuals (396 samples)
Jul 23-30, 2019	Fish	-	-	-	492 individuals
Oct 07-09, 2019	Water	42	-	-	-

2.3 Sample collection

2.3.1 Surface water

Surface water samples were obtained by filtration using a Manta trawl (opening 32 cm × 82 cm) equipped with a 500 μm mesh size polyamide net and a removable cod-end with the same mesh size (Faure et al., 2012; Galgani et al., 2013; Hidalgo-Ruz et al., 2012). 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. Each sampling event consisted in attaching the Manta trawl to the bridge guardrail over the river and by immersing it in a fast flowing and deep part of each site for approximately 10 min (sampling duration was precisely recorded to the nearest second for each sampling). Sampling was performed in successive triplicates.

The most commonly used method for flow measurement is the flow meter (Zhang, 2007). The flow meter measures the water flow velocity, in meters per second, which is then converted to the discharge by using the cross-sectional area sampled. At this study, a propeller type of flowmeter (Hydro-Bios, Germany) was placed at the center of net entrance. After each sampling, the cod-end content was sieved into a 500 μm mesh using river water and transferred to sealable plastic bags made of polyethylene (Cheung et al., 2019; J. K. H. Wong et al., 2020) (Fig. 2.6). Due to the particle size range considered in the present study, contamination from these bags was very unlikely. Samples were stored in a cooler in the field and stocked at 4 °C in the laboratory before subsequent analyses.

2.3.2 Sediments

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 (Fig. 2.7a). Surber nets were used in microhabitats composed of gravels and cobbles as the main substrate and the area delimited by the Surber net (Fig. 2.7b) (subsequently used to calculate microplastic concentration) was gently washed to remove settled particles, which were subsequently collected in the cod-end of the Surber net. The contents of the cod-end were filtered in the field through a 500 μm sieve (Fig. 2.7c), rinsed with river water (previously filtered at 500 μm), and transferred to sealable plastic bags. All samples were stored in a cooler in the field and subsequently stored at 4 °C in the laboratory before analyses.



Figure 2.6: Water surface sampling with (a) manta trawl equipped with (b) a removal cod-end. Samples were filtered in the field (c) using a 500 μm sieve and stored in a plastic bag before processing in the laboratory.

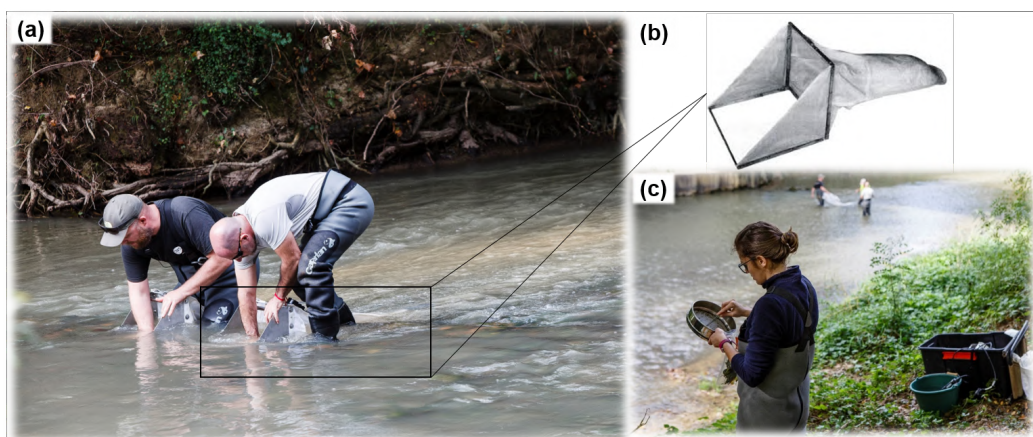


Figure 2.7: Sediments sampling (a) in the riffle areas of each site using the (b) surber net. Samples collected in the cod-end were (c) filtered in the field.

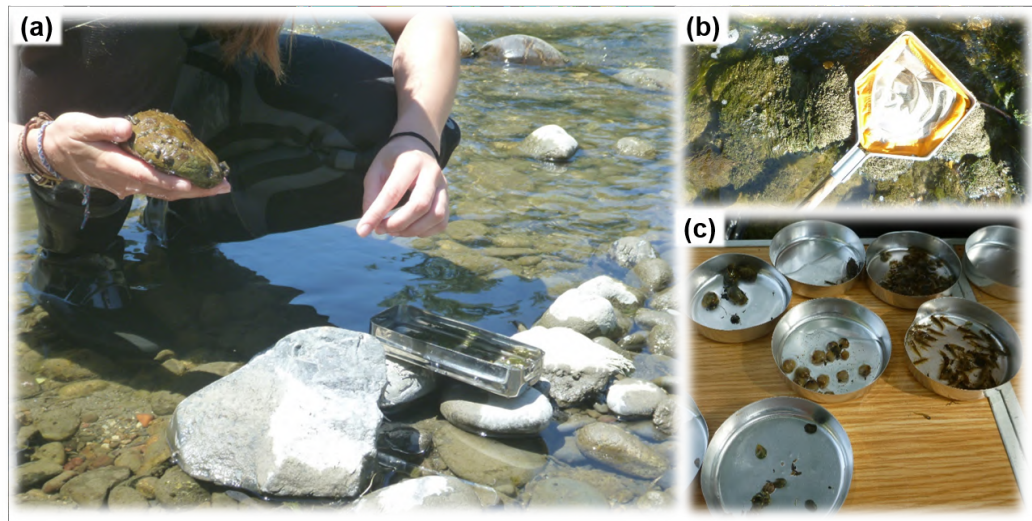


Figure 2.8: Macroinvertebrates sampling (a) manually and (b) using a surber net. Individuals were (c) aggregated in the field.

2.3.3 Macroinvertebrates

Macroinvertebrates were collected using Surber and kick (500 μm mesh size) netting, performed representatively of microhabitats (Fig. 2.8a and b). Specimens were collected as a representative sample for each site, which reflected feeding modes (shredders, collectors, predators, and scrapers) (Tachet et al., 2015) 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) (Fig. 2.8c). 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).



Figure 2.9: Fish sampling by (a) electrofishing, followed by (b) dissection in the laboratory to obtain the entire digestive tract.

2.3.4 Fish

Fish sampling was performed by electrofishing (model FEG 1500 and 5000, EFKO GmbH, Germany) (Fig. 2.9a). To limit the potential effect of diel 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 of 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 (Fig. 2.9b). Crayfish were dissected using the same approach of fish and the entire digestive tract was retrieved 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 ($-20 \text{ }^{\circ}\text{C}$) before analyses.

2.3.5 Angling baits selection

We purchased some of the most popular commercially-available angling baits used in Europe, i.e. groundbaits, boilies and pellets, to target freshwater cyprinid fish. Angling baits were purchased in two angling shops and online from a popular angling website in France. In total, 16 different products were purchased (6 for groundbaits,



Figure 2.10: Examples of angling baits purchased (a) grounbait, (b) boilies and (c) pellets.

6 for boilies and 4 for pellets, Fig. 2.10), produced by 6 different companies, therefore including multiple bait categories from some companies. The products differed from each other by their commercial name or packaging and, in total, 27 commercial bags were purchased). Each sample consisted of 10 g of angling bait, with 10 replicates analysed for each angling bait product, resulting in 160 analysed samples. Depending upon the packaging of angling baits and the number of bags purchased, samples were collected to maximise the number of bags used. When several samples were collected in the same bag, they were collected in different locations within the bag

2.4 Sample treatment

The detection of microplastics in environmental matrices faces two crucial challenges: (i) reduction of matrices without altering the target particle, and (ii) unequivocal identification of microplastic particles (Valcárcel Cases et al., 2018). A general overview of different sample processing applied in this thesis is showed in Figure 2.11. In this protocol, microplastic was defined as particle with a major axis larger than $700 \mu\text{m}$ (i.e. diagonal of the $500 \mu\text{m}$ mesh net of sampling device) and smaller than 5 mm, and with composition defined as plastic, comprising synthetic polymers, petroleum-based waxes, tire and wear particles and, paint resins (Hartmann et al., 2019).

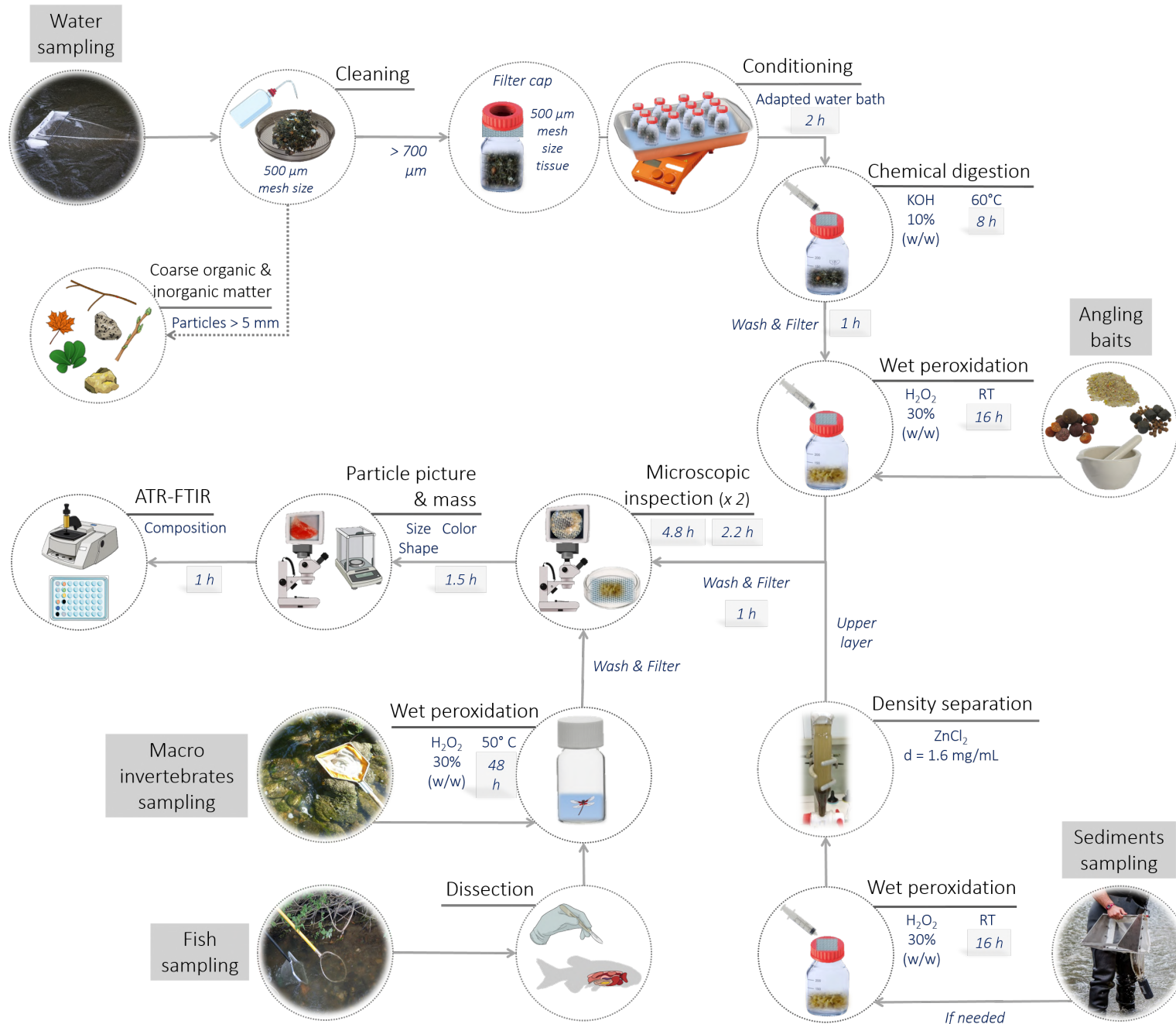


Figure 2.11: An overview of sample processing of all matrices analyzed

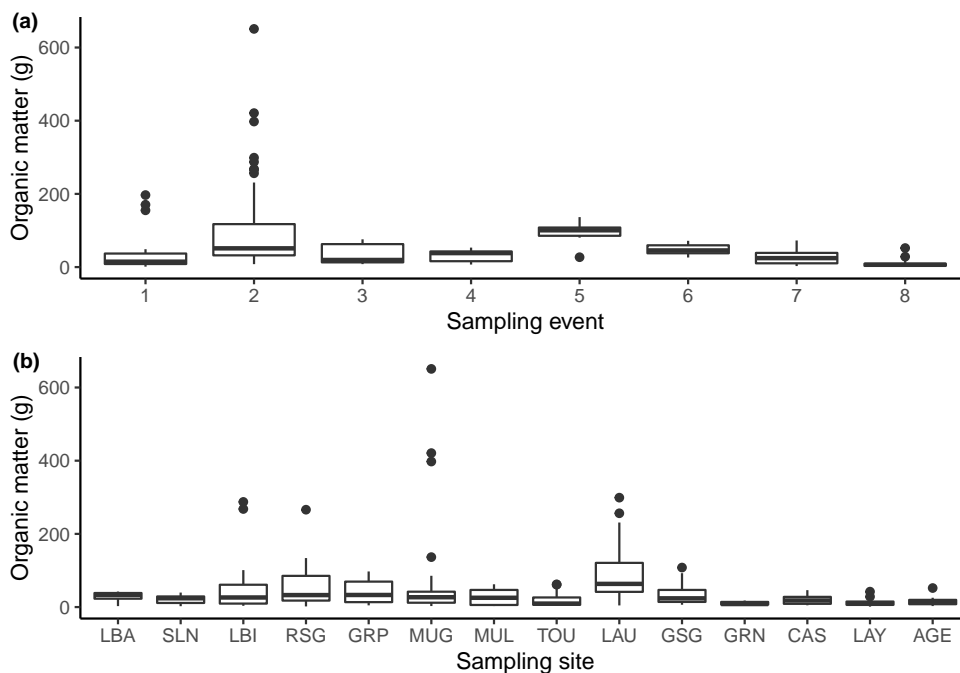


Figure 2.12: Variation in wet organic matter mass (g) collected across (a) sampling events and (b) sampling sites

2.4.1 Surface water

Protocol validation:

In this study, a protocol consisting of a double-step digestion through two different reagents, potassium hydroxide (KOH) (pellets, Sigma-Aldrich, USA) 10% (w/w) and hydrogen peroxide (H_2O_2) 30% (w/w) (Merck KGaA, Germany) solutions, the two main reagents used for digestion purposes in microplastic monitoring studies (Renner et al., 2018), was proposed to optimize the digestion of the rich and diverse organic matter content in freshwater samples. The temporal and spatial variation in the wet organic matter content collected during sampling campaigns is displayed at figure 2.12. The efficacy of organic matter digestion was quantified using samples collected from the same catchment ($n = 35$) and randomly submitted to three different digestion protocols: two single-reagent digestion (single step; chemical digestion: KOH 10% 60 °C, 24 h and wet peroxidation: H_2O_2 30% room temperature - RT, 24 h) and one double-step digestion (KOH 10% 60 °C followed by H_2O_2 30% RT, totalizing 24 h). We measured sample wet mass before and after digestions and calculated digestion efficiency as the percentage of wet mass loss. We found that the double digestion protocol ($n = 6$) allowed the elimination of, on average, 65.8% (± 9.59 SD) of mass,

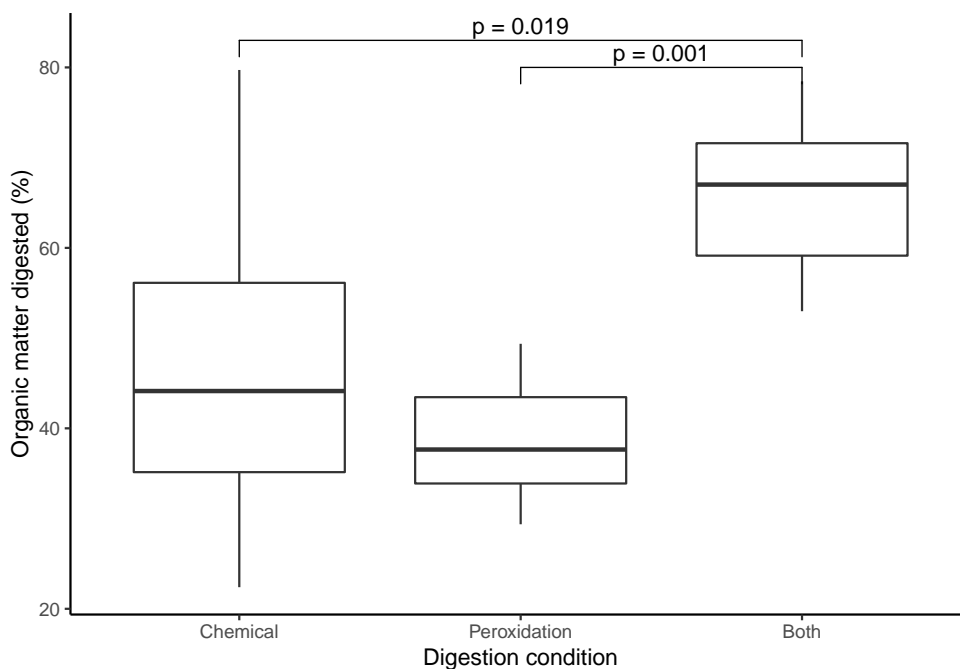


Figure 2.13: Percentage of organic matter digested by the digestion protocols tested

significantly more efficient than the single ones, with 43.5% (± 15.2 SD) digested for KOH ($n = 19$) and 39.4% (± 7.29 SD) for H_2O_2 protocol ($n = 9$) (Kruskal test, $\chi^2 = 10.845$, $p = 0.004$). No difference was found between the two single protocols (Fig. 2.13). The reduction of the organic matter content together with the bleaching effect caused by the wet peroxidation step greatly facilitate the subsequent visual inspection of samples (Fig. 2.14). Because a multi-step digestion protocol would require the inclusion of washes and filtrations steps, a customized filter-cap was specifically designed to facilitate content drain-out. The glass bottle was covered with a Nitex tissue (500 μm , similar to the water sampling net), and a commercially available screw open-cap (Fig. 2.15). A syringe was used to facilitate liquid addition through the tissue.

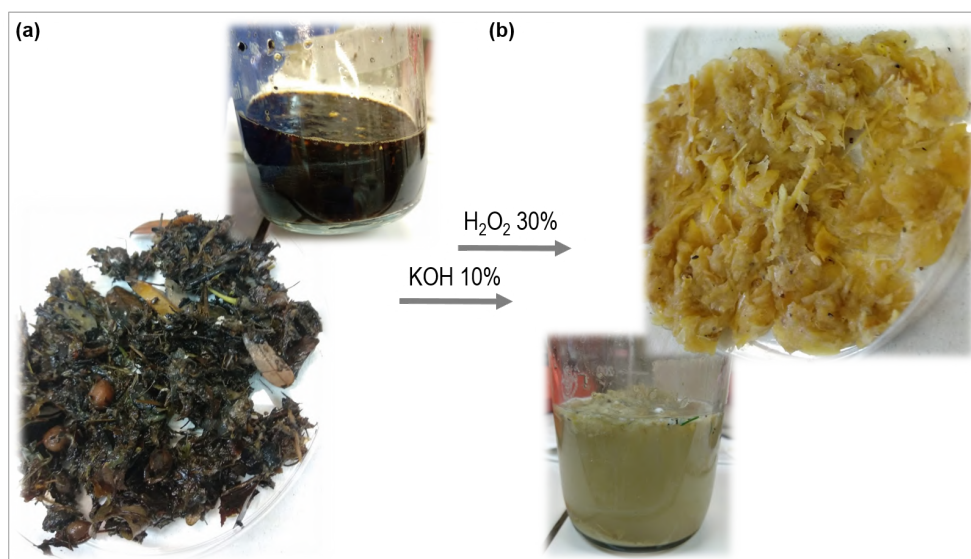


Figure 2.14: Changes in sample characteristics induced by the double digestion protocol (a) before and (b) after incubation with potassium hydroxide solution followed by hydrogen peroxide solution

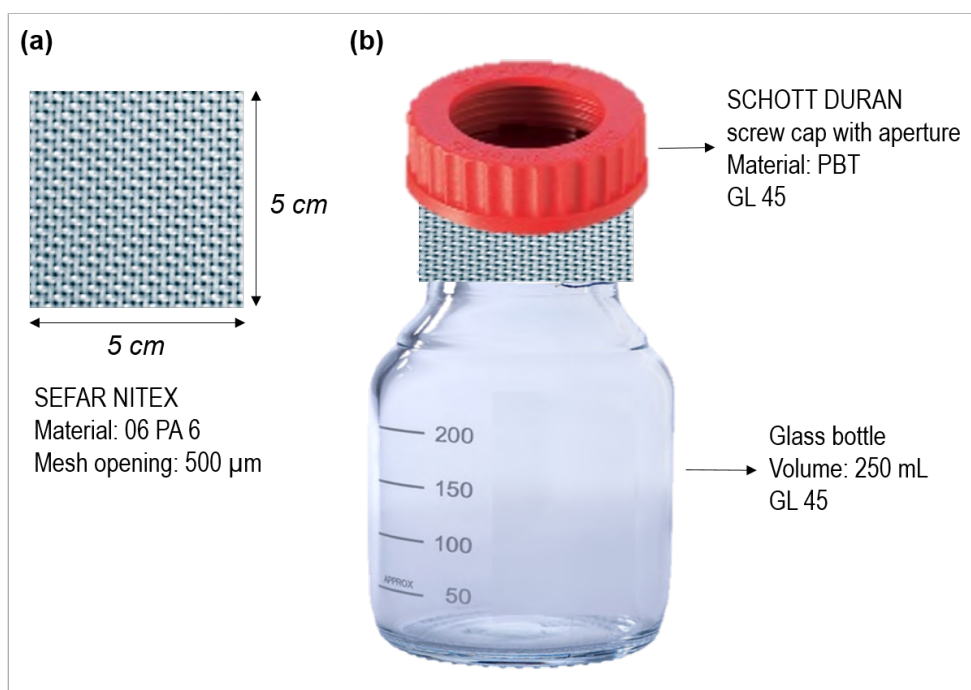


Figure 2.15: Customized bottles used for sample digestion where (a) a piece of tissue is placed (b) between the screw open and the glass bottle.

Although it has been reported that virgin microplastic pellets are not affected by these single protocols (Dehaut et al., 2016; Karlsson et al., 2017), we quantified potential physical damages through changes in mass and chemical modification (as-

essed by ATR-FTIR) arising from each step of the double digestion protocol. Three to five virgin pellets (1–5 mm) from 12 different synthetic polymers were tested in triplicates: polyethylene (PE) of three different densities, polystyrene (PS), expanded polystyrene, polypropylene (PP), polyethylene terephthalate (PET – from two different manufactures, Sigma and GoodFellow), polyamide 6 and 12, ethylene vinyl acetate, polycarbonate and polyetherimide (Table 2.5). The polymers tested represented the main microplastic composition found in environmental samples (Skalska et al., 2020).

Table 2.5: Polymers references for resistance test

Polymers	CAS	Source	Code	Lot #
Polypropylene	9003-07-0	GoodFellow	LS464294	PP306320/4
Polyethylene low density 1	9002-88-4	Aldrich (d = 0.918 g/mL)	428078	07730MEV
Polyethylene low density 2	9002-88-4	Aldrich (d = 0.925 g/mL)	428043	MKBL3627V
Polyethylene high density	9002-88-4	Aldrich (d = 0.952 g/mL)	427985	MKBQ2137V
Polystyrene	9003-53-6	GoodFellow	LS464294	ST316310:4
Expanded polystyrene (ePS)	-	packaging	-	-
Polyethylene terephthalate (PET)	25038-59-9	Sigma	429254	MKBV4092V
Polyethylene terephthalate (PET)	25038-59-9	Sigma	429254	MKCF1408
Polyethylene terephthalate (PET)	25038-59-9	GoodFellow	LS511753 IM	ES306312/1
Ethylene vinyl acetate	24937-78-8	GoodFellow	LS508242	ET346300/1
Polycarbonate	25037-45-0	GoodFellow	LS467893	CT306310/3
Polyamide 6/6	32131-17-2	Sigma	429171	MKBX2257V
Polyamide 12	24937-16-4	Sigma	181161	MKBQ2716V
Polyetherimide	9002-98-6	GoodFellow	LS403713	EI316300/1

No significant alteration that could lead to misidentification was observed in the infrared spectra of particles submitted to digestion protocol when compared with two control conditions, virgin particles and particles treated with distilled water (Fig. 2.16).

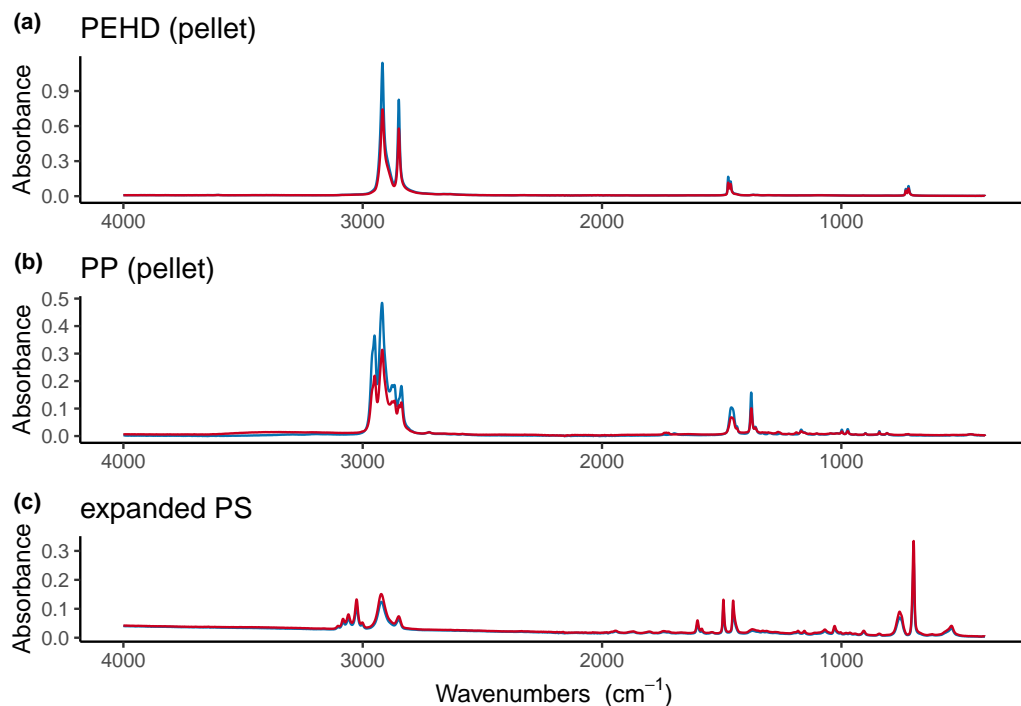


Figure 2.16: Examples of ATR-FTIR spectra in control condition (blue line) and after digestion protocol (red line) for (a) PE high density (HD, $d = 0.952 \text{ g.cm}^{-3}$), (b) expanded PS, (c) PP.

Despite the FTIR spectra of PET after digestion protocol showing a distinct peak at wavenumber 3320 cm^{-1} (Fig. 2.17), only for pellets from Sigma Aldrich), indicating carboxylic acid and alcohol functional groups (R-OH stretching, $3000\text{--}3500 \text{ cm}^{-1}$) (Sammon et al., 2000), all particles were unequivocally identified (Fig. 2.16). Similarly, no significant mass changes occurred (Kruskal test, $\chi^2 = 1.495$, $p = 0.474$), excepted for the two PET batches from Sigma Aldrich (Table 2.5), where a significant mass loss of 17.0% ($\pm 5.18 \text{ SD}$) was observed (Kruskal test, $\chi^2 = 15.699$, $p = 0.003$). Tests with PET pellet from a different manufacture - GoodFellow (Table 2.5) showed no significant mass variation following the treatment ($98.2\% \pm 1.81 \text{ SD}$). We highlight that the diversity among plastic formulation might interfere in their chemical stability and further studies regarding potential impacts of this treatment on smaller and/or chemically-altered microplastics are needed.

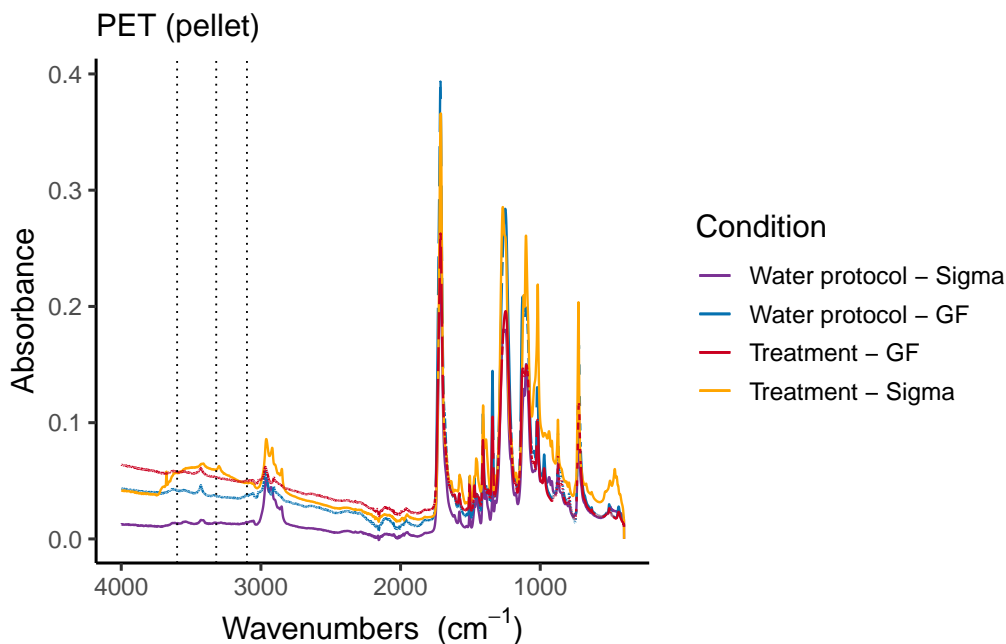


Figure 2.17: Examples of ATR-FTIR spectra in control condition (blue and purple lines) and after digestion protocol (red and orange lines) for PET from GoodFellow and Sigma manufacturers. Dotted lines represent wavenumbers 3600, 3320 and 3100 cm^{-1} .

Microscopic analysis of samples is a critical step for particles detection and we tested the gains obtained with a second and third inspections by different operators (Fig. 2.18). We found that, on average, 23 min (± 10.4 SD) were needed for the first inspection of a sample and that it allowed to recover 91.1% of particles found in the sample. The second and third inspections lasted 5.6 min (± 1.9 SD) and 6.4 min (± 2.0 SD), respectively, and allowed to recover 6.7% and 2.3% of detected particles, respectively. We concluded that two stereomicroscope analyses, by two different operators, represent a good compromise between analyses time and particles recovery, both in terms of quantity and characteristics of microplastics. Considering the instrumental size limitation associated with the detection and quantification of particles by visual inspection using a microscope (Filella, 2015), the selected size range (700 μm – 5 mm) favors an optimal chemical identification by attenuated total-reflectance Fourier-transformed infra-red (ATR-FTIR) spectroscopy, in which a minimum score of 60% of library match was applied.

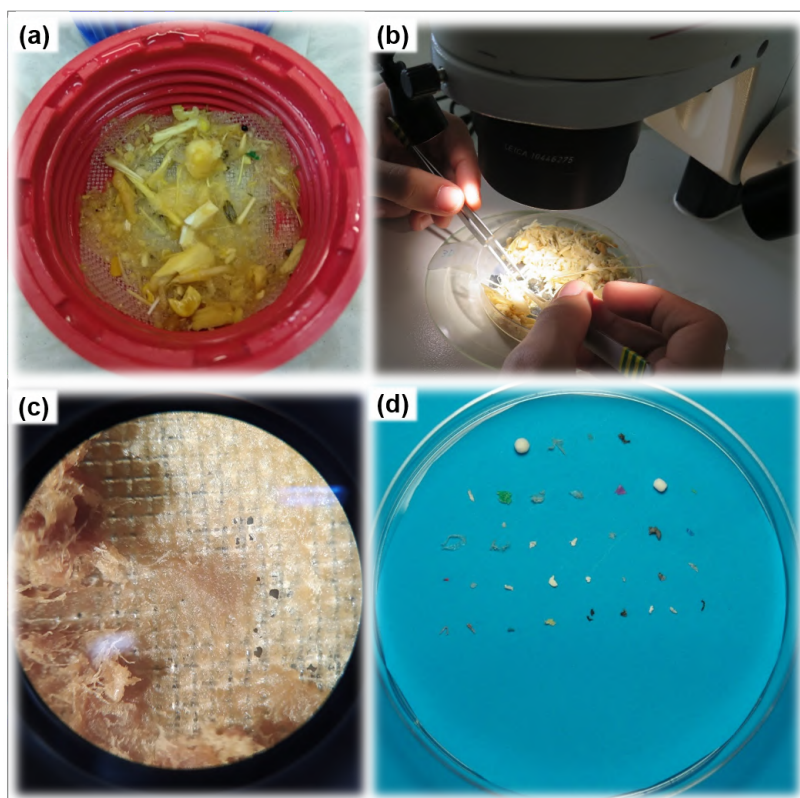


Figure 2.18: Sample processing following the digestion of organic matter, firstly the (a) transfer to the open cap, then (b) microscopic inspection with (c) magnified view and an example of (d) recovered particles.

Applied protocol:

After validating the critical aspects, the protocol applied for surface water samples is described at Figure 2.19.

2.4.2 Sediments

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) ($d = 1.6 \text{ g} \cdot \text{cm}^{-3}$). 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 (Fig. 2.11). 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 polystyrene petri dishes at room temperature for further analyses.

2.4.3 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 (Fig. 2.11). 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 polystyrene petri dishes at room temperature for further analyses.

2.4.4 Angling baits

Samples were first gently homogenised with a mortar and pestle, and then incubated in glass bottles equipped with aluminium caps for 48 h with H_2O_2 (w/w) (30%) solution to digest organic matter. Then, samples were filtered through a 500 μm mesh Nitex and washed with distilled water and ethanol (70% solution in water). The retained

Box 1 | Protocol for surface water samples

1. At the laboratory, wet mass of samples were measured
2. Over a 500 µm sieve, coarse organic and inorganic debris, such as branches, pebbles, leaves and gravels, and particles larger than 5 mm, were removed by rinsing with distilled water.
3. The retained content was transferred into labelled glass bottles of 250 mL.
4. KOH 10% (w/w) solution was added in a proportion of 4 units of volume (mL) for 1 unit of mass of sample (g).
 - If sample wet mass > 40 g, leading to > 160 mL of reagent, sample was splitted into several glass bottles to avoid overflow.
5. Pre-cut fabrics of Nitex tissue in square format (5 cm x 5 cm) were placed on the top of bottles and use open screw caps to lose (**Fig. 2.15**)
6. Bottles were covered with aluminum (foil or tray).
7. Samples were incubated in a water bath at 60 °C for 8 h.
 - Heating at 60 °C was proposed to reduce incubation period ⁽¹⁾
 - An adapted aluminum tray bath was employed, although an inox tray is recommended due to its higher resistance to oxidation.
 - Temperature was monitored with a thermometer immersed in a similar glass bottle filled with water only.
 - Water level in the bath was verified at every 2 h and refill when needed.
8. The bottles content was removed by pouring through the tissue.
9. 40 mL of distilled water was added with a syringe through the tissue.
10. Washing was enhanced through shaking and stirring the bottle.
11. The bottles content was removed by pouring through the tissue.
12. Steps 8 to 11 were repeated three times minimum or until obtaining a clear rinsing liquid.
13. H₂O₂ 30% (w/w) solution was added until fully cover the whole sample.
 - This step was performed with caution once this process may result in a highly reactive mixture.
14. Samples were incubated overnight at room temperature (16 h equivalent).
 - Due to the reactive mixture, samples were not heated. Then, the incubation period was slightly longer.
15. Steps 8 to 11 were repeated.
16. The filter-cap was removed, placed upside-down and sample was filtered through the tissue, adding water to remove all remaining content in the bottle (**Fig. 2.18a**).
17. The tissue with the retained sample was placed in labelled Petri-dish (8 cm diameter) and store at room temperature.

Figure 2.19: Box 1: Protocol for processing surface water samples (¹: Dehaut et al. (2016)).

content in the Nitex was stored in polystyrene petri dishes.

2.5 Sample analysis and microplastic identification

Following the sample treatment protocol, samples were analysed for the presence of microplastic in the size range from 700 μm to 5 mm. Figure 2.20 describe the protocol applied for microplastic for all samples of this work.

2.6 Quality control and contamination assessment

Although low risks of over or under estimation within the size range of microplastics targeted ($> 700 \mu\text{m}$) (Bruge et al., 2020), some procedures were set up to avoid cross contamination.

During field sampling, all the equipment was rinsed with river water. In addition, “100% cotton” clothing was used whenever possible to minimize potential contamination.

In the laboratory, all material was previously rinsed with distilled water and ethanol. Metal and glassware were used whenever possible and rinsed with ethanol before use. All the procedure was performed under a fume-hood and samples recipients remained covered with original caps or aluminum foil. A cotton lab-coat and nitrile gloves were always worn, and work surfaces were cleaned with ethanol.

Fibers particles were not included as microplastic particles considered in the study of Chapters 3 and 4, given the considerable amount of remaining organic matter that hampered the particles recovery.

In Chapter 5 the solvents, distilled water, ethanol and H_2O_2 solution were previously filtered through 8 μm polyethersulfone membranes (Sterlitech, EUA) to avoid external contamination. The original packaging of each angling bait was sampled and submitted to the same ATR-FTIR analysis to determine its polymer composition. A total of 10 replicates of silica powder (50 μm , Interchim), with around 10 g each, were used as blanks and submitted to the same entire process in the same sample batch. The microscopic inspection indicated the absence of suspicious particles in the size range of this study and contamination was therefore considered negligible.

For Chapter 6, 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)

Box 2 | Protocol for microplastic identification

1. The petri-dish was analysed under a stereomicroscope (14-fold magnification suggested) (Leica MZ 75 and Nikon SMZ 800) and potential plastic particles were selected using metallic tweezers, placed temporarily in a new identified petri-dish (Fig. 2.18).
 - The time of analysis may strongly vary depending on the amount of remaining organic matter and microplastic concentration.
2. Step 1 was repeated.
 - To reduce the risk of missing microplastic through manual selection and to avoid potential bias in detection (e.g. particle color, visual appearance), a second stereomicroscope analysis was performed by a different operator.
 - The order of processed samples was randomized.
 - For macroinvertebrates, a single inspection was performed due to very small amounts of organic matter remaining
3. Each particle was pictured together with a ruler or size reference using a stereomicroscope (Leica MZ16) equipped with a digital camera (DP20, Olympus, Japan). They were then stored individually.
 - A 96 well-plate was used for storing, at room temperature, individually all particles until further analyses.
4. The shape of each particle into one of five predefined categories (adapted from ⁽¹⁾):
 - 4.1 line or fibre: thin elongated items with one dimension significantly greater than the other two;
 - 4.2 film: sheets, with their thickness significantly lower than other two dimensions;
 - 4.3 fragment: pieces of thick plastics of irregular shape with all three dimensions comparable;
 - 4.4 pellet: pieces of regular and non-rounded shape or primary produced particles;
 - 4.5 sphere: three dimensional items of spherical shape.
5. Particles were classified into predefined categories of colors, as black, white, blue, green, grey, red and yellow ⁽²⁾.
6. ImageJ picture software was used ⁽³⁾ to measure the two main orthogonal axes in the picture of each particle (i.e. maximal length and height) as its perimeter and area (Fig. 2.21).
 - The particle width may be estimated considering each particle shape category.
7. The mass of each particle was measured individually (AT21 Comparator, d = 0.001 mg, MettlerToledo) (nearest 0.001 mg) and they were stored back in the same location within the well plate.
 - For **Chapter 5**, there were four particles of mass < 0.001 mg that were not included in calculations of mass concentration.
8. Each particle was analysed by attenuated total reflectance Fourier transformed infrared (ATR-FTIR) spectroscopy (Thermo Nicolet 6700, Thermo Fisher Scientific) equipped with a diamond crystal.
 - The ATR crystal was cleaned with ethanol with a background scan prior to analysis of a set of 24 particles.
 - The IR spectra were obtained with a resolution of 4 cm⁻¹ over the wavenumber range from 400 to 4000 cm⁻¹ applying at least 8 scans.
 - The spectrum of a sample obtained using an ATR accessory is not identical to the spectrum obtained by transmission. As most of commercially available spectral libraries and published peak tables contain and refer to transmission spectra, each spectrum was ATR-corrected (OMNIC software) and then compared with the reference spectra of synthetic polymers from commercially available libraries using OMNIC software (Thermo Fisher Scientific).
 - The similarity threshold of 60% was settled for the chemical composition to be assigned to the particle ⁽⁴⁾ otherwise it was considered as non-identified.
 - The identified particle was then classified as the polymer type (or polymer artificial additive), based on the Polymer Properties Database ⁽⁵⁾ when available, or as non-plastic.
 - The final categories of MP composition included: polyethylene (PE), polypropylene (PP), polystyrene (PS), polyester (including polyethylene terephthalate, PET), polyacrylate, polyvinylester, polyamide, polyurethane, polydiene, polysiloxane, polyethersulfone, tire and bitumen MP particles (TBMP) ⁽⁶⁾ and artificial additives (considered here as polyolefin-based or alkyd resins, as waxes, oils and coatings lubricants ⁽⁷⁾). The last two categories were considered as microplastics ⁽⁸⁾, with the latter showing similarities with paint particles ⁽⁹⁾.

Figure 2.20: Box 2: Protocol for microplastic identification (¹: Zobkov et al. (2020), ²: Mani and Burkhardt-Holm (2019), ³: Rasband (1997), ⁴: Masura et al. (2015), ⁵: (“Polymer Database,” 2020), ⁶: Leads and Weinstein (2019), ⁷: Su et al. (2019), ⁸: Hartmann et al. (2019), ⁹: Verschoor et al. (2016)).

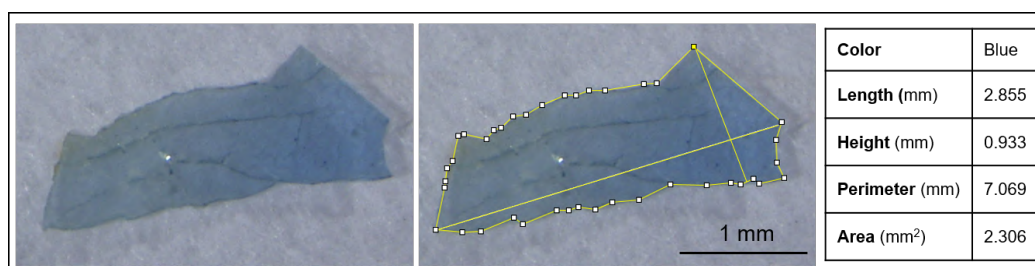


Figure 2.21: Measurement of microplastic length using ImageJ. The color was assigned based on existing color pallet (Mani and Burkhardt-Holm, 2019)

to avoid contamination. Control samples were subjected to the same protocol as the other samples. Overall, a single fiber was found among the 81 control samples and contamination was therefore considered as null.

3 | Environmental drivers of the spatial and temporal changes in microplastic pollution in surface water

Adapted from: de Carvalho, Aline Reis, Flavien Garcia, Louna Riem-Galliano, Loïc Tudesque, Magali Albignac, Alexandra ter Halle, and Julien Cucherousset. 2021. “Urbanization and Hydrological Conditions Drive the Spatial and Temporal Variability of Microplastic Pollution in the Garonne River.” *Science of The Total Environment*. 769: 144479. <https://doi.org/10.1016/j.scitotenv.2020.144479>

Abstract

Microplastic (MP) pollution represents a novel environmental pressure acting on freshwater ecosystems. Improving our understanding of the dynamics of MP pollution in freshwater ecosystems is therefore a prerequisite for managing and limiting this pollution. In this study, we quantified the spatial and temporal variability of MP (size range 700 μm – 5 mm) pollution in surface water in 14 sites located across the Garonne river catchment (Southwestern France, 6 in the main river and 8 tributaries). MP concentration averaged 0.15 particles.m⁻³ (\pm 0.46 SD) and strongly varied both in space and in time. We found that the spatial variation in MP concentration was driven by urbanization and that the temporal variation in MP concentration and MP size was driven by hydrological conditions, with higher concentrations and smaller particles sizes in warm seasons with low discharge. Polyethylene (44.5%), polystyrene (30.1%) and polypropylene (18.2%) were the main polymers and their proportion did not vary significantly across sampled sites. Particle color was associated with polymer type, with a high proportion of white particles in polystyrene. We also found a significant and negative relationship between MP size and the distance to the source in sites located in the main stream. MP pollution across watershed, from headwater tributaries to lowland rivers, is dynamic, and further studies are needed to improve the resolution of our knowledge of spatial and temporal patterns of MP pollution in freshwater ecosystems.

3.1 Introduction

Freshwater ecosystems provide countless services to humans, but they are facing multiple disturbances induced by global changes (Vörösmarty et al., 2010). Habitat fragmentation (Morita et al., 2009), water pollution (Couceiro et al., 2007), climate changes (Magnuson et al., 1997) and biological invasions (Gallardo et al., 2016) are among the multiple factors threatening freshwater ecosystems and their rich biodiversity. Microplastic (MP) pollution has recently emerged as a novel source of concern with potential effects on freshwater biodiversity and ecosystems that remain to be quantified (Eerkes-Medrano and Thompson, 2018; Eerkes-Medrano et al., 2015). Rivers are at the heart of the dynamic of plastic pollution (Rochman, 2018), notably because they convey 70–80% of the plastics observed in marine ecosystems (Horton, Walton, et al., 2017). In aquatic environment, plastics undergo a degradation process through mechanical abrasion, photochemical alteration and other weathering processes (Andrady, 2011; Gewert et al., 2015; Halle et al., 2017) which leads to the production of MP, i.e. plastic fragments smaller than 5 mm (Arthur et al., 2009; Thompson et al., 2009). In addition, primary MPs (i.e. those not originated by fragmentation of larger debris) often found as cosmetics additives and drug vectors (Cole et al., 2011) can directly enter freshwater ecosystems.

MP pollution is a ubiquitous phenomenon (Lusher et al., 2015; Rochman, 2018; Woodall et al., 2014) and the presence and accumulation of MP in ecosystems represent an important toxicological risk for organisms through direct and indirect ingestion (Prata, J. da Costa, Lopes, et al., 2020; Smith et al., 2018). The study of MP properties, such as composition, density, size and color, can not only contribute to elucidate their origins, but also provides insights into the drivers of their consumption by aquatic organisms (Collard et al., 2019; Garcia et al., 2021; T. Wang et al., 2019). MP can be ingested by many freshwater consumers taxa, from invertebrates (Windsor, Tilley, et al., 2019) to fish (McNeish et al., 2018; Roch et al., 2019; Sloomakers et al., 2019) and the consequences of MP consumption on individual are highly variable (Foley et al., 2018). Although there has been a recent increase in the number of studies investigating MP pollution in freshwater ecosystems, improving our understanding of the dynamics of this pollution in these ecosystems is essential (Eerkes-Medrano and Thompson, 2018; Horton, Walton, et al., 2017).

In rivers, MP pollution varies spatially and is strongly affected by land use (Skalska et al., 2020). Urbanization is a key driver of MP pollution in freshwater ecosystems (Baldwin et al., 2016; Cable et al., 2017; Grbić et al., 2020), and, in highly urbanized

areas, MP contamination levels are comparable to those observed in marine environments (Horton, Walton, et al., 2017). However, our knowledge of the effects of different land use practices on the characteristics of MP pollution remains limited, and MP composition has already been identified as an approach to identification of microplastic sources (Chen et al., 2020). MP pollution can also vary temporally through changes in hydrological and meteorological conditions. Indeed, flood and rainfall can regulate the mobilization of particles previously settled in sediments or on land (Zhang et al., 2017). For instance, MP pollution can be affected by weather conditions and increase after precipitation events (Eo et al., 2019) and several studies have demonstrated a positive correlation between rainfall rates and MP pollution (Cheung et al., 2019; Dris, Gasperi, et al., 2015; G. Wong et al., 2020; Yonkos et al., 2014). The effects of seasonal variability are more ambiguous, with studies showing either the presence (Han et al., 2020; Wu et al., 2019) or the absence (Mani and Burkhardt-Holm, 2019; Rodrigues et al., 2018) of seasonal patterns. Despite their importance for the development of efficient management strategies on MP pollution in freshwater ecosystems, integrative quantification and characterization of MP pollution and comprehensive analyses of its spatial and temporal drivers are lacking (Lebreton et al., 2017; C. Li et al., 2020).

The present study aims to fill this gap of knowledge by quantifying the environmental determinants of the spatial and temporal variability of MP pollution (particles with a size range from 700 μm to 5 mm) in surface water of the Garonne river (Southwestern France). We first quantified the changes in MP concentration across sampling sites and sampling events. We tested the hypothesis that MP concentration was variable spatially and temporally and associated to changes in environmental conditions. The variability in environmental conditions between sampling sites and events was quantified using a multivariate approach. Second, we investigated the spatial and temporal changes in MP composition. We tested the hypothesis that MP composition was different between sampling sites but not between sampling events and correlated with changes in spatial environmental conditions. Third, we explored changes in MP size and hypothesized that MP size varied in time and space, and that this variation was related with changes in environmental conditions. We also tested if there was an overall size difference between MP polymers. Finally, we quantified changes in MP size along the upstream-downstream gradient in the main river.

3.2 Material and methods

Sampling strategy and sample treatment are described at Chapter 2.

3.2.1 Environmental conditions

Environmental conditions in each sampling site and at each event were summarized using multivariate analyses based on a series of spatial and temporal descriptors. Environmental parameters related to water characteristics, as temperature and turbidity (NTU – Nephelometric Turbidity Unit), were measured with a DO probe (ProDSS Multiparameter Water Quality Meter, YSI, USA) at each sampling event and for each site (Table 3.1). Daily discharge of each site (except SLN that had no gauge) were obtained from the Agence de l'Eau Adour-Garonne (Hydro Eau France, 2020) (Table 3.1). Daily air temperature and precipitations were obtained from Meteo France (Meteo France, 2020) (Table 3.2). River width was measured at each site using aerial pictures (Géoportail, 2020). A Geographic Information System (ArcGIS v.10.6, ESRI, Redlands, CA, USA) was then used to calculate, for each site, the distance to the Garonne river source, drainage area, land cover (urban and agricultural) and human population. The distance to the Garonne river source (km) was calculated between each sampling site and the source of the Garonne river following the main river bed and the drainage area (km²) represented the area of land drained in each site. Urban and agricultural land cover (%) was calculated at a predetermined buffer scale of 5 km long with 1 km large upstream of the each sampling site using the Corine Land Cover database (European Environment Agency, 2018). Human population (numbers of inhabitants) was calculated using the same buffer (INSEE, 2018) which described the population in each municipality. Because municipality did not exactly overlap with our buffer, we calculated the percentage of municipalities' area included in the buffer to assess the numbers of inhabitants.

Table 3.1: Water characteristics of each sampled site at the four sampling events: discharge ($\text{m}^3 \cdot \text{s}^{-1}$), temperature (Temp, $^{\circ}\text{C}$) and turbidity (NTU, Nephelometric Turbidity Unity).

Code	February 2019				April 2019				July 2019				October 2019		
	Dis-charge	Temp.	NTU	pH	Dis-charge	Temp.	NTU	pH	Dis-charge	Temp.	NTU	pH	Dis-charge	Temp.	NTU
LBA	25.3	6.4	4.1	7.59	34.1	9.4	5.4	7.8	28.7	14.2	4.8	8.38	11.8	11.6	2.3
SLN	-	7.2	3.1	7.94	-	10.9	1.8	8.3	-	16.2	1.5	8.84	-	11.9	1.8
LBI	46.7	8.3	5.7	7.87	48.4	11.9	2.5	8.2	37.2	18.1	2.9	9.1	16.9	14.9	1.8
RSG	48.6	7.9	2.1	7.96	38.1	12.6	2.9	8.3	22.9	20.7	1.6	9.8	13.3	16.2	1.4
MUL	5.91	9.3	7.6	8.18	3	13.2	6.7	8.07	1.99	21	1.9	8.5	2.22	16.8	6.9
MUG	179	9.4	3.4	8.08	198	13	4.8	7.94	82.4	22.5	3	8.91	45.6	17.4	2.6
GRP	60.3	8.1	6	7.88	63.5	12.4	3.6	7.77	21.5	21.1	2.9	8.3	17.1	16	2.9
LAU	4.54	8.9	26.4	7.91	1.9	16.2	16.2	7.99	0.95	24.2	28.4	8.81	0.9	15.8	35.3
TOU	2.25	8.5	10.6	8.06	1.21	15	6.1	8	0.613	24.2	5.8	9.02	0.53	16.3	10.1
GSG	216	7.7	7.3	7.81	174	13.9	6.8	7.86	80.1	22.6	5.9	8.4	44	16.6	2
CAS	224	8.3	10.7	7.84	162	14.4	6.1	7.78	69.7	26.6	0.9	9.11	49.3	-	-
GRN	4.71	7.6	13.7	7.96	2.56	14.4	9.1	8.03	1.46	21.8	13.8	8.26	1.35	15.6	18
LAY	4.38	7.7	22.2	7.75	3.2	14.5	5.3	7.89	2.86	24	8	8.64	1.09	16.6	8.7
AGE	480	8.1	9.6	7.48	263	14.6	5.2	7.79	110	25.3	1.7	8.14	75.7	18.5	5

Table 3.2: List of sampled sites with the correspondent meteorological station (MeteoFrance, 2020), used to obtain precipitation (mm) and air temperature ($^{\circ}\text{C}$), and river station (Hydro Eau France, 2020), used to obtain river discharge ($\text{m}^3.\text{s}^{-1}$).

Code	Meteorological station	River station
LBA	Clarac	La Garonne à Chaum
SLN	Clarac	La Garonne à Chaum
LBI	Clarac	La Garonne [partielle] à Saint-Gaudens [Valentine]
RSG	Palaminy	Le Salat à Roquefort-sur-Garonne
MUL	Muret	La Louge à Muret
MUG	Muret	La Garonne à Portet-sur-Garonne
GRP	Muret	L'Ariège à Auterive
LAU	Toulouse	L'Hers Mort à Toulouse [Pont de Périole]
TOU	Toulouse	Le Touch à Plaisance-du-Touch
GSG	Toulouse	La Garonne à Verdun-sur-Garonne
CAS	Castelsarrasin	La Garonne à Verdun-sur-Garonne
GRN	Ondes	La Save à Larra
LAY	Agen	Le Gers à Montestruc-sur-Gers
AGE	Agen	La Garonne à Lamagistère

3.2.2 Statistical analyses

We first conducted two Principal Component Analyses (PCA) to summarize the spatial and temporal variability in environmental conditions and avoid collinearity among variables used to assess the environmental determinants of MP pollution. Regarding environmental variability across sampling sites, 6 variables (namely river width, drainage area, mean yearly discharge, human population, urban land cover and agricultural land cover) were used. The first two axes of the PCA (eigenvalues of 3.06 and 2.31, respectively) represented 89.39% of the total inertia and were selected for subsequent analyses. The first axis or principal component (PC)1 was defined as **river size** as it was strongly associated ($r > 0.60$) with river width, drainage area and mean yearly discharge (Fig. 3.1a). This axis discriminated large sites located downstream in the Garonne river with high discharge (e.g. AGE, CAS, GSG) from smaller sites, located upstream in the Garonne river and its tributaries displaying lower discharge (e.g. LBA, GRN, LAY). The second axis (PC2) was defined as the level of **urbanization** as it was strongly associated ($r > 0.60$) with human population density, urban

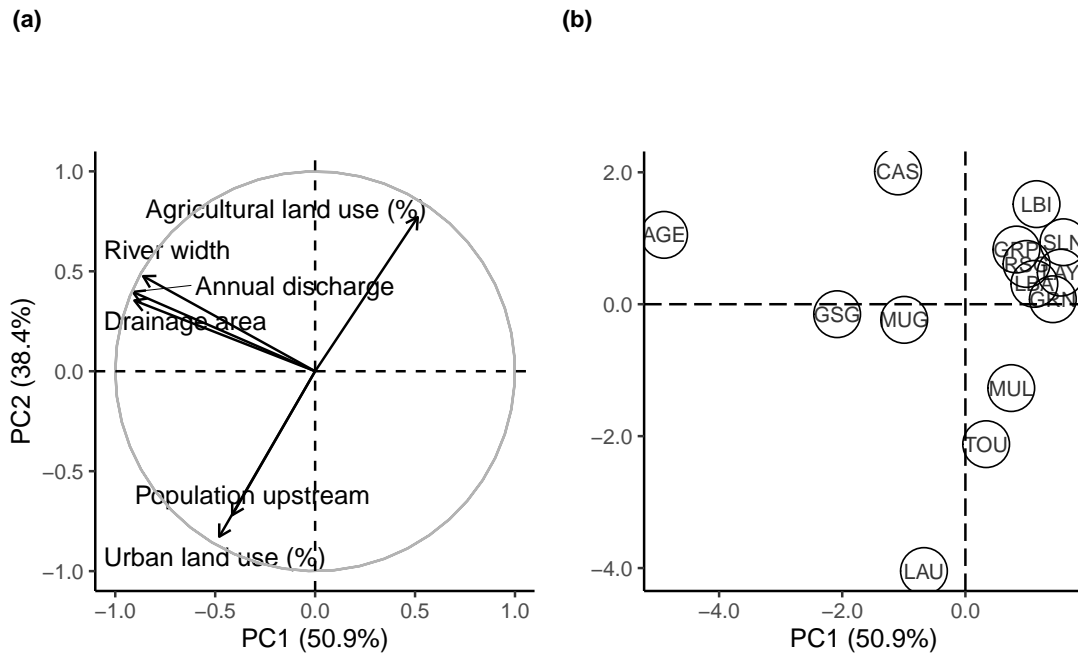


Figure 3.1: Summary of environmental conditions on the PCA across sampling sites: (a) correlation circle for spatial variables and (b) distribution of sampling sites along PC1 (**river size**) and PC2 (**urbanization**) axes.

land cover and agricultural land cover (Fig. 3.1b). This axis discriminated sites located in an agricultural landscape with low human population density (e.g. LBI, SLN, GRP) from sites with high population density located in highly urbanized area (e.g. LAU, TOU, GSG).

Regarding environmental variability across sampling events, we used 7 variables in the PCA representing the temporal changes in environmental conditions: water temperature, air temperature (day before sampling), precipitations the day before sampling and accumulated across three days before sampling, relative turbidity (calculated as the relative values across the 4 sampling events within each site), relative discharge (calculated as the relative values across the 4 sampling events within each site), and discharge fluctuation (relative change in discharge observed within 3 days before sampling). The first two axes of the PCA (eigenvalues of 3.06 and 2.28, respectively) represented 76.33% of the total inertia and were selected for subsequent analyses. The first axis (PC1) represented seasonal **hydrological conditions** as it was strongly associated ($r > 0.60$) with relative discharge, relative turbidity and air and water temperatures (Fig. 3.2a). This axis discriminated sampling events performed

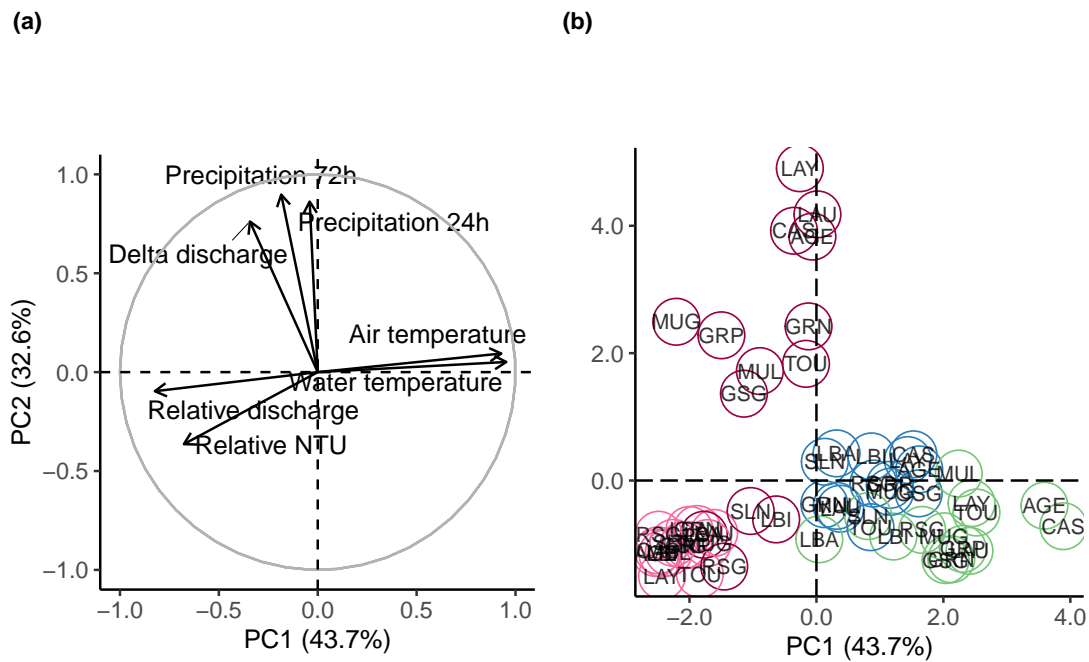


Figure 3.2: Summary of environmental conditions on the PCA across sampling events: (a) correlation circle for temporal variables and (b) distribution of sampling events along PC1 (**seasonal hydrological conditions**) and PC2 (**weather changes**) axes, with february represented in light pink, april in dark pink, july in green and october in blue.

in low discharge and turbidity conditions and with high air and water temperatures (e.g. July) from events performed when discharge and turbidity were high, and air and water temperatures were low (e.g. February). The second axis (PC2) was strongly associated ($r > 0.60$) with precipitation that occurred 24 h and 72 h before sampling and changes in discharge, and was therefore defined as **weather changes**. This axis discriminated sampling events that occurred in dry conditions (e.g. July) from events that occurred with some rainfall and increased stream discharge (e.g. April) (Fig. 3.2b).

MP concentration was calculated as the number of microplastics particles divided by the volume of filtered water ($\text{MP}\cdot\text{m}^{-3}$). We used a linear mixed-effects model (lmm) with sampling event or sampling site as a random factor to test if MP concentration (log-transformed) differs between sampling sites or between sampling event, respectively. A similar model was then used to test the effects of spatial environmental conditions (PC axes: **river size** and **urbanization**) on MP concentration, with sampling event as random factor. We then tested the effects of temporal environmental

conditions (PC axes: **seasonal hydrological conditions** and **weather changes**) on MP concentration, with sampling site as random effect. Fisher Exact test was then used to compare MP colors between MP composition.

Spatial and temporal variations in proportion of the three main polymers types (polyethylene, polystyrene, and polypropylene) were tested using generalized mixed-effects models (glmm), considering sampling event and sampling sites as random factors, respectively, using a quasibinomial family. We tested the effects of spatial environmental conditions (PC axes: **river size** and **urbanization**) on polymers proportion with sampling event as random factor. Then we tested the effects of temporal environmental conditions (PC axes: **seasonal hydrological conditions** and **weather changes**) on polymers proportion, with sampling site as random effect.

Linear mixed-effects models with sample code plus sampling event or sampling site as random factor were then used to test for differences in MP size (log-transformed) between sampling sites and sampling events, respectively. Similar models were then used to test the relationship between MP size (log-transformed) and spatial and temporal environmental conditions. The relationship between MP size (log-transformed) and the distance to Garonne source was tested using a linear mixed-effects model with sample code and polymer type as random factors. Finally, a linear mixed-effects model (lmm) was used to test differences in MP size (log-transformed) between polymer types, with sample code as random factor.

All statistical analyses were performed using R v.4.0.2 (Team, 2019) and linear mixed effects models were performed using the package lme4 v.1.1.10 (Bates et al., 2015). Generalized linear mixed-effects models-PQL were performed using the package MASS (Venables et al., 2002). Significant levels of mixed effects models were obtained using the ‘Anova’ function in the car package (Fox and Weisberg, 2019). All explanatory variables were scaled (mean of zero and standard deviation of one) prior to analyses. Assumptions of linearity and homogeneity of variances on residuals from all models were checked visually. All full models were initially run with two-way interactions. As no interaction was significant, models were simplified.

3.3 Results

Some particles examples are described in Fig. A total of 1887 particles were visually detected. Among them, 1283 were within the studied size range (700 μm to 5 mm) and successfully identified by ATR-FTIR as microplastics (Fig. 3.3 and 3.4).

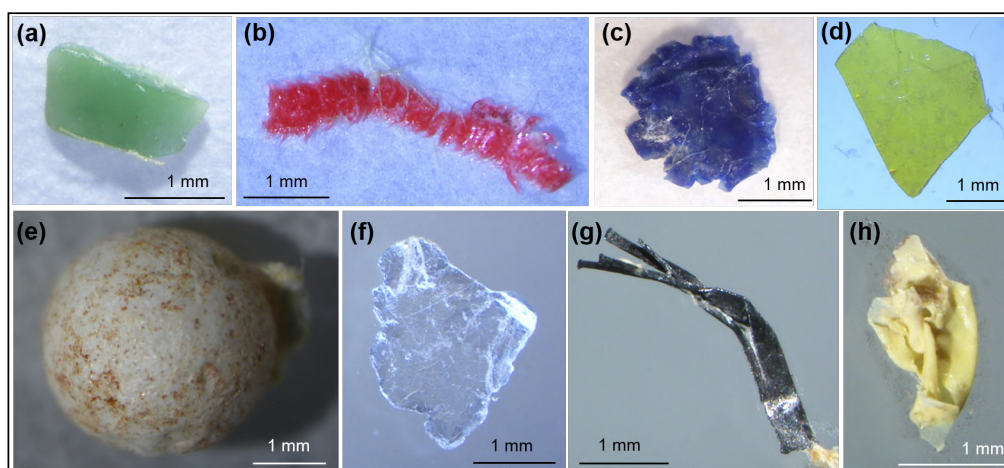


Figure 3.3: Examples of microplastics collected in surface water in the Garonne River: (a) green polystyrene; (b) red polyester; (c) blue polyethylene; (d) yellow polyethylene; (e) white polystyrene; (f) white polypropylene; (g) black polypropylene and (h) yellow polyurethane.

3.3.1 Spatial and temporal variation of MP concentration

MP concentration averaged 0.15 MP.m^{-3} ($\pm 0.46 \text{ SD}$) and ranged from 0 to 3.4 MP.m^{-3} across all sampled sites and events. There was overall a significant difference in MP concentration between sampling sites (lmm, $\chi^2 = 170.51$, $p < 0.001$). Specifically, we found that MP concentration was significantly higher in LAU than in TOU site, two sites highly urbanized (at Chapter 2 Table 2.2), that had higher MP concentrations compared to all other sites (post-hoc pairwise comparisons, $p < 0.05$, Fig. 3.5). There was a significant effect of the level of urbanization on MP concentration (lmm, $\chi^2 = 108.84$, $p < 0.001$), with MP concentration increasing with urbanization (Fig. 3.7a). There was no significant effect of river size on MP concentration (lmm, $\chi^2 = 3.43$, $p = 0.064$).

There was a significant difference in MP concentration between sampling events (lmm, $\chi^2 = 16.53$, $p < 0.001$) with significantly higher MP concentration in July than in February and October (post-hoc pairwise comparisons, $p < 0.05$, Fig. 3.6). MP concentration was significantly and positively related to seasonal hydrological conditions with MP concentration increasing in periods of low discharge (lmm, $\chi^2 = 11.20$, $p < 0.001$) (Fig. 3.7b). There was no significant effect of weather changes on MP concentration (lmm, $\chi^2 = 2.56$, $p = 0.109$).

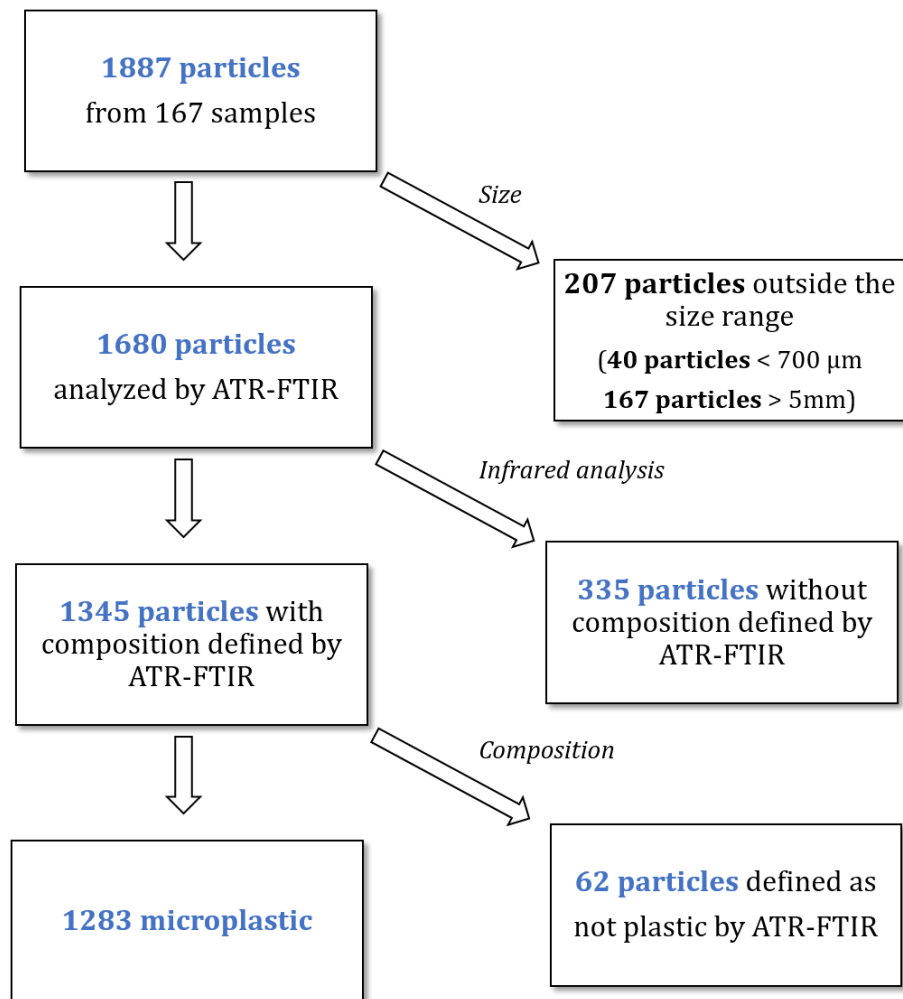


Figure 3.4: Flowchart of microplastic selection for data analyses.

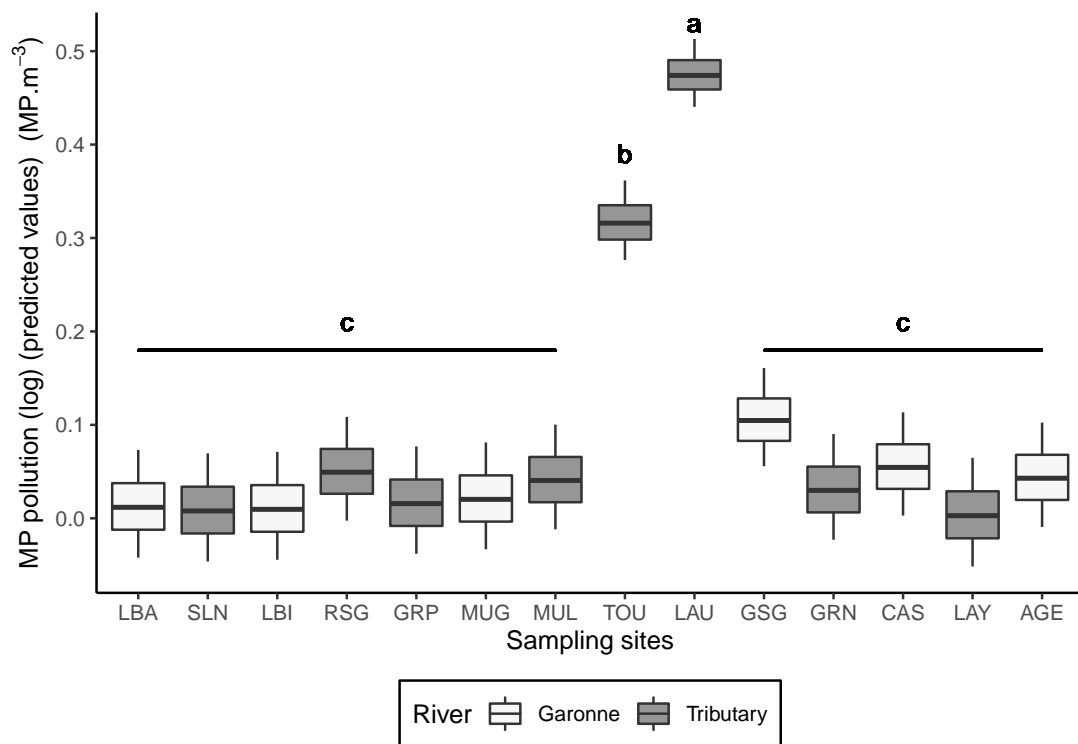


Figure 3.5: Microplastic concentration (log-transformed, MP.m⁻³) across sampling sites (from upstream to downstream). Different letters indicate significant differences ($p < 0.05$).

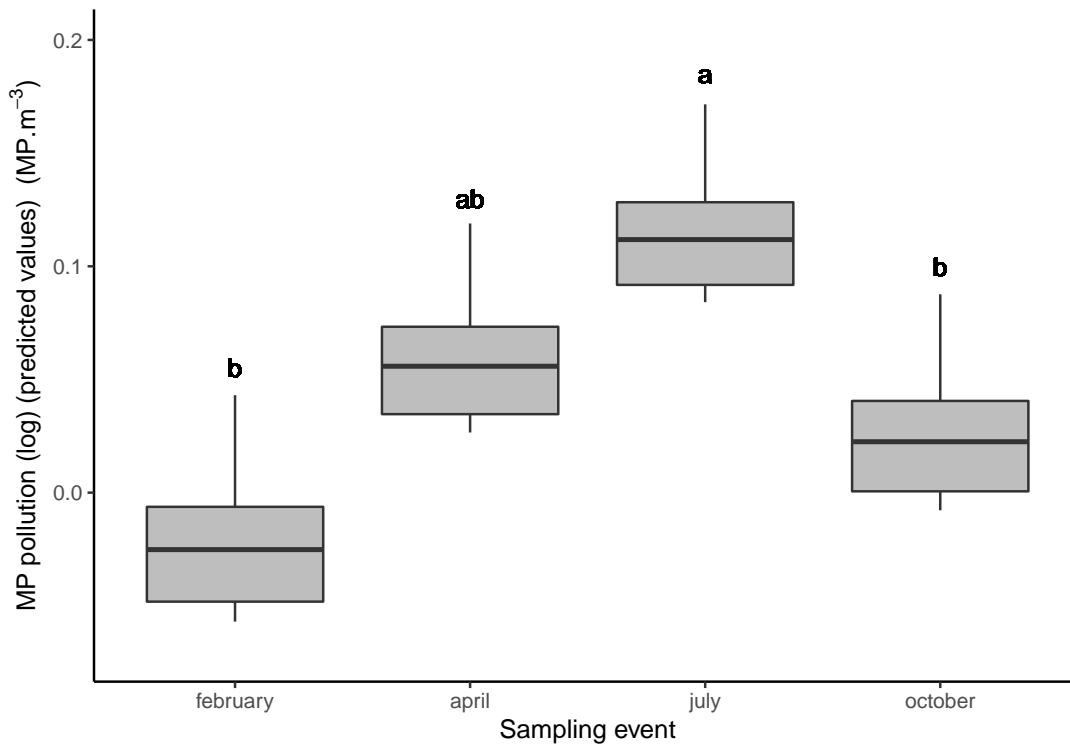


Figure 3.6: Microplastic concentration (log-transformed, MP.m⁻³) across sampling events. Different letters indicate significant differences ($p < 0.05$).

3.3.2 Spatial and temporal variation of MP composition

Three main types of polymers were collected, namely polyethylene (PE), polystyrene (PS) and polypropylene (PP), representing 44.5%, 30.1% and 18.2% of the total number of particles, respectively (Fig. 3.8). The other MP particles represented 7.2% of all microplastics. The three main colors of the MP particles were white, black, and blue, and represented 32.4%, 31.1% and 14.3% of particles, respectively. The other MP particles colors were red (7.6%), green (6.5%), yellow (4.5%) and grey (3.6%). The distribution of particle color significantly differed between polymer types ($p < 0.001$) with a main contribution of the higher proportion of white particles in the PS (37.9%) and smaller proportion of white particles in PE (12.7%) and black particles in PS (10.4%) (Fig. 3.8). The proportion of PE, the most abundant polymer, among the sampled MP particles was not significantly different between sampling sites (glmm, $\chi^2 = 12.67$, $p = 0.474$, Fig.3.9a). There was a significant difference between sampling events (glmm, $\chi^2 = 11.05$, $p = 0.011$, Fig. 3.9b), with a significantly lower proportion of PE measured in October compared to April and July (post-hoc pairwise comparisons, $p < 0.05$). There was no significant relationship between environmental drivers

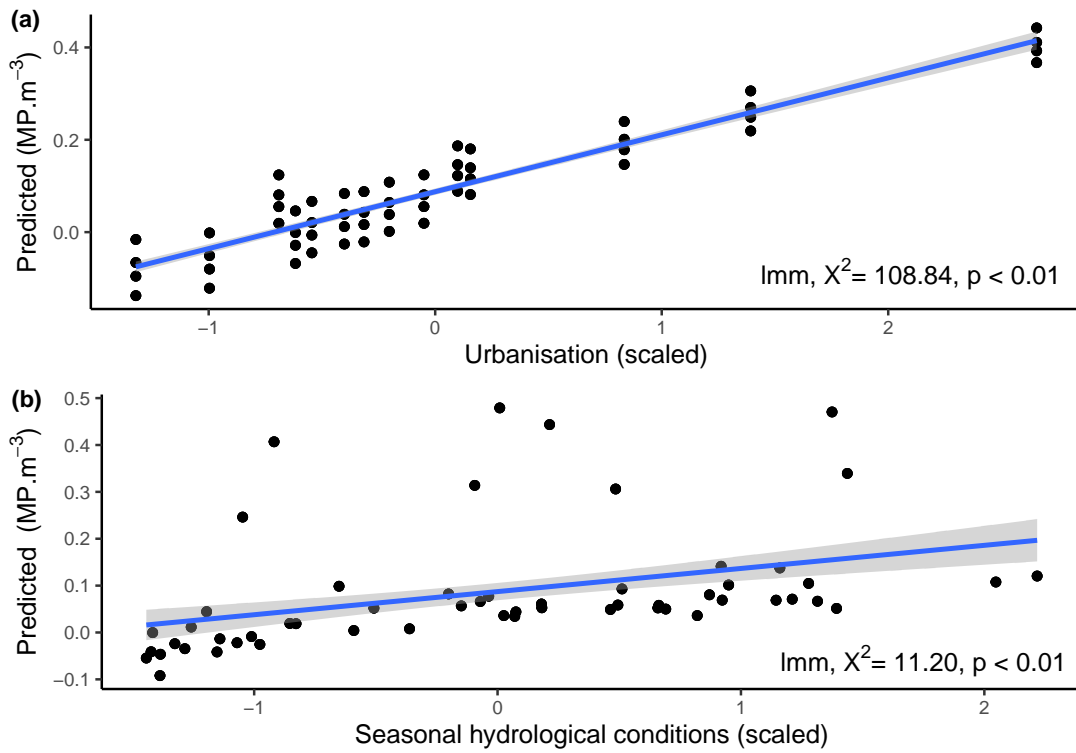


Figure 3.7: Predicted effects of (a) urbanization and (b) seasonal hydrological conditions on microplastic concentration (log-transformed, $\text{MP}\cdot\text{m}^{-3}$).

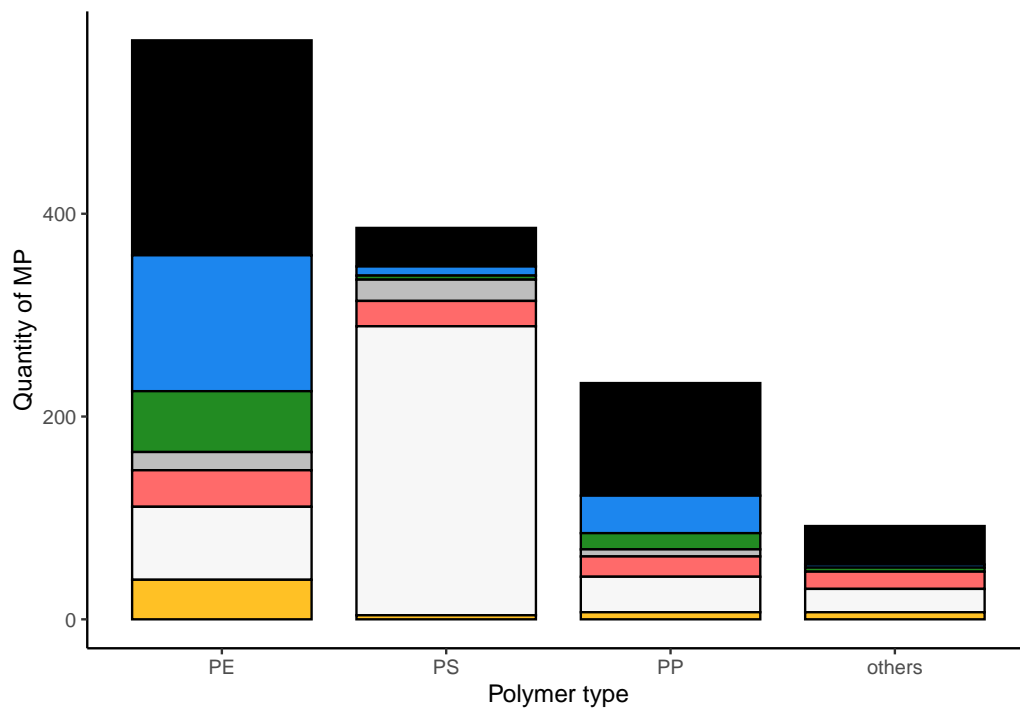


Figure 3.8: Distribution of particles colors for each polymer type. Displayed colors represent particles colors (black, blue, green, grey, red, white and yellow, respectively).

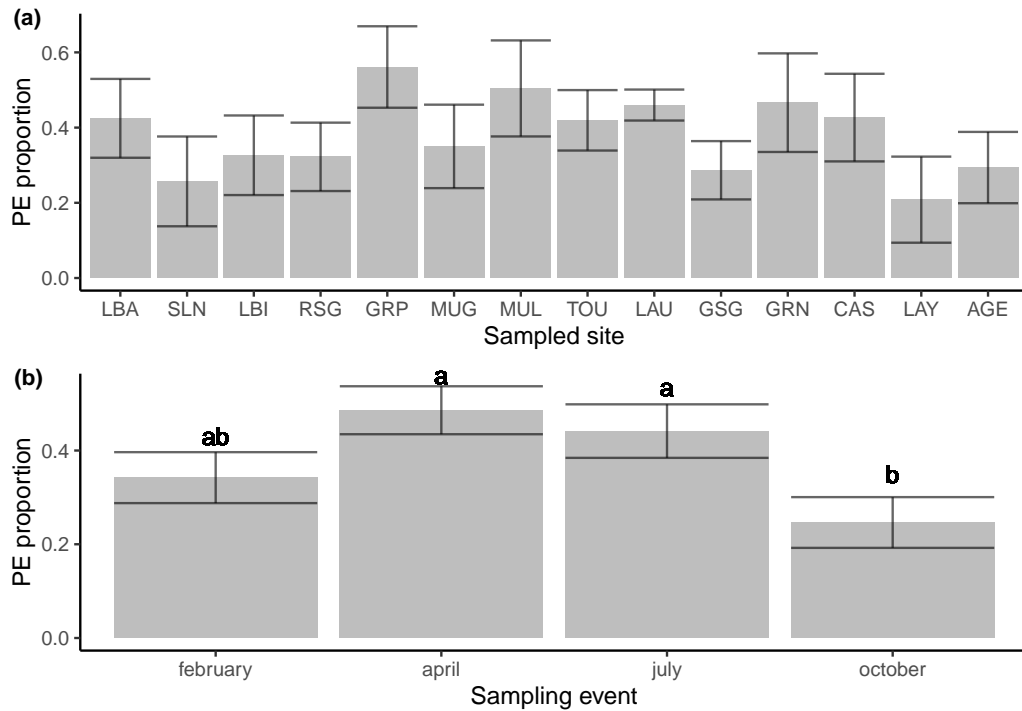


Figure 3.9: Spatial (a) and temporal (b) variability in the proportion of PE among sampled microplastics. Different letters indicate significant differences ($p < 0.05$).

and proportion of PE (Table 3.3). The proportion of PS was not significantly different between sampling sites (glmm, $\chi^2 = 11.60$, $p = 0.561$, Fig. 3.10a) and between sampling events (glmm, $\chi^2 = 2.87$, $p = 0.411$, Fig. 3.10b). There was no significant effect of environmental variables on the proportion of PS (Table 3.3). There was no significant difference in the proportion of PP between sampling sites (glmm, $\chi^2 = 19.40$, $p = 0.111$, Fig. 3.11a) and between sampling events (glmm, $\chi^2 = 6.126$, $p = 0.106$, Fig. 3.11b). The proportion of PP was significantly related to river size, with the proportion of PP increasing in larger sites, mainly located more in downstream of the drainage (Table 3.3). There was no significant effect of the other environmental variables on the proportion of PP (Table 3.3).

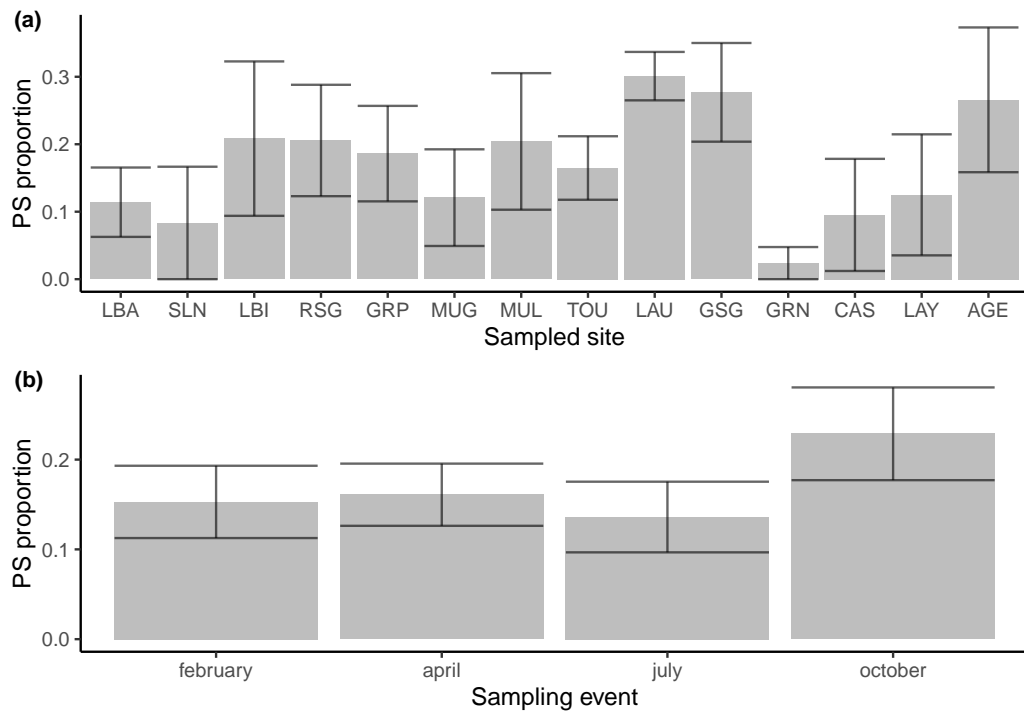


Figure 3.10: Spatial (a) and temporal (b) variability in the proportion of PS among sampled microplastics.

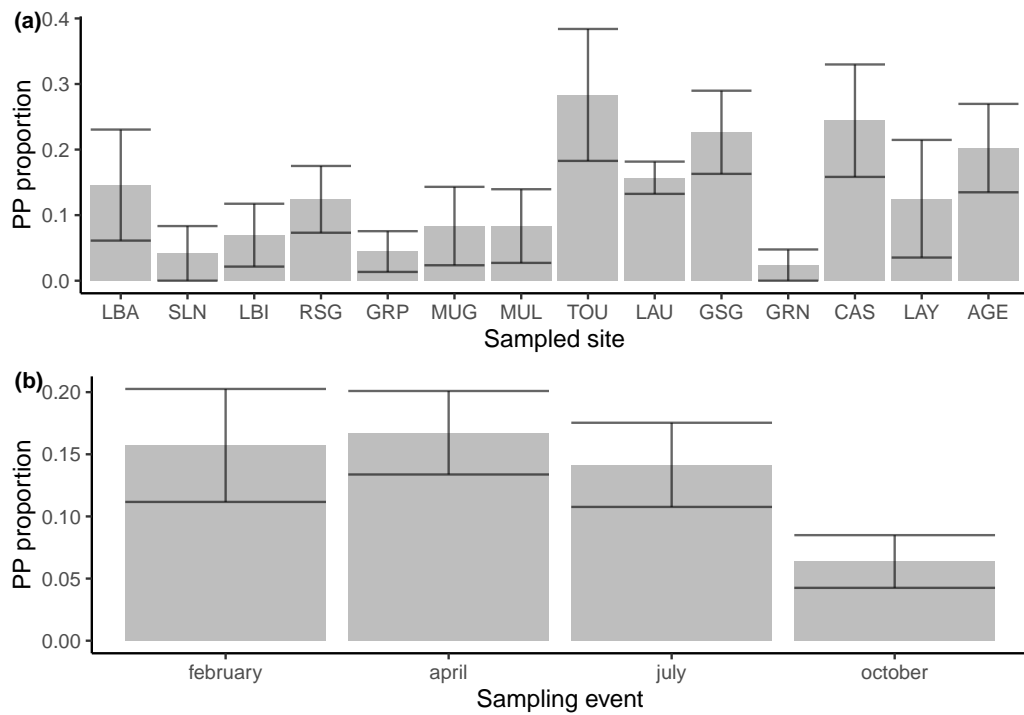


Figure 3.11: Spatial (a) and temporal (b) variability in the proportion of PP among sampled microplastics.

Table 3.3: Results of the mixed effect models testing the effects of environmental conditions of the proportion of the three main polymer types (PE, PS and PP).

Response	Spatial parameter	t	p	Temporal parameter	t	p
PE proportion	Urbanization	1.176	0.241	Seasonal changes	-0.014	0.988
	River size	-0.589	0.557	Weather changes	-0.664	0.507
	Intercept	-2.484	0.014	Intercept	-2.266	0.024
PS proportion	Urbanization	1.468	0.144	Seasonal changes	-0.119	0.905
	River size	1.929	0.055	Weather changes	-0.383	0.702
	Intercept	-10.254	0.000	Intercept	-10.605	0.000
PP proportion	Urbanization	1.001	0.318	Seasonal changes	-0.466	0.642
	River size	2.485	0.014	Weather changes	0.898	0.370
	Intercept	-10.225	0.000	Intercept	-11.441	0.000

3.3.3 Spatial and temporal variation of MP size

MP size averaged 2.31 mm (± 1.01 SD). There was no significant difference in MP size distribution between sampling sites (lmm, $\chi^2 = 19.34$, $p > 0.05$) (Fig. 3.12), and no significant effect of urbanization and river size on MP size (lmm, $p > 0.05$). There was a significant difference in MP size between sampling events (lmm, $\chi^2 = 12.91$, $p = 0.005$) (Fig. 3.13), with larger MP in February compared to other sampling events (post-hoc pairwise comparisons, $p < 0.05$). A significant effect of seasonal hydrological conditions on MP size was observed, with MP size decreasing in low hydrological conditions (lmm, $\chi^2 = 8.64$, $p = 0.003$). There was a significant difference in MP size between polymer types (lmm, $\chi^2 = 19.38$, $p < 0.05$), with PS being significantly larger than PE and PP (post-hoc test, $p < 0.05$) (Fig.3.14). In the main stream of the Garonne river, MP size significantly decreased when increasing the distance from the Garonne source (lmm, $\chi^2 = 3.91$, $p = 0.048$).

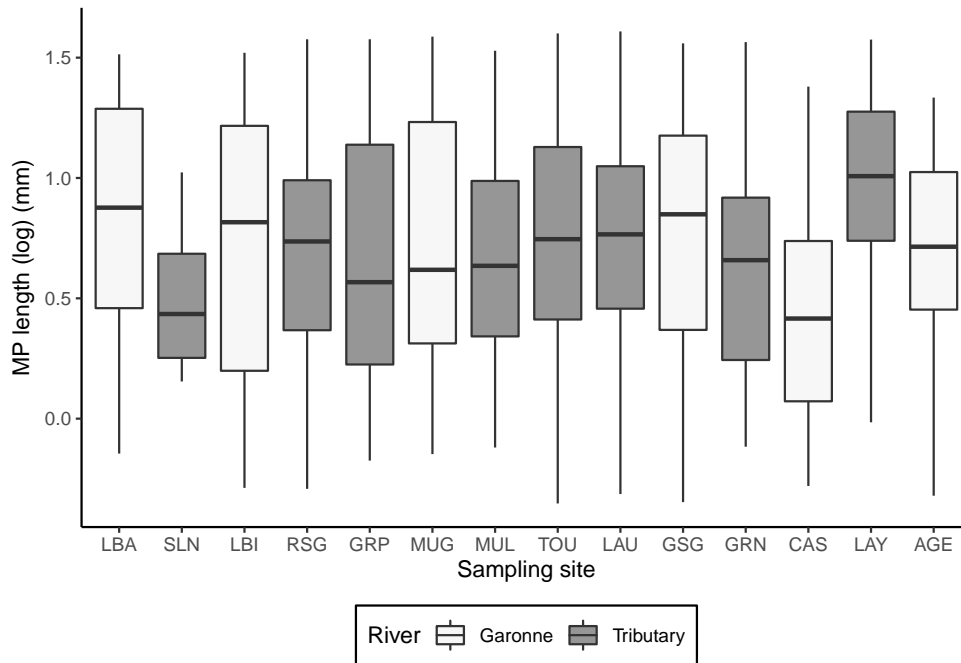


Figure 3.12: Microplastic size (log-transformed, mm) between sampling sites.

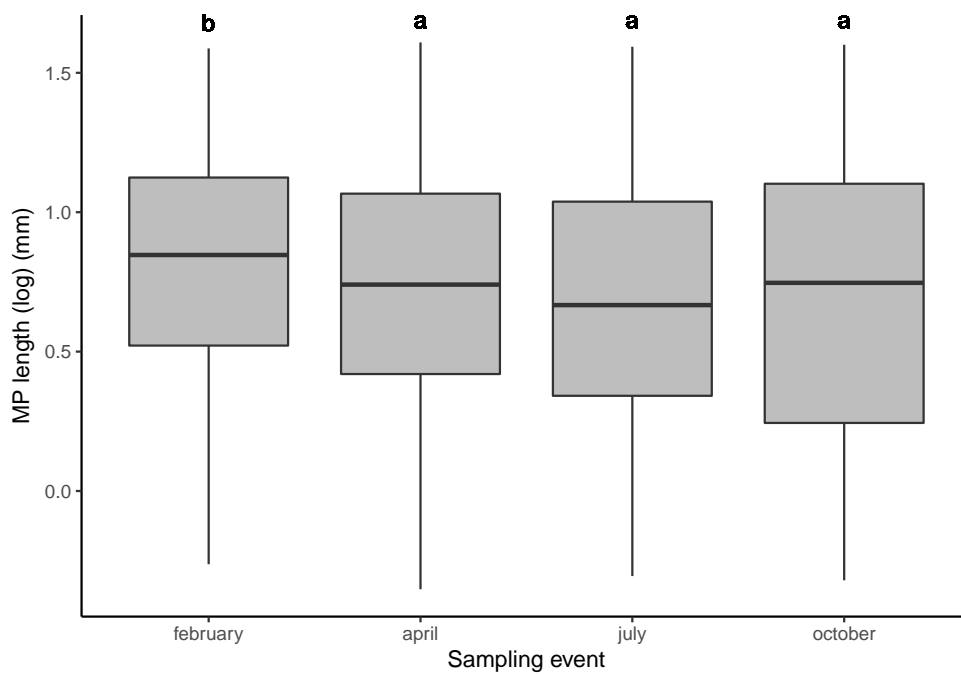


Figure 3.13: Microplastic size (log-transformed, mm) between sampling events. Different letters indicate significant differences ($p < 0.05$).

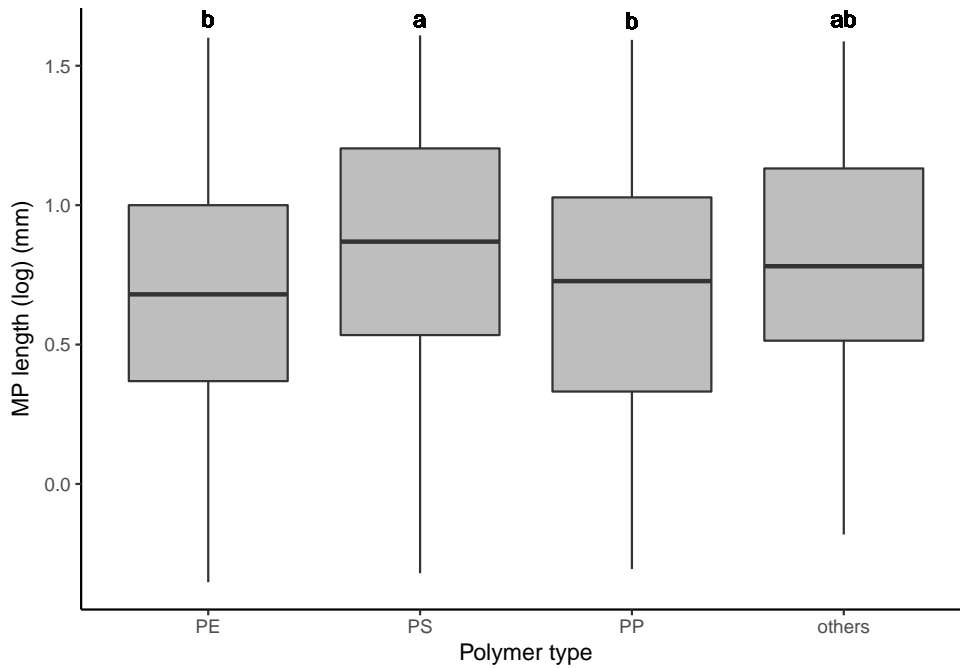


Figure 3.14: Microplastic size (log-transformed, mm) between polymer type. Different letters indicate significant differences ($p < 0.05$).

3.4 Discussion

The spatial and temporal dynamics of MP pollution in freshwater ecosystems are complex. We demonstrated that spatial variability in MP concentration observed at the catchment level was driven by urbanization, with MP concentration increasing with urbanization. Temporal variability in MP concentration was strong and driven by seasonal hydrological changes, with higher concentration observed in low flow conditions. We then observed that MP polymers differed in term of color distribution, with higher proportions of white PS and black PE. There was also a temporal variability in the proportion of PE, the most common polymer. The size distribution varied among each polymer, with PS particles being larger. Finally, we found that the temporal variability in MP size was driven by seasonal hydrological changes, with smaller MP encountered in low flow conditions and MP size decreased with the distance to the source only in the main stream of the Garonne River.

3.4.1 Spatial and temporal variability in MP concentration and its determinants

In general, MP pollution in European rivers is highly variable (C. Li et al., 2020; J. K. H. Wong et al., 2020). The mean MP concentration found in this study ($0.15 (\pm 0.46) \text{ MP}\cdot\text{m}^{-3}$) was within the range of values reported elsewhere (Li et al., 2018), such as in the Rhine catchment ($0.04\text{--}9.97 \text{ MP}\cdot\text{m}^{-3}$) (Mani and Burkhardt-Holm, 2019). However, comparisons between studies are limited by differences in the size range considered and the use of different methodological approaches, from water sampling to extraction of MP particles. MP pollution is also highly variable within catchments, with difference up to a factor of 250 times (Rodrigues et al., 2018). As predicted, a strong spatial variability in MP pollution was observed within the Garonne catchment and driven by urbanization but not by river size. This result is in agreement with previous findings showing that MP concentration was driven by upstream population size rather than watershed size (Christensen et al., 2020), although in the present study a multivariate approach of environmental conditions was applied. We also found that MP concentration displayed a significant temporal variability, with higher levels of MP pollution observed in low flow conditions. Lower MP abundance in water has previously been associated with weak hydrodynamics conditions in a reservoir and were explained by the reduction in the vertical mixing of MP within the water column (Zhang et al., 2017), with deposition of suspended particles. However, a different mechanism may prevail in our study, probably related with particles size and shape, where low flow conditions result in prevalence of MP in the upper layer of water column, consequently increasing their measured concentration. Therefore, independently of the global flux of MP, MP pollution level in water surface was reduced in high discharge conditions. Weather changes associated to precipitation prior to field sampling did not affect MP concentration. This is consistent with results observed in the Rhine catchment with no relationship between precipitations and MP concentrations (Mani and Burkhardt-Holm, 2019). A relationship between precipitation and MP concentration was observed in Tamsui catchment only in some of the studied rivers (G. Wong et al., 2020). While precipitation might move MP from land to the rivers, this increase in MP quantity might not translate into significant changes in concentration due to an increased discharge and changes in suspension-settlement dynamics of particles. Studies are therefore still necessary to elucidate the relationship between precipitations, hydrological conditions and MP concentration to understand how the position within the catchment could modulate this relationship.

3.4.2 Spatial and temporal variability in MP type

The diversity of polymer types composing MP are driven by a high diversity of input sources, including plastic industries, littering, roads and wastewater effluent (Grbić et al., 2020; Zhang et al., 2017). In the present study, we found that 92.8% of all MP in the Garonne catchment were composed of three main polymers, PE, PS and PP. This finding is very similar to results observed elsewhere (Mao et al., 2020). These polymers are the most common plastics found in the environment (J. K. H. Wong et al., 2020) and are largely applied in the food packaging, reusable bags, and toys, for example (PlasticsEurope, 2019). Interestingly, this distribution differs from the total European plastic demand, in which these three polymers types represents only 55.4% (PlasticsEurope, 2019), suggesting a difference between the production and this fraction of freshwater MP pollution. The overall low density of these polymers, commonly lower than the water, might explain their presence in surface water (Andrady, 2017; J. K. H. Wong et al., 2020). However, as these particles are subject to different degradation process while in the environment, which are temporally dynamic and polymer -dependent (Boyle and Örmeci, 2020), their prevalence in water surface might be reduced due to a sedimentation process. For instance, biofouling is known to affect microplastic density, altering their floatability and causing their sedimentation (Karlsson et al., 2017) , with studies demonstrating the presence of microplastics composed of low density polymers in river sediments (Van Cauwenberghe et al., 2015) . The proportion of PE was higher in low flow conditions, when MP concentration was the highest. Because PE was the main type of polymer observed in the sampled MP, representing almost half of all particles (44.5%), the variation of MP concentration seems to be influenced by the presence of PE particles. We found a significant higher proportion of white PS particles, which is compatible with the higher proportion PS in its foam type (that is, 98% gas and 2% of polystyrene on a volume basis, (Song et al., 2017)) and typically used in packaging or containers (T. Wang et al., 2019). As most of the PS foam had a spherule shape, their presence in the upper layer of the water column was expected (Van Melkebeke et al., 2020). Moreover, as these particles were weathered only on their surface, a hypothesis of relatively recent emission could be made, as they are expected to easily fragment under mechanical factors (Mani and Burkhardt-Holm, 2019; Song et al., 2017). A comprehensive study of plastic pollution across ecosystem types is essential to identify the potential land sources and transport mechanisms, including the long-term dynamics of MP in the environment.

3.4.3 Variability in MP size

In streams, MP can be continuously deposited in sediments and resuspended with hydrological dynamics (Rochman and Hoellein, 2020). River banks and floodplains represent a temporal sink of plastics, where larger MP are more easily trapped (Christensen et al., 2020). Contrary to our predictions, there was no spatial variability of MP size across all sampled sites but MP size was affected by seasonal hydrological conditions with smaller MP in low flow conditions. This finding could be caused by the hydrodynamic processes with larger MP needing higher discharge conditions to be resuspended and transported. At the opposite, the proportion of smaller MP particles, that need less force to be resuspended and/or moved, increased in low discharge conditions. Regarding size distribution among polymers types, PS particles were, on average, larger than the PP and PE particles which is in line to the hypothesis of a recent emission of PS particles. The size distribution among polymer types was similar to a previous study (Serranti et al., 2018). Temperature and ultraviolet (UV) radiation play an important role in plastic degradation, at a rate largely depending on its exposure (Christensen et al., 2020; Weinstein et al., 2016) and polymer type, that may also be influenced by the manufacturing process (Julienne et al., 2019). Further studies are needed to better understand the specificities of fragmentation mechanisms within rivers.

Independently of MP composition and sampling event, a negative correlation between MP size and the distance to the Garonne source was observed. Two mutually non-exclusive hypotheses could explain this finding. First, a possible fragmentation of MP particles could occur along the stream (Garvey et al., 2020; Kataoka et al., 2019). Second, and although it was not measured systematically in the present study, water depth differ between sampling sites. Although water surface was always sampled, this fraction represents a proportion of the water column that varies between each sampling site and event. This may have affected the average size of sampled MP particles in surface water because they are not uniformly distributed through the water column (Kooi et al., 2017; Kukulka et al., 2012; Law, 2017). MP particles density, sizes and shapes impact their suspension-settlement dynamics (Daily and Hoffman, 2020). Further studies investigating the vertical (Choy et al., 2019; Liu et al., 2020), through the water column, and lateral (Dris, Gasperi, et al., 2015) variability in MP pollution are still needed. The temporal variability in both MP concentration and MP size was driven by hydrological conditions. The increase of MP concentration with decreasing MP size was already reported in water and sediments

in coastal metropolis (Su et al., 2020). As particle size decreases, they spread over greater distances, and a wider range of organisms are likely to ingest them (Auta et al., 2017). In addition, because MP abundance increases when their size decreases (Morét-Ferguson et al., 2010; Roch et al., 2019), the size limit ($> 700 \mu\text{m}$) used in the present study likely underestimates MP pollution. Because MP size is linked to some of their characteristics, it is important to quantify the characteristics (shape, size and polymer composition) of smaller MPs to fully appreciate how MP pollution is linked to their potential effects on freshwater organisms.

This study identified the main environmental drivers of the variability in MP pollution in a large temperate river and revealed that urbanization and hydrology were the main drivers of spatial and temporal variability, respectively. We highlight that not only the concentration or polymer type should be quantified in the analysis of MP pollution because variation in MP properties such as size, density and color, can provide a better understanding of the sources and dynamics of this pollution. The dynamic MP pollution across watersheds, from headwater tributaries to lowland rivers and to its final sink, the marine environment, is complex and multifaceted, and efforts should still be made to improve the spatial and temporal resolution of our understanding of MP pollution in aquatic ecosystems for the management of this pollution (Cable et al., 2017; Rochman, 2018; Skalska et al., 2020).

4 | Urbanisation modulates microplastic pollution during flood episodes

Abstract

The relevance of freshwater ecosystems in transporting and accumulating microplastics have been revealed in the last years. Spatial and temporal variability of microplastic pollution lead to critical spot and moment of contamination, however the effect of their interaction is still poorly understood. The aim of this work was to assess the interaction between urbanisation and river discharge as drivers of microplastic pollution in freshwater. Water surface was collected in two sites in the Garonne river, upstream and downstream Toulouse, during two flood episodes. Samples were chemically digested to facilitate microplastic isolation and microplastic were characterized by infrared spectroscopy. A greater increase in microplastic concentration was observed in the downstream site for both episodes and was driven by river discharge. Through multivariate analysis, microplastic physical and chemical properties were resumed, and an overall profile of bigger particles was noticed in water surface during the flood for both sites. For polyethylene, the flood resulted in an increase of particles with higher oxidation state, and a larger abundance of white particles only in the downstream site. Urbanisation regulated the microplastic pollution variation in the studied freshwater ecosystem during a flood episode. This interactive effect resulted in greater deviation on microplastic concentration and general profile, which should be further integrated within toxicity studies to evaluate risks potentially elevated to animal and human health.

4.1 Introduction

Rivers play an important role in the transport and temporal dynamics of the global microplastic pollution (He et al., 2020; Reisser et al., 2015; Roebroek et al., 2021). In general, the concentration of microplastic particles, i.e. particles smaller than 5 mm (Andrady, 2011), in a river is expected to increase from upstream to downstream, with additional inputs from tributaries or from inland activities in the main stream (Napper et al., 2021). The catchment land use is an important driver of the spatial distribution of microplastic. For instance, urbanized areas have been consistently identified as important sources of microplastic to rivers, resulting in ‘hotspots’ of microplastic pollution (Chen et al., 2020; Dris, Gasperi, et al., 2015; Grbić et al., 2020). Furthermore, temporal variations in freshwater microplastic pollution might also occur, modulated mainly by hydrological conditions. High discharge events disturb the state of microplastic pollution in a river, for instance, resulting in an abrupt remobilization of settled particles accumulated in river bed (Hurley, Woodward, et al., 2018). Consequently, an increased microplastic concentration in the water column was observed, corroborated by additional inputs from the floodplain (Christensen et al., 2020). Therefore, the temporal dynamic of microplastics pollution lead to the occurrence “hot moments” of pollution (Hitchcock, 2020; McClain et al., 2003). Alterations in this pollution to an increased river discharge seem to be regulated by the catchment land-use (Wagner et al., 2019), but further investigations are needed to better understand this interactive effect in regulating microplastic pollution profile in streams.

Flood events influence not only changes in microplastic concentration, but also on the general pattern of this pollution (Christensen et al., 2020; Gündoğdu et al., 2018). Microplastics are considered a complex contaminant with variable combination of physical (i.e. size, morphology, color) and chemical (i.e. polymer, oxidation level, chemicals associated content) properties. Temporal changes in the diversity of microplastic composition might represent the predominance of a specific source, as for example, an increased proportion on tire wear particles in stream after a flood, reflecting the proximity with a highway (Horton and Dixon, 2018). Although not specifically tested, an interaction between spatial and temporal descriptors might be observed in describing microplastic pollution. The remobilization of particles settled in sediments, retained due to their greater size or density higher than the water, also results in an altered profile of microplastic in streams (Cheung et al., 2019; Gündoğdu et al., 2018). The predominance of film-like particles in water surface following an

increased river discharge reflects the importance of a particle hydrodynamic in regulating its behaviours face hydrological changes (Wang et al., 2021). Microplastic hydrodynamics is, in turn, regulated by its physical and chemical properties, which are inter-dependent (Kowalski et al., 2016; Liu et al., 2020). Efforts to embrace the complexity of describing microplastic behaviour in a turbulence are necessary to further investigate qualitative changes in microplastic profile to hydrological changes (Rochman et al., 2019; Waldman and Rillig, 2020).

In the environment, microplastics undergo a weathering process that results in changes in their physical and chemical properties (Barnes et al., 2009; Christensen et al., 2020; Gewert et al., 2015). For instance, higher oxidation states, obtained through the measurement of carbonyl, hydroxyl and methylene contents, are observed in environmental microplastic when compared to virgin ones (Canopoli et al., 2020; Dong, Q. Zhang, et al., 2020; Gewert et al., 2015). Several factors influence this photo-induced oxidative process, as the polymer type, presence of additives, residence time in the environment and location within a river (Delorme et al., 2021). The higher exposure to ultraviolet radiation and consequent increase in photo-initiated oxidation reactions might result in microplastics more oxidized in floodplain than the ones in the water (Bond et al., 2018; Christensen et al., 2020; Xiong et al., 2017), where a slowed oxidative process is observed (Karlsson et al., 2018; Liu, 2020; Tang et al., 2019). The role of microplastics as vector of chemicals and pathogens is influence by its weathered condition, and an increased embrittlement, surface cracks, biofilm and oxidation state are commonly associated with a greater concentration of chemicals (Hossain et al., 2019; Kirstein, 2016; Naik, 2019; Wagner et al., 2014). Investigating changes in the chemical profile of microplastics found in diverse hydrological conditions might contribute to understand particles dynamics and moments of accentuated risks to aquatic life.

The general aim of this study was to understand the interactive effects between flood events and urbanization on the dynamic of microplastic pollution in rivers. This was achieved by quantifying the variability in microplastic pollution during flood episodes upstream and downstream of a large urban area (Toulouse, France). The first objective was to quantify the changes in the concentration of microplastics in water surface during a flood event. Our hypothesis was that the urban area modulates the changes in microplastic concentration during a flood, with a greater increase in microplastic concentration caused by increased in water discharge in the downstream site. The second objective was to quantify the changes in microplastic characteristics during a flood event. We hypothesized that these changes were affected by urbani-

sation, with a higher diversity of polymer type and particle colours downstream of the urban area that represents a higher diversity of microplastic sources. We also hypothesized an overall increase in particles size in water surface, independently of urbanization, that would prevails on water surface driven by increased river discharge. Finally, the third objective was to analyse chemical changes at microplastics through their infrared spectra, under the hypothesis that an increase in oxidation profile during the flood represents the predominance of land-based microplastics.

4.2 Material and methods

Sampling strategy and sample treatment are described Chapter 2.

4.2.1 Microplastic characterization

The protocol described at Chapter 2 (Box 2) was adapted for measurement of further microplastic properties. All particles collected during flood event B were placed with tweezers in a petri dish under the stereomicroscope and photographed along with a scale rule. The length (L) of largest particle axis and of the second largest axis perpendicular to the first one (denominated ‘height’, H), the perimeter and the particle projected area were measured using a software of image processing, ImageJ (Rasband, 1997). The mass of each particle was measured (AT21 Comparator, Mettler Toledo, d=0.001 mg) and particles weighting less than 0.001 mg was considered as null weight due to balance sensitivity. Through their pictures, particles were classified into one of five morphology categories: line, film, fragment, pellet and sphere (Gündoğdu et al., 2018), that was therefore used to estimate the particle width (W). For lines, width was considered equal to their height; for films and fragments, width was estimated as 0.08 μm and 0.33 μm , respectively. Pellet had their width considered as half of the length and spheres’s width was considered the average of length and height. Also, sphericity descriptor was also calculated according with the following formula (Kooi and Koelmans, 2019; Krumbein, 1941):

$$Sphericity = \left(\frac{W*H}{L^2}\right)^{1/3}$$

A sphericity value close to 1 represents a high spherical particle and a small sphericity value a film-like particle. Each particle had their composition defined by attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR) (Nicolet 6700, Thermo Fisher Scientific) as described at Chapter 2. Of all particles obtained in flood B, 78.5 % were of plastic composition of the following categories: polyethylene (PE), polystyrene (PS), polypropylene (PP), polydienes, asphalt, artificial additives (mainly polyolefin-based, lubricants and waxes) (Hartmann et al., 2019; “Polymer Database,” 2020).

4.2.2 Measurement of microplastic concentration

Regarding the first objective, microplastic concentration was calculated as number of microplastic per volume of filtered water ($\text{MP}\cdot\text{m}^{-3}$). Microplastic concentration in flood A was corrected by the percentage of collected particles in flood B that were

of plastic composition (78.5 %) because particles were not analysed by ATR-FTIR. The effects of the interaction between sampled sites and sampling events on the microplastic concentration (log-transformed) was tested using linear mixed effect models (lmm), considering replicate as a random factor. Independent models were applied for each flood event. To further investigate the drivers of changes in microplastic concentration across sampling events, a similar model was applied to test the interaction between sampled sites and river discharge. Due to the high correlation between water turbidity and river discharge (Spearman correlation, $r = 0.61$, $p < 0.001$), only the water discharge was used in further analyses. Models were simplified when the interactions were not significant.

4.2.3 Variance in microplastic characteristics

Regarding the second objective, microplastic properties (length, height, area, perimeter, weight, sphericity, color and composition) were summarized using a factorial analysis of mixed data (FAMD). Instead of analysing individual microplastic properties, that are highly correlated, a multivariate analysis allow the integration of all properties and originate new variables (called principal components), each one accounting for an exclusive variance in data. As microplastic properties are a mixed of continuous and categorical data, a FAMD approach was applied. Components 1, 2 and 3 were mainly composed by size descriptors, with only the fourth one representing variance in microplastic color and composition. Therefore, the first and fourth principal components (Comp. 1 and Comp. 4) were selected and presented an eigenvalue of 3.92 and 1.17, accounting for 26.17% and 7.78% of the total variance, respectively. Comp. 1 was better represented by the height, area, and perimeter of microplastics and was named as “**microplastic size**” axis, where bigger particles presented a higher value (Fig. 4.1a). Also, white and red polystyrene particles presented a higher value in this axis and were distinguished from particles from other colors and composition (Fig. 4.1b). Comp. 4 was chosen for accounting for the remaining microplastic characteristics composition and colour, as was named as “**microplastic quality**” axis, distinguishing between black polypropylene and other polymeric composition, with higher values, from polyethylene of diverse colors, with smaller values. Microplastics were then analysed considering their coordinates in this new (Comp. 1 x Comp. 4) plot.

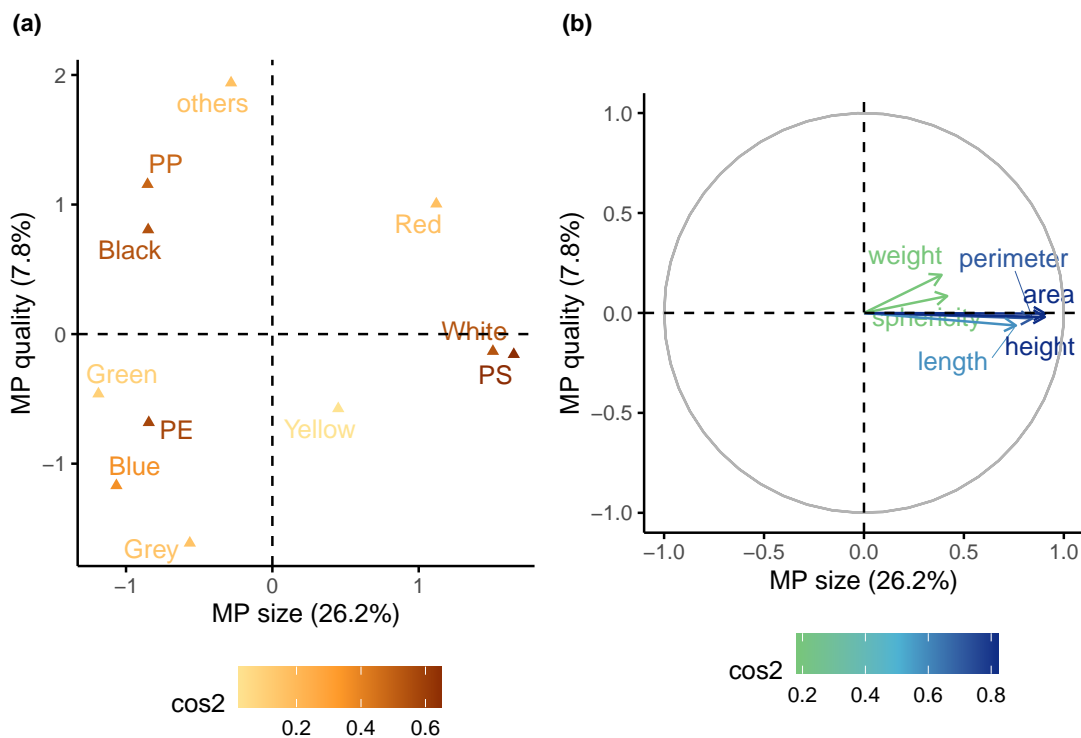


Figure 4.1: Quality of representation (\cos^2) of microplastic properties (a) area, weight, perimeter, length, sphericity, height and (b) color and composition variables to FAMD analysis.

The effects of the interaction between sampled sites and flood in microplastic size and in microplastic quality was tested using linear mixed-effect models (lmm), considering replicate as a random factor. To further investigate the drivers of changes in microplastic size and quality, a similar model was applied to test the interaction between sampled sites and river discharge in microplastic size and quality.

4.2.4 Assessment of changes in infrared spectra of polyethylene microplastics

Changes in the ATR-FTIR spectra of polyethylene microplastics, the main polymer type found during the flood episode B at GSG ($n = 59$), were evaluated through principal component analysis (PCA). First, spectra were pre-processed through baseline correction, normalized by the sum and scaled using pareto scaling (Jung et al., 2018; Kedzierski et al., 2019; Liland, 2015). To reduce the influence of great variance in spectral bands inherent in polyethylene, i.e. related with methylene vibrations, only absorbance bands presented in wavenumber ranges 800 - 1200, 1500 - 1900 and 3000 - 3400 cm^{-1} were selected. PCA was performed with absorbance data at each

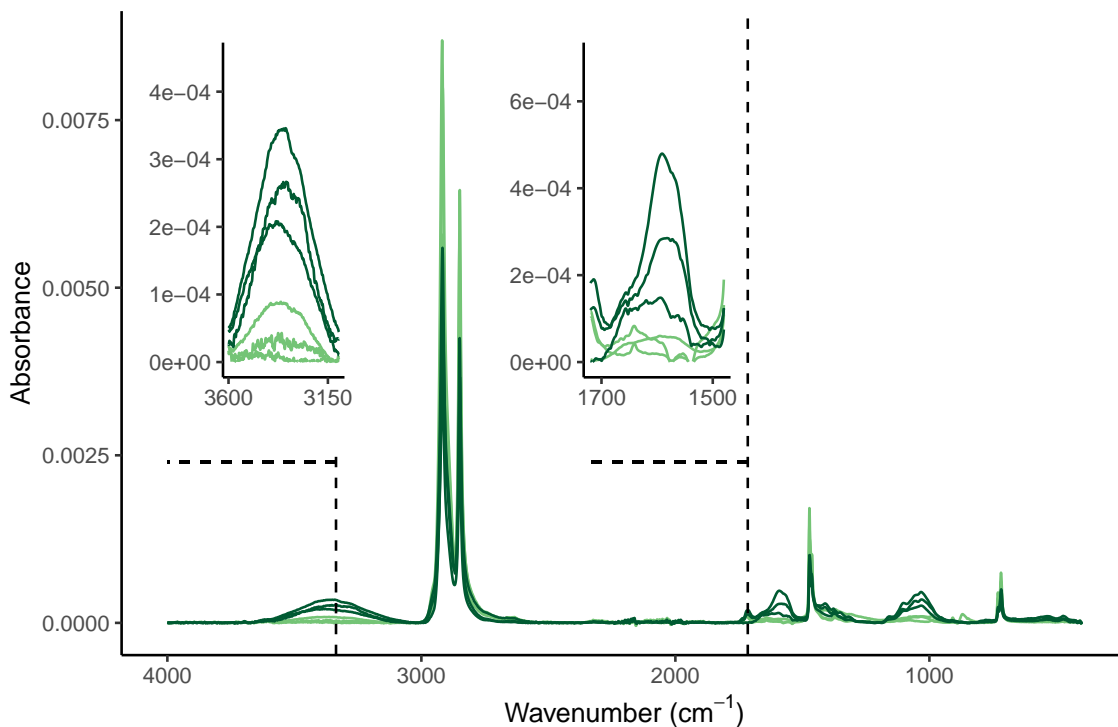


Figure 4.2: Examples of ATR-FTIR spectra of PE microplastics submitted to PCA analysis. Microplastics collected without and with flood are displayed in light and dark green lines, respectively. Dashed lines represent wavenumbers 3336 and 1715 cm^{-1} , regions of greatest variances among spectra after wavenumber selection, zoomed at upper spectra.

wavenumber, thus considered as one variable (total of 2158 wavenumbers). Instead of studying selected wavenumbers, multivariate analysis allow to synthetize the main variance in the data into new variables, which should be further interpreted. The first two principal components (PC1 and PC2) presented an eigenvalue of 0.081 and 0.007 and accounted for 73.0% and 6.23% of the total variance, respectively. PC1 represents absorbance bands in the range of 3336 - 3345 cm^{-1} , mainly related with the presence of hydroxyl group (Gardette et al., 2013; Grigoriadou et al., 2018), and was named as “**hydroxyl content**” axis (Fig. 4.2). PC2 represents bands in the range 1715 to 1780 cm^{-1} , related with carbonyl group, as ketones (1720 cm^{-1}), carboxylic acids (1713 cm^{-1}), esters (1735 cm^{-1}) and lactones (1780 cm^{-1}) (Almond et al., 2020; Gardette et al., 2013; Karlsson et al., 2018), and was named as “**carbonyl content**” axis (Fig. 4.2). Microplastic hydroxyl and carbonyl contents were therefore compared in conditions with and without flood.

4.2.5 Statistical analysis

All the analysis were performed using R software v. 4.0.4 (Team, 2019). Linear mixed effects models were performed using the package lme4 v.1.1-26 (Bates et al., 2015). Significant levels of mixed effects models were obtained using the ‘Anova’ function in the package car v. 3.0-10 (Fox and Weisberg, 2019). FAMD and PCA were performed through the package FactoMineR v.2.4 (Lê et al., 2008) while graphs were generated through package Factoextra v.1.0.7 (Kassambara, 2015). Baseline correction of spectra were performed with ‘baseline’ function from package baseline v. 1.3-1 (lambda=4, hwi=50, it=10, int=740, method=‘fillPeaks’) (Liland, 2015), while pareto scaling was performed with ‘scaling’ function from package MetabolAnalyze v.1.3.1 (Gift et al., 2010). Changes in hydroxyl and carbonyl contents of polyethylene microplastics in flood B were also tested through Wilcoxon tests. Assumptions of linearity and homogeneity of variances on residuals from all models were checked visually.

Table 4.1: Summary of measured microplastic properties from particles collected during sampling event B.

Microplastic property	Mean \pm SD (n = 1580)	Microplastic color (n / % of total)	Microplastic polymer (n / % of total)
Length (mm)	2.33 \pm 0.97	Black (528 / 33.4%)	Polyethylene (650 / 41.1%)
Width (mm)	1.28 \pm 0.86	White (462 / 29.2%)	Polystyrene (521 / 33.0%)
Perimeter (mm)	6.34 \pm 3.05	Blue (227 / 14.4%)	Polypropylene (342 / 21.6%)
Area (mm ²)	2.73 \pm 2.97	Red (117 / 7.4%)	Polydienes (20 / 1.3%)
Weight (mg)	0.52 \pm 1.24	Green (100 / 6.3%)	Artificial additives (17 / 1.1%)
Sphericity	2.49 \pm 0.22	Grey (83 / 5.4%)	Asphalt (17 / 1.1%)
-	-	Yellow (63 / 3.9%)	Other (13 / 0.8%)

4.3 Results

A total of 456 and 1580 microplastics were collected during sampling events A and B, respectively. During event A, microplastic concentration averaged 0.032 MP.m⁻³ (\pm 0.035 SD) in MUG and 0.401 MP.m⁻³ (\pm 0.330 SD) in GSG. During event B, microplastic concentration averaged 0.031 MP.m⁻³ (\pm 0.058 SD) in MUG, 0.252 MP.m⁻³ (\pm 0.307 SD) in GSG and 1.219 MP.m⁻³ (\pm 0.490 SD) in LAU (Table 4.1).

4.3.1 Increased microplastic concentration during a flood

During the sampling events A and B (Fig. 4.3a and b, respectively), a significant interaction between sampling event and sampled site in explaining variation in microplastic concentration (MP.m⁻³) was observed (lmm, $\chi^2 = 9.230$, p = 0.026, for flood A and lmm, $\chi^2 = 42.906$, p < 0.001, for flood B). Specifically, a higher microplastic concentration was measured in sampling events A2 or A3 and flood event B4 in the two sampled sites, but this increase was stronger in GSG, located downstream of Toulouse (Fig. 4.3).

The interaction between river discharge (m³.s⁻¹) and sampled sites in explaining

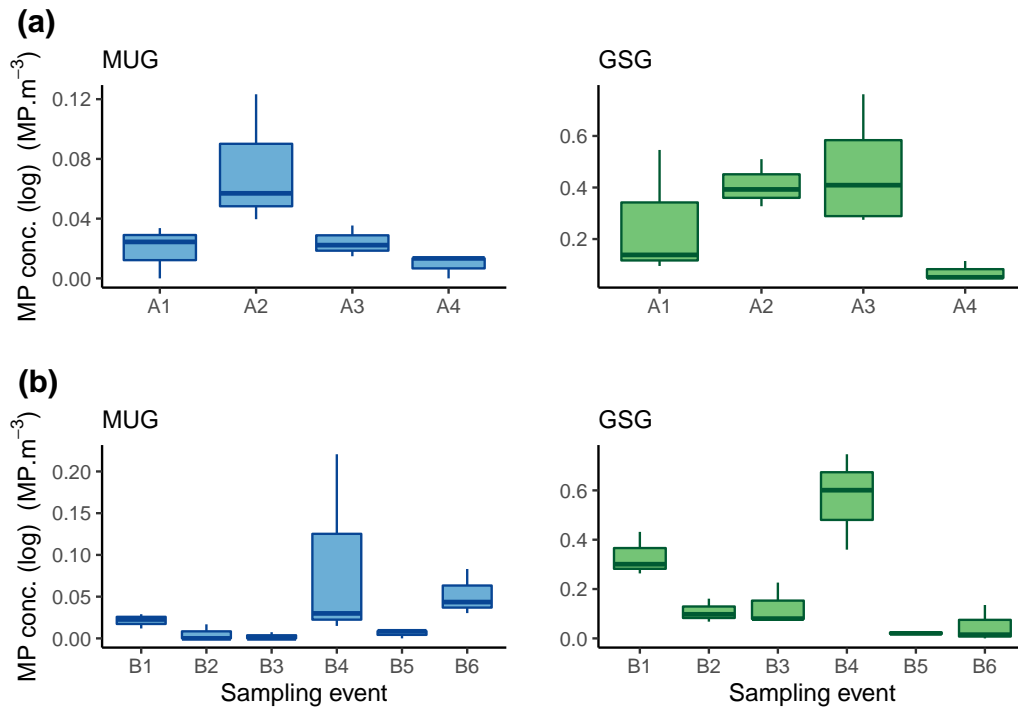


Figure 4.3: Microplastic concentration (log-transformed, $\text{MP}\cdot\text{m}^{-3}$) during sampling events in the sites MUG and GSG in (a) flood A and (b) flood B.

microplastic concentration ($\text{MP}\cdot\text{m}^{-3}$) variation was significant for both floods (lmm, $\chi^2 = 4.313$, $p = 0.038$, for flood A and lmm, $\chi^2 = 20.101$, $p < 0.001$, for flood B). Specifically, increased river discharge had a stronger effect on microplastic concentration in GSG (Fig. 4.4a and b).

Microplastic concentration ($\text{MP}\cdot\text{m}^{-3}$) in tributary LAU was significantly lower in event B2 than in B1, B4, B5 and B6 (lmm, $\chi^2 = 29.014$, $p < 0.001$). However, there was no significant relationship between microplastic concentration and river discharge.

4.3.2 Variation in microplastic characteristics

In both sites, the dimension of microplastic particles significantly varied during a flood (MUG: lmm, $\chi^2 = 4.40$, $p=0.036$ and GSG: lmm, $\chi^2 = 4.60$, $p=0.032$) (Fig. 4.5a), with overall larger particles during the flood, driven by river discharge (lmm, $\chi^2 = 9.373$, $p=0.002$). Particles quality did not vary in MUG, but presented significantly smaller values during the flood in GSG (lmm, $\chi^2 = 10.402$, $p=0.001$), also driven by river discharge (Fig. 4.5b). Smaller microplastic quality values represent an increase in colour diversity and a greater incidence of polyethylene microplastics. A significant interaction between river discharge and sampled sites in explaining the variation in

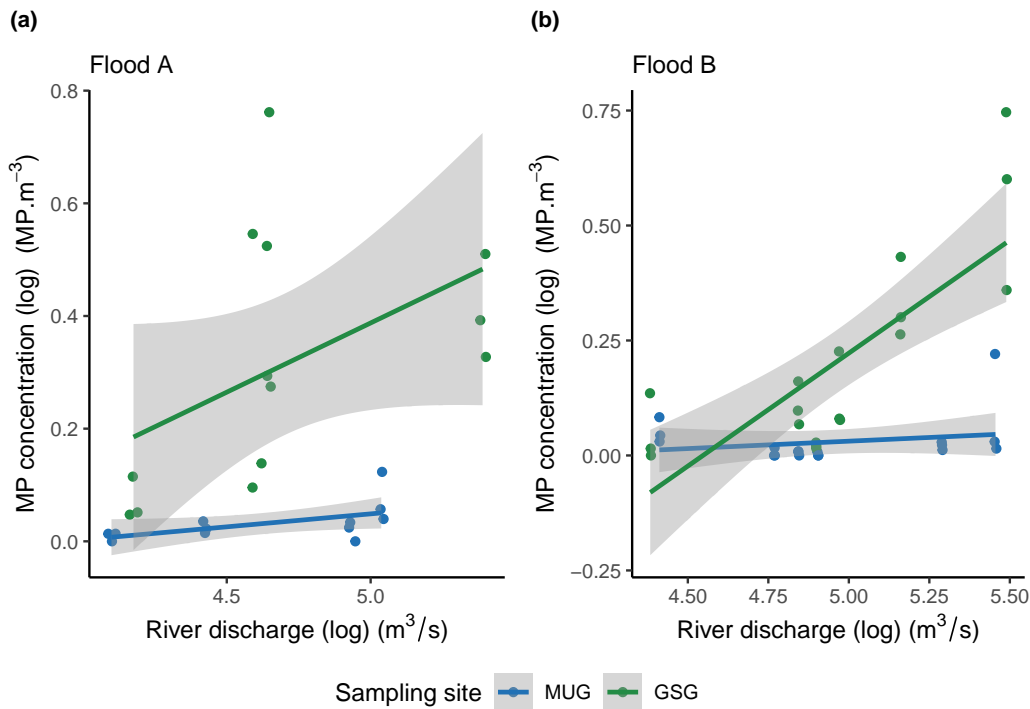


Figure 4.4: Relationship between microplastic concentration and river discharge (log-transformed, MP.m⁻³ and m³.s⁻¹, respectively) in the sites MUG (displayed in blue) and GSG (in green) for (a) flood A and (b) flood B.

microplastic quality was observed (lmm, $\chi^2 = 6.271$, $p=0.012$). Specifically, the increase in river discharge was negatively correlated with microplastic quality in GSG (Fig. 4.5b), and positively correlated with microplastic quality in MUG. Microplastic size in tributary LAU was significantly smaller in event B2 than B1 and B3 (lmm, $\chi^2 = 16.969$, $p = 0.005$), however no significant relationship was observed between microplastic size and river discharge. There was no significant relationship between microplastic quality, sampling events and river discharge in tributary LAU.

4.3.3 Main chemical changes at polyethylene microplastics

The hydroxyl content in the polyethylene microplastics did not vary during the flood episode (Fig. 4.6a). However the carbonyl content in particles collected during a flood was significantly higher than without flood (lmm, $\chi^2 = 10.748$, $p = 0.001$, Fig. 4.6b). River discharge was positively correlated with the carbonyl content only (lmm, $\chi^2 = 6.974$, $p = 0.008$) (Fig. 4.6c and d).

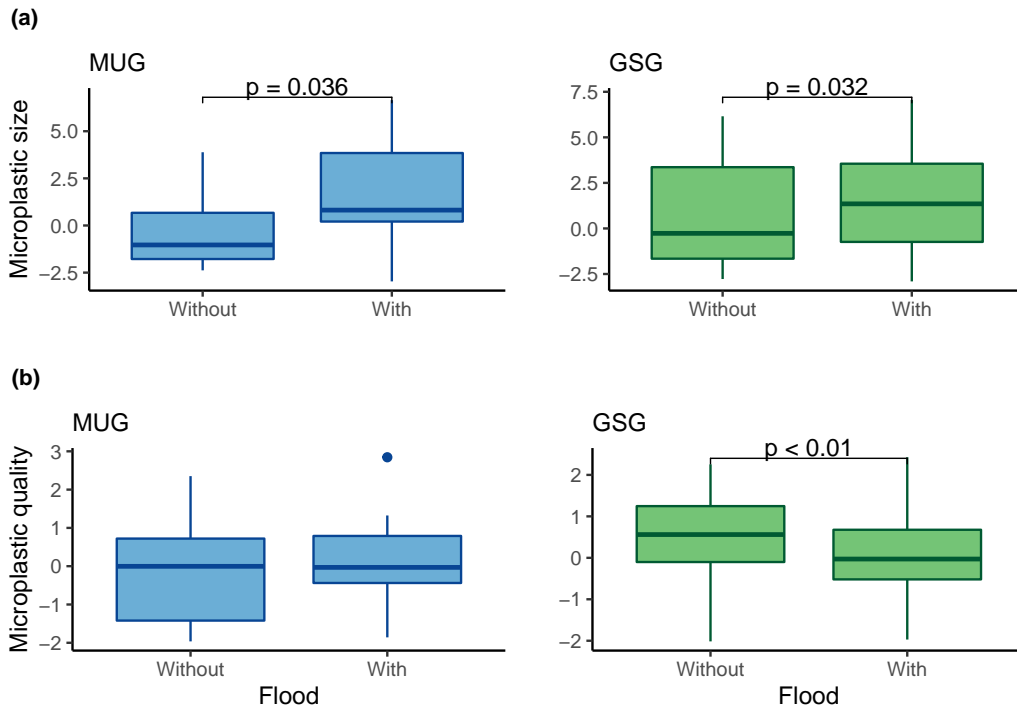


Figure 4.5: Microplastic (a) size and (b) quality with and without flood during event B in sites MUG (left) and GSG (right).

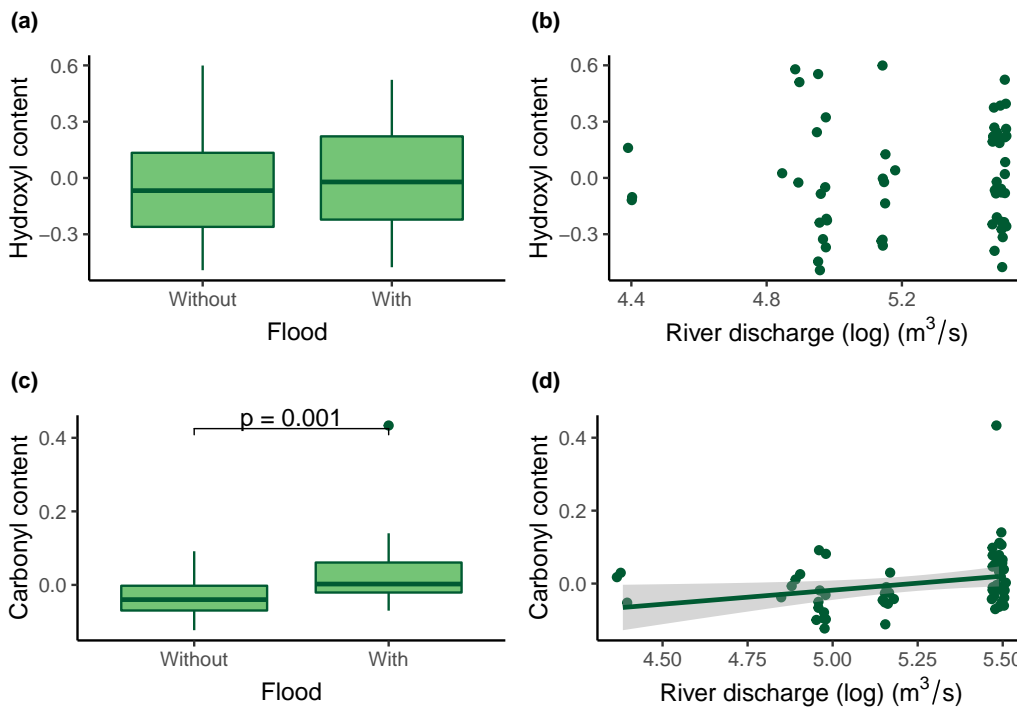


Figure 4.6: Hydroxyl and carbonyl contents in polyethylene microplastics (a and b) with and without flood and changes in these contents (c and d) with river discharge during event B in the site GSG.

4.4 Discussion

In the present study, we found strong evidence for the existence of interactive effects between flood and urbanization on microplastic pollution. Specifically, our results highlighted that the increase in microplastic concentration during flood episodes was stronger in the site affected by urbanisation (downstream of the city) and driven by river discharge. Microplastic size significantly varied during flood episode both upstream and downstream of the city, with larger particles in period of higher discharge. However, microplastic quality was significantly different only on the downstream, with higher proportion of coloured polyethylene microplastics during a flood. River discharge was positively correlated with microplastic quality in the upstream and negatively correlated on the downstream site. Finally, a significant higher carbonyl content was observed at polyethylene microplastics during the flood on the downstream site, driven by river discharge.

Microplastic concentration in the downstream site was always higher than the upstream one and showed a stronger increase with a flood episode. Three distinct and non-exclusive mechanisms could lead to these findings: the indirect microplastic inputs from tributaries, the direct inputs into the main stream, related with catchment land-use, and the remobilization from river beds and banks. As no variation in river discharge was observed in the tributaries between the two sites, it is unlike that the greater increase in microplastic concentration in the downstream site was caused by tributaries input. The hydrodynamics of microplastic is a complex process, driven by microplastic intrinsic properties and hydrological conditions (Nizzetto et al., 2016). Regarding the latter, a similar profile of river discharge was observed at the two sampled sites. Moreover, both sites showed similar microplastic pollution profile (Chapter 3). Therefore, the higher increase in microplastic concentration in the downstream was considered mainly driven by the urban area, as also described by recent findings (Napper et al., 2021). When comparing an urban and a rural catchment, plastic concentration only increased with river water discharged in the urban catchment (Wagner et al., 2019). The greater proportion of impervious area in urban catchments, together with increased anthropogenic activity, contribute to surface runoff and consequently, greater microplastic input into the stream (Calianno et al., 2013; Cheung et al., 2019; Kaiser et al., 2021). This flux, however, might be locally controlled by natural barriers, as the type and extension of river banks vegetation might influence the retention of this pollution (Delorme et al., 2021). The time spend by a plastic debris on land next to river might exceed the time inside the river and a

period of flood might contribute to the removal of this accumulated pollutant (Christensen et al., 2020; Tramoy et al., 2019). Additional studies are needed to further understand the role of local-scale process regulating microplastic pollution dynamic and their interaction with catchment-scale process.

During the flood, bigger particles overall prevailed in water surface. The need of higher energy flow to mobilize them, their prevalence in floodplain rather than in streams and their higher rise velocity than smaller ones (Cheung et al., 2019; Christensen et al., 2020; Kooi et al., 2016) likely explain this finding, although further laboratory experiments integrating and interacting these factors are needed. Albeit the input of white polystyrene particles, mainly foam, from an urban agglomeration into a stream was previously described (Chapter 3 and Mani and Burkhardt-Holm (2019)), this seemed to be intensified in a flood episode. We found that the flood episode had an opposite impact in terms of microplastic quality in the upstream and downstream sites. Particles distribution revealed a prevalence of coloured polyethylene on the downstream site, while a reduction in its proportion on the upstream. Polyethylene is greatly used in disposable items and single-used materials, as packaging, which incidence are correlated with anthropogenic activity (PlasticsEurope, 2020). Although the absence of data regarding colour distribution of produced plastics, it is reasonable to expect that an increase diversification of colour types is aligned with greater multiplicity of sources. Studies about the effects of microplastic on animal and human health are still on their infancy. They are currently not representative of the plastic particles found in the environment, mainly due to the impossibility for considering all available combinations of microplastic properties (Bucci et al., 2020; Latchere et al., 2021). In this sense, efforts were made to account for the diversity of plastic particles in the environment and investigate their spatial and temporal changes. In order to explain the complexity of microplastic particles and the high correlation between their characteristics (Chapter 3 and Rochman et al. (2019); Waldman and Rillig (2020)), eight microplastic properties were measured and resumed through a factorial analysis approach. ‘Hot’ spots and ‘hot moments’ of microplastic pollution represent not only an increased concentration, but also a distinct pollution profile that should be take into account in risk assessment studies. This study highlight the interaction between spatial and temporal variation in explaining the overall changes in microplastic profile, but their implications to freshwater ecosystems functioning remain to be investigated.

The multivariate analysis of infrared spectra granted the recognition of the most important wavenumbers responsible for differences between samples (Yun et al., 2019). Regarding the polyethylene spectra, a significant difference was observed in its car-

bonyl content with and without a flood, which was increased with a flood. Carbonyl bands, between 1700 and 1760 cm^{-1} classically appear when the polymer ages, providing valuable insight into degradation profile (Andrady, 2011; Karlsson et al., 2018; Kedzierski et al., 2019). Degradation of plastics in the environment results in visible (e.g. discolouration, brittleness) and invisible (e.g. bond scission, formation of new functional groups) effects (Canopoli et al., 2020). Because these degradation mechanisms are highly dependent on polymer type (Fernández-González et al., 2020; Gewert et al., 2015), we decided to perform a within-polymer analysis with the main polymer type, PE, which enough particles were available for a robust assessment. The importance of good quality spectra cannot be understated in the comparison of spectra using statistical methods (Lavine et al., 2020). Weathering may not happen uniformly across samples and we recommend that further studies perform an average of spectra acquired at several particle spots. Misinterpretation might occur due to the appearance of additional bands through a biofouling process (Maquelin et al., 2002). At this study, samples were submitted to a double chemical digestion protocol, likely attenuating this fouling effect. Moreover, intrinsic plastic components, as formulation additives, might either prolong environmental lifetime or increase the susceptibility to material degradation (Sait et al., 2021). Their influence on the respective oxidation profile of microplastics should be further integrated in experimental protocols to contribute to our understanding of factors influencing environmental degradation of plastic material. The consequences of chemical digestion of samples together with the presence of diversified natural organic matter, protecting or promoting further degradation of microplastics, remain to be further investigated (**Appendix A** and Prata, J. da Costa, A. C. Duarte, et al. (2019)).

In conclusion, we found that urbanization can strongly modulate river microplastic pollution during a flood. A hot spot and hot moment of microplastic pollution occurred simultaneously, representing an accentuated disturbance from a steady pollution state and a particular moment of greater risk for aquatic organisms. To better understand the impacts of microplastic pollution on organism's health and ecosystems, realistic risk assessment studies should be performed. This study highlights the importance of integrating multiple stressors in time and space and accounting for the complexity of an environmental microplastic particle to conceive a reliable experimental scenario.

5 | Microplastic in angling baits as a cryptic source of contamination in freshwater

Adapted from: de Carvalho, Aline Reis, Alexis Imbert, Ben Parker, Axelle Euphrasie, Stéphanie Boulêtreau, J. Robert Britton, and Julien Cucherousset. 2021. "Microplastic in Angling Baits as a Cryptic Source of Contamination in European Freshwaters." *Scientific Reports* 11 (1): 11255. <https://doi.org/10.1038/s41598-021-90468-0>

Abstract

High environmental microplastic pollution, and its largely unquantified impacts on organisms, are driving studies to assess their potential entry pathways into freshwaters. Recreational angling, where many anglers release manufactured baits into freshwater ecosystems, is a widespread activity with important socio-economic implications in Europe. It also represents a potential microplastic pathway into freshwaters that has yet to be quantified. Correspondingly, we analysed three different categories of industrially-produced baits ('groundbait', 'boilies' and 'pellets') for their microplastic contamination (particles 700 μm to 5 mm). From 160 samples, 28 microplastics were identified in groundbait and boilies, with a mean concentration of 17.4 (\pm 48.1 SD) MP.kg^{-1} and 6.78 (\pm 29.8 SD) mg.kg^{-1} , yet no microplastics within this size range were recorded in the pellets. Microplastic concentrations significantly differed between bait categories and companies, but microplastic characteristics did not vary. There was no correlation between microplastic contamination and the number of bait ingredients, but it was positively correlated with C:N ratio, indicating a higher contamination in baits with higher proportion of plant-based ingredients. We thus reveal that bait microplastics introduced accidentally during manufacturing and/or those originating from contaminated raw ingredients might be transferred into freshwaters. However, further studies are needed to quantify the relative importance of this cryptic source of contamination and how it influences microplastic levels in wild fish.

5.1 Introduction

Microplastic pollution (plastic particles < 5 mm in size) represents a growing and ubiquitous threat to ecosystems (Demeneix, 2020; Rochman, 2018). In freshwater, microplastics primarily originate from the fragmentation of larger plastic items (Skalska et al., 2020), and their prevalence in lakes (Dong, Luo, et al., 2020; Grbić et al., 2020) and rivers (Dris, Gasperi, et al., 2015; Mani et al., 2015) can be high. Microplastics are consumed by aquatic organisms across trophic levels and taxa (Campbell et al., 2017; Pinheiro et al., 2017; Roch et al., 2019), representing toxicological threats to individuals and subsequently affecting community composition and the functioning of freshwater ecosystems (López-Rojo et al., 2020; Redondo-Hasselerharm et al., 2020). Microplastic characteristics, such as colour and shape, can modulate their consumption by aquatic organisms (Hoellein et al., 2019; Roch et al., 2020), with their consumption being either direct (occurring both intentionally or accidentally (Collard et al., 2019) or indirect through the consumption of food resources contaminated with microplastics (McGoran et al., 2018; Welden et al., 2018). Identifying microplastic sources and their pathways into freshwater ecosystems is therefore important for reducing their potential impacts (Rochman and Hoellein, 2020).

Angling is a widespread recreational activity practiced by more than 10% of the global population (Cooke and Cowx, 2004) and by up to 20% of populations in some European countries (Arlinghaus et al., 2020). While angling is multifaceted in the way anglers capture a fish, most techniques release angling baits into the water to attract fish into a restricted spatial area in order to maximise the chance of fish capture (Wolos et al., 1992). Baits are introduced into freshwater ecosystems by anglers, either by hands and/or using devices such as a catapult. Anglers use, on average, 7.3 kg of baits per year (Arlinghaus, 2004), with some specialised anglers using at least 200 kg of angling bait per year (Arlinghaus and Mehner, 2003). Evidence suggests that angling baits can represent an important food resource to wild fish, contributing to over half of the diet of fish in some ecosystems, and nearly 80% for some individuals (Bašić et al., 2015). This dietary contribution by bait tends to increase with fish size (De Santis et al., 2019), with larger specimens often being targeted by anglers more than smaller fish and they can be more vulnerable to capture (Gutmann Roberts et al., 2017). Angling baits represent important trophic subsidies to freshwater ecosystems that can additionally contribute to eutrophication through the addition of phosphorous (Amaral et al., 2013; Arlinghaus and Niesar, 2005).

Angling baits are usually purchased by anglers from commercial sources and can

be categorised as ‘groundbaits’ (composed of relatively fine particles, often used for attracting smaller fish and mixed with water to obtain a compact ball), ‘boilies’ (circular, hardened baits of up to 24 mm diameter that are designed to select for larger fish) and ‘pellets’ (usually pelletized fish meal products of 3 to 24 mm diameter). These angling baits differ in their composition but generally contain various flours (plant- and/or animal-based) mixed with additional ingredients. Because commercially-available angling baits are primarily produced industrially, there is potential that they also contain substantial quantities of microplastics, either present in the raw materials or introduced during manufacture. Microplastics have already been reported in other industrially-produced and packaged wines (Prata, Paço, et al., 2020), pet foods (Zhang et al., 2019) and canned fish for human consumption (Akhbarizadeh et al., 2020; Karami et al., 2018). Therefore, angling baits could represent an unknown pathway of microplastic contamination within freshwater ecosystems that requires quantification, especially given their ubiquitous use in angling in many European countries (Arlinghaus and Mehner, 2003; Arlinghaus and Niesar, 2005).

This study aimed to investigate the presence of microplastics within angling baits as a potential source of microplastic to freshwater pollution. The objectives were to firstly quantify the number, mass and characteristics (size, colour and polymeric composition) of microplastics within commercially-available, industrially-produced, angling baits (several products of three main bait categories: groundbaits, boilies and pellets), and to determine if contamination levels varied between bait categories and companies. This study also explored whether differences in microplastic number or characteristics could be related to the number of ingredients, as well as the origin of ingredients. This was assessed using the carbon to nitrogen (C:N) ratio to determine the relative amount of animal- versus plant-based ingredients (smaller C:N ratio with high proportion of animal-based ingredients) (Gibb et al., 2015; Martin, 2007). Specifically, we tested the hypothesis that (i) the number of ingredients in angling baits was positively related to microplastic concentration, as it likely represent an increased diversity of the potential sources of contamination; and (ii) the ingredients of the angling baits are a major determinant of their microplastic concentration, with animal-based baits containing more microplastic than plant-based baits.

5.2 Material and methods

Selection of angling baits samples and sample treatment are described at Chapter 2.

5.2.1 Elemental and stable isotopes composition

The elemental composition (carbon and nitrogen) of each bait was quantified and the C:N ratio calculated. A relatively high C:N value indicates that the bait has a higher proportion of plant-based ingredients, whereas a relatively low C:N ratio indicates that the bait has a higher proportion of animal-based ingredients (Gibb et al., 2015; Martin, 2007). About 3 g of each angling bait, in triplicates, was oven-dried at 60°C for 72 h before being ground (Retsch MM200) and analysed at the Cornell Isotope Laboratory (COIL, USA) by isotope ratio mass spectrometry (IRMS, Delta V, Thermo Fisher Scientific).

5.2.2 Statistical analyses

Microplastic occurrence in angling baits was calculated as the proportion between products containing microplastic and the total products available per category. Microplastic concentration in angling baits was calculated as the number of microplastics per unit of ground bait dry mass (number concentration in MP.kg⁻¹) and as the mass of microplastics per unit of ground bait dry mass (mass concentration in mg.kg⁻¹). Fisher exact tests were first used to compare the occurrence of microplastics between angling bait categories and between companies. Generalized linear mixed-effects models (glmm) were used to test the difference in microplastic concentration (number and mass) between the categories of angling baits and between the companies (fixed effects) using angling bait product as a random factor and gamma distribution as family. Fisher exact tests were then used to compare the polymer composition and colour distribution of microplastics between angling bait categories. Spearman correlations tested the relationship between the microplastic concentration (number and mass) in angling baits (averaged value across replicates), number of reported ingredients and C:N ratio (mean value across replicates) across all products (n=16). All statistical analyses were performed using R v.4.0.2 (Team, 2019) and generalized linear mixed effects models were performed using the package lme4 (Bates et al., 2015). Significant levels of generalized mixed effects models were obtained using the ‘Anova’ function in the car package (Fox and Weisberg, 2019). Error reported around mean values are standard deviation.

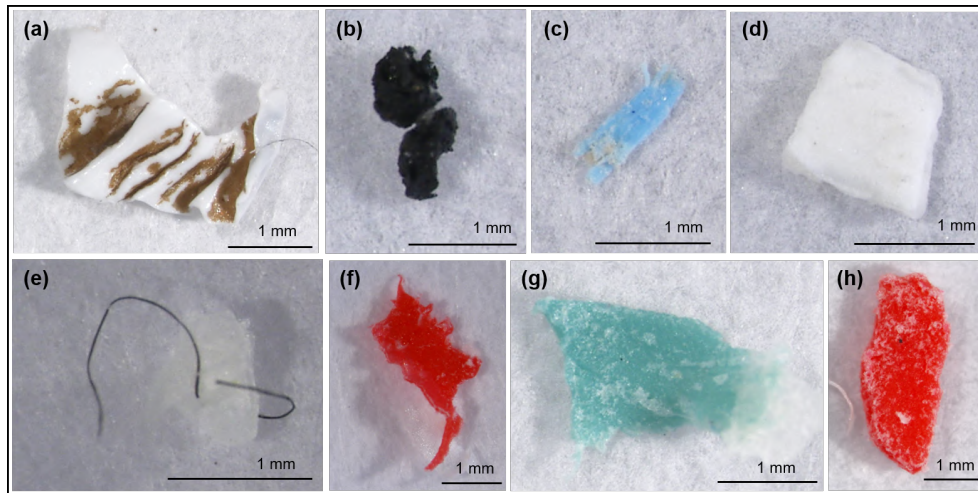


Figure 5.1: Examples of microplastics (colour, polymer composition and shape) found in angling baits (category, G = groundbait and B = boilies, and product, 1 to 6): (a) white polypropylene fragment (B6); (b) black additive fragment (G1); (c) blue polyethylene fragment (B2); (d) white additive fragment (B2); (e) black additive fiber (G1); (f) red polyethylene fragment (G2); (g) blue polyethylene fragment (G6) and (h) red polyethylene fragment (G6).

5.3 Results

5.3.1 Microplastic contamination level

Microplastics (700 μm - 5 mm) were investigated within 16 commercially available angling baits products (6 groundbaits, 6 boilies and 4 pellets) that were purchased in France, with each bait replicated 10 times. Across the 160 analysed samples, a total of 86 particles were collected. Infrared spectroscopy analyses revealed that 39 particles were plastic, of which 28 were within the selected size range of microplastics, i.e. 700 μm to 5 mm (Fig. 5.2 and 5.1, Table 5.1). Therefore, 11 plastic particles were excluded from further analyses, and 20 microplastics were collected in groundbaits and 8 in boilies. Correspondingly, microplastic contamination of pellets within the selected size range was considered as null.

The mean occurrence of microplastics across all angling bait samples was $13.7 \pm 17.01\%$, with occurrence varying between bait categories (groundbait: $26.7 \pm 19.7\%$; boilies: $10.0 \pm 10.9\%$) (Table 5.1). There was a significant difference in the occurrence of microplastics between bait categories (Fisher Test, $p = 0.032$) and companies (Fisher Test, $p < 0.001$). The microplastic concentration level ranged between 0 and 300 MP kg^{-1} (Table 5.1), where the mean concentration over all samples was $17.4 \pm$

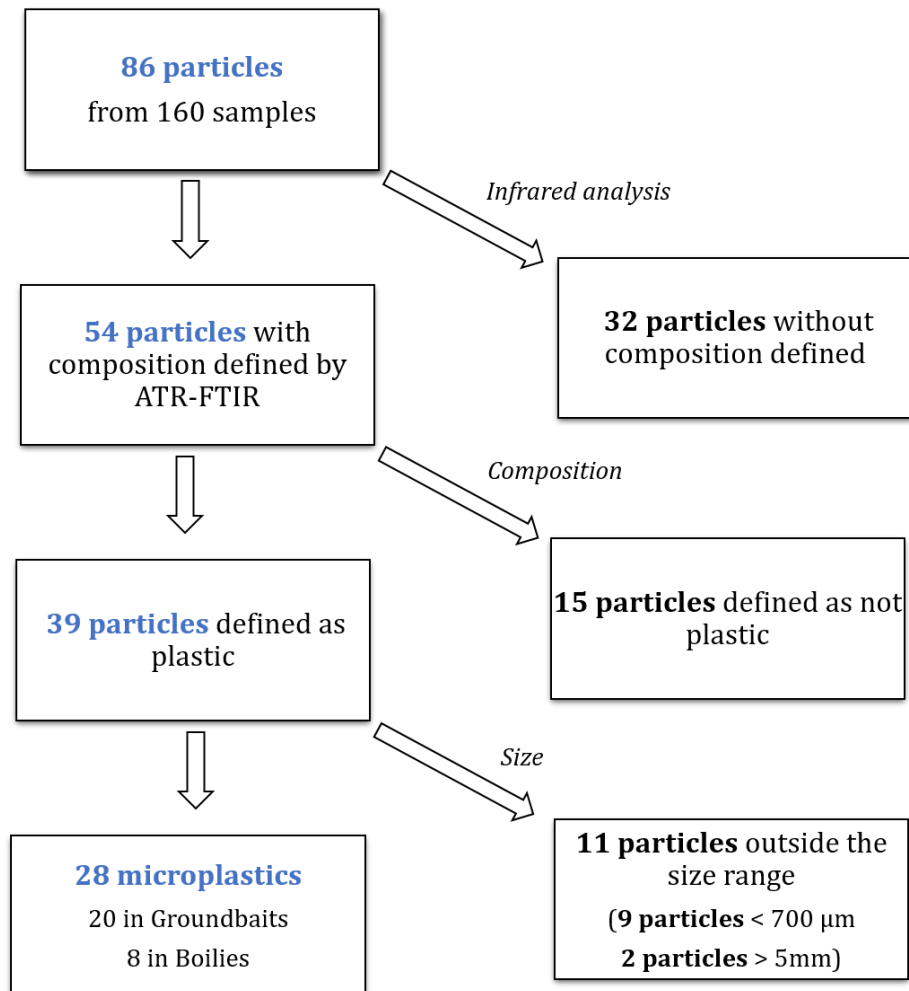


Figure 5.2: Flowchart of microplastic selection for data analyses

48.1 MP kg⁻¹. The difference in MP levels between groundbait and boilies were not significant (groundbait: range 0 - 300 MP.kg⁻¹; mean 33.3 ± 62.8 MP.kg⁻¹; boilies: range: 0 – 199 MP.kg⁻¹, mean 13.2 ± 42.8 MP.kg⁻¹, glmm: $\chi^2 = 15468.2$, $p < 0.001$, post-hoc pairwise comparison, $p = 0.082$, Fig. 5.3a). A significant difference in MP levels was detected between companies (glmm: $\chi^2 = 2863.1$, $p < 0.001$).

Table 5.1: List and characteristics of the angling baits analyzed in the present study.

Category	Product	Company	Bags purchased (n)	Bag mass (kg)	Reported ingredients	C:N ratio (mean \pm sd) (n =3)	MP concentration (mean \pm sd) (MP.kg-1 / mg.kg-1) (n = 10)
Groundbaits	G1	A	1	5	6	23.91 \pm 0.92	59.8 \pm 84.1 / 7.99 \pm 13.9
	G2	B	2	2.5	3	23.70 \pm 1.79	60.0 \pm 96.6 / 6.92 \pm 11.3
	G3	C	5	1	2	24.66 \pm 3.58	9.99 \pm 31.6 / 2.01 \pm 6.35
	G4	A	1	2	10	21.91 \pm 0.14	20.0 \pm 42.2 / 42.7 \pm 90.5
	G5	C	1	1	2	20.52 \pm 0.68	0 \pm 0 / 0 \pm 0
Boilies	G6	B	1	0.85	3	22.15 \pm 0.33	49.7 \pm 52.4 / 25.4 \pm 62.0
	B1	A	1	10	0	25.62 \pm 1.79	9.93 \pm 31.4 / 4.27 \pm 13.5
	B2	D	1	5	15	8.82 \pm 0.13	49.8 \pm 84.6 / 10.7 \pm 22.4
	B3	E	1	10	6	20.52 \pm 1.42	0 \pm 0 / 0 \pm 0
	B4	E	1	1	3	7.40 \pm 0.06	0 \pm 0 / 0 \pm 0
Pellets	B5	A	1	1	3	23.79 \pm 0.19	9.95 \pm 31.5 / 5.19 \pm 16.4
	B6	F	1	2	8	15.97 \pm 0.16	9.67 \pm 30.6 / 3.30 \pm 10.4
	P1	C	5	0.75	6	8.21 \pm 0.42	0 \pm 0 / 0 \pm 0
	P2	A	1	10	6	7.21 \pm 0.08	0 \pm 0 / 0 \pm 0
	P3	E	3	2	2	8.22 \pm 0.25	0 \pm 0 / 0 \pm 0
	P4	C	1	1	4	18.12 \pm 0.38	0 \pm 0 / 0 \pm 0

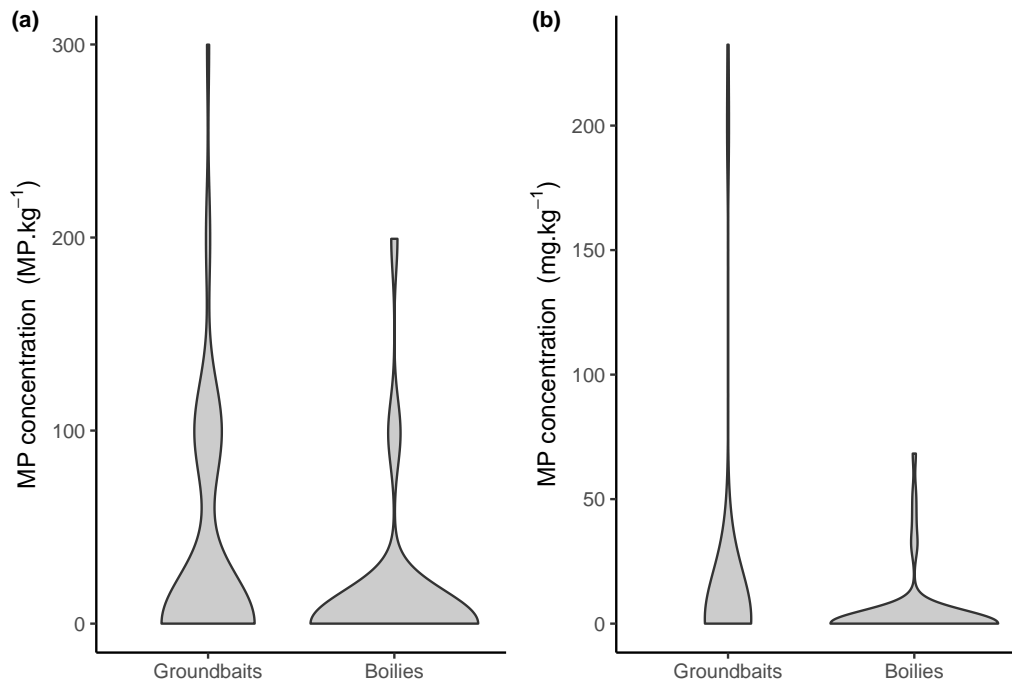


Figure 5.3: Microplastic concentrations in angling baits in (a) number (MP.kg⁻¹) and (b) mass (mg.kg⁻¹).

The range in MP concentration by mass across all samples was 0 to 232 mg.kg⁻¹ (mean 6.78 ± 29.8 mg.kg⁻¹) (Table 5.1). There were also significantly higher concentrations in groundbaits than in boilies (groundbait: range 0 to 232 mg.kg⁻¹, mean 14.2 ± 46.1 mg.kg⁻¹; boilies: range 0 to 68.3 mg.kg⁻¹, mean 3.91 ± 13.3 mg.kg⁻¹; glmm: $\chi^2 = 29758.1$, $p < 0.001$, post-hoc pairwise comparison, $p < 0.001$, 5.3b). Significant differences were also found between companies (glmm, $\chi^2 = 4027.6$, $p < 0.001$).

5.3.2 Microplastic characteristics

The microplastics detected in groundbaits and boilies were almost exclusively comprised of fragments, with only one fibre detected (Fig. 5.1e). Polyethylene was the main polymer found across these two categories (35.7%), followed by artificial additives (32.1%) (mainly alkyd resins), polyvinylester (21.5%), polypropylene (7.1%) and polyacrylate (3.6%) (Fig. 5.4a). Red and white were the main colours detected (both 28.6%), but with blue (17.9%), yellow (10.7%), black and green (7.1% each) also present (Fig. 5.4b). The mean MP size was 2.25 ± 1.26 mm (Fig. 5.4c). Overall, there was no significant difference between groundbaits and boilies in microplastic composition and colour (Fisher tests, $p > 0.05$), and microplastic size (Wilcoxon test,

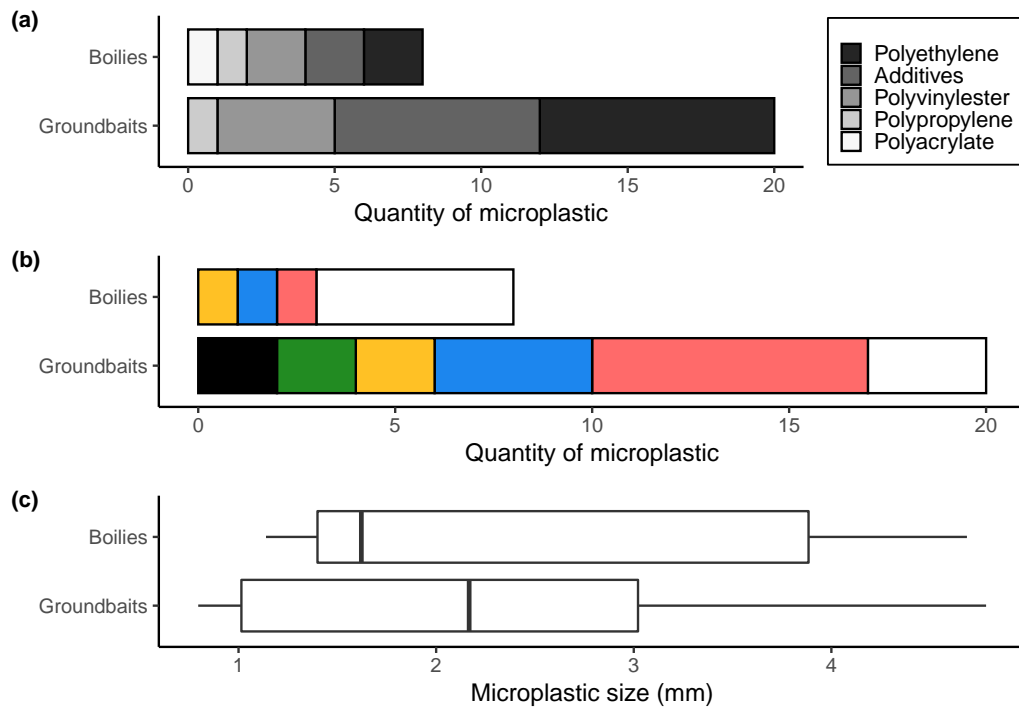


Figure 5.4: Microplastic characteristics in boilies ($n = 8$) and groundbaits ($n = 20$): (a) polymer composition, (b) colour (as displayed) and (c) size (mm).

$p > 0.05$). The bait packaging was composed of two polymers, polyethylene terephthalate and polyethylene (Table 5.2). Polyethylene terephthalate was never detected as a contaminant of angling baits and only 37.5% of packages were polyethylene. Other microplastics present in the baits were also not associated with their packaging (e.g. polyvinylester (G2 and B2) and artificial additives (B2)). Thus, microplastics found in these baits were apparently not derived from their packaging.

Table 5.2: Polymer composition of angling bait packaging and of microplastics observed in each model.

Category	Product	Packaging composition	Microplastic
Groundbaits	G1	polyethylene terephthalate	polypropylene (n = 1), polyvinylester (n = 2), additives (n = 3)
	G2	polyethylene	polyethylene (n = 4), polyvinylester (n = 2)
	G3	polyethylene terephthalate	additive (n = 1)
	G4	polyethylene terephthalate	additives (n = 2)
	G5	polyethylene terephthalate	None
Boilies	G6	polyethylene terephthalate	polyethylene (n = 4), additive (n = 1)
	B1	polyethylene	polyethylene (n = 1)
	B2	polyethylene	polyethylene (n = 1), polyvinylester (n = 2) additives (n = 2)
	B3	polyethylene	None
	B4	polyethylene terephthalate	None
Pellets	B5	polyethylene terephthalate	polyacrylate (n = 1)
	B6	polyethylene terephthalate	polypropylene (n = 1)
	P1	polyethylene terephthalate	None
	P2	polyethylene	None
	P3	polyethylene terephthalate	None
	P4	polyethylene	None

5.3.3 Relationships of microplastic levels with bait ingredients and C:N ratios

When analysing all angling products together (n = 16), the correlations between the number of ingredients reported on packages and microplastic concentrations were not significant (Spearman correlations, $\rho = 0.15$, $p = 0.572$ and $\rho = 0.24$, $p = 0.375$ for number and mass concentration, respectively). A significant and positive correlation was observed between microplastic concentrations and C:N ratio (Spearman correla-

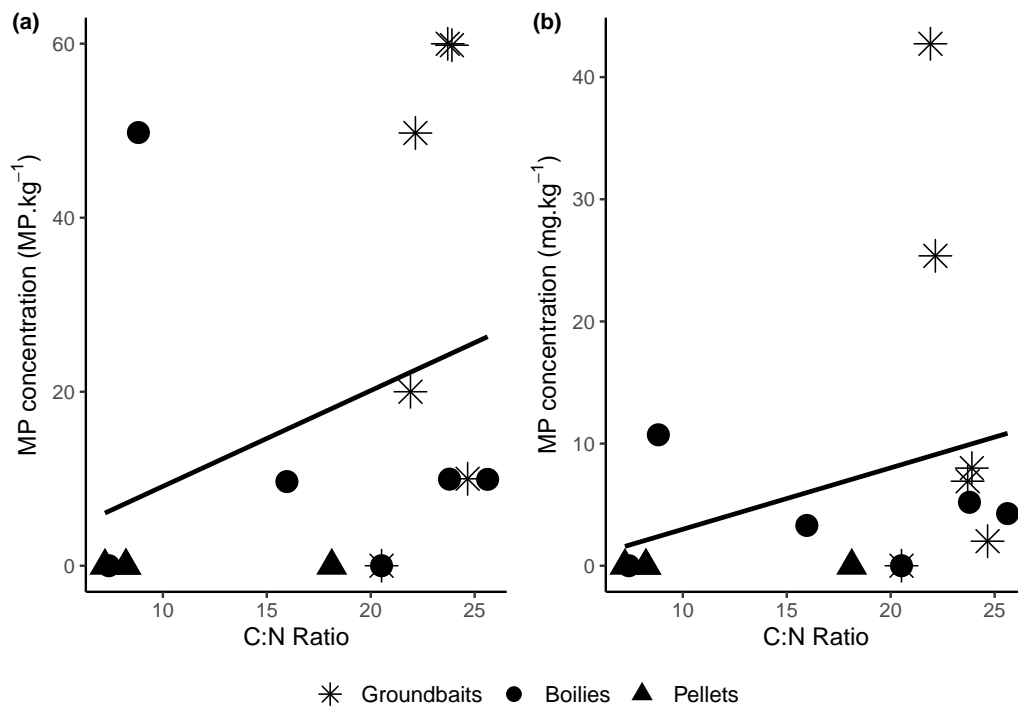


Figure 5.5: Relationship between average microplastic concentration (across all replicates) in (a) number (MP.kg⁻¹) and (b) mass (mg.kg⁻¹), and C:N ratio in angling baits. A lower C:N ratio indicates a higher proportion of animal-based components.

tions, $\rho = 0.62$, $p = 0.018$ and $\rho = 0.55$, $p = 0.028$ for number and mass concentration, respectively) (Fig. 5.5). This suggests that microplastic concentration was higher in angling baits with higher C:N ratios

5.4 Discussion

This investigation into microplastic contamination in angling baits revealed microplastic particles (700 μm – 5 mm) contaminated two out of three studied bait categories; groundbaits and boilies, with significant differences between categories and companies. Microplastics were mainly composed of polyethylene and artificial additives such as alkyd resins and paint additives, and were mainly white, red and blue. There was no correlation between the number of bait ingredients and their microplastic concentration and the C:N ratio of the baits was positively correlated with contamination level.

Given the incidence and number of microplastics per unit of bait mass, they could represent a significant source of microplastics to freshwater fish when fishing pressure is high. Once in the water, fish may consume the microplastics derived from angling baits either directly, i.e. microplastic released from bait, or indirectly through the ingestion of contaminated bait or other biota that have themselves consumed bait. The fact that anglers tend to target larger individuals (Gutmann Roberts et al., 2017), whose diets may also depend heavily on angling baits (Bašić et al., 2015; De Santis et al., 2019), suggests that larger fish might be most exposed to microplastics via this pathway. While several studies have already identified correlations between fish body size and microplastic loads in fish (Dantas et al., 2012; Garcia et al., 2021; Pegado et al., 2018; Ramos et al., 2012; Ryan et al., 2019), it is currently unknown whether this might be related to the consumption of contaminated baits.

Species of the Cyprinidae family are the primary target for the angling baits investigated and microplastics have previously been detected in common carp (*Cyprinus carpio*) from several rivers (Jabeen et al., 2017; Merga et al., 2020; Park et al., 2020; Warrack et al., 2018). Microplastic incidence and counts within cyprinid fishes have generally been high compared to other fish within the same system, ranging from 2.5 to 48 pieces per individual in the gastrointestinal tract (Merga et al., 2020; Park et al., 2020; Warrack et al., 2018), with many of these studies implicating the benthic foraging habits of carp as a likely explanation. Fish feeding largely on baits, particularly large individuals, could reasonably achieve the levels of microplastic contamination observed in the wild, although it is acknowledged that identifying the sources of specific microplastics is difficult. While the potential effects of ingestion of contaminated baits have also yet to be determined, microplastic exposure has been shown to adversely affect *C. carpio* biochemistry, immunological activity, growth and oxidative pathways within the laboratory (Banaee et al., 2019; Nematdoost Haghi and Banaee,

2017; Xia, 2020).

The diverse size range of microplastic particles detected in studies limits comparisons between the microplastic concentrations in the baits and those that are generally found in the biota (Hartmann et al., 2019). However, comparisons of bait microplastics with those detected in wild fish have shown that both are dominated by particles of varying colours (Merga et al., 2020; Park et al., 2020). Polymer compositions in baits were also similar to those in wild fish, with polypropylene and polyethylene common (Garcia et al., 2021; Merga et al., 2020), although other studies have also identified polymers such as polytetrafluoroethylene and rayon (Park et al., 2020), which were absent from our angling baits. The most commonly identified microplastics in the angling baits included some of the five most frequently used plastics that account for 90% of polymers used (Andrady and Neal, 2009). As such, it is difficult to draw inferences about microplastic contributions via angling baits by comparing polymer data alone.

The absence of microplastics in the selected size range (700 μm – 5 mm) in the pelletized angling baits suggests some key differences in their ingredients and/or manufacturing process compared with groundbait and boilies. Polyethylene microplastics and additives such as polyolefin and alkyd resins found in the angling baits are commonly present in machinery paints (Hofland, 2012) that might gradually fragment over time. Various heating, milling and filtering processes during manufacturing may also alter, and fragment microplastic particles, potentially producing smaller particles falling outside the minimal threshold used in the present study (Hanachi et al., 2019; Karbalaei et al., 2020). This higher level of industrial manufacturing might explain the absence of larger particles in the pelletized angling baits. Considering that particle size is an important factor determining microplastic ingestion and impacts on organisms (Hartmann et al., 2019; Jung et al., 2021), further investigations focusing on smaller fragment sizes (< 700 μm) are needed. That the microplastics identified in the baits were of a different composition to their packaging further suggests contamination existing within the raw ingredients and/or introduced during their manufacture, although further work is necessary to identify the exact stage(s) and source(s) of contamination.

Commercial fishmeal has previously been shown to contain microplastics (Hanachi et al., 2019; Karbalaei et al., 2020), with often higher concentrations occurring in those with particular ingredients and/or manufacturing processes. Fishmeal was found to contain mostly fragments 100-1000 μm in size that were composed of polypropylene, polyethylene and polystyrene (Hanachi et al., 2019; Karbalaei et al., 2020), which are

largely comparable to those particles found in our angling baits. Hanachi *et al.* (2019) additionally found higher microplastic concentrations in salmon and sardine than kilka-derived fishmeal, whereas, contrary to our first hypothesis, we found a positive correlation between bait C:N ratio and microplastic contamination, suggesting lower contamination in animal-based baits. Nevertheless, the similarity in microplastic features to those recovered from industrialized food (Prata, Paço, *et al.*, 2020) suggests at least some procedural contamination from the manufacture process.

The estimates of the extent of cryptic microplastic emissions from angling baits were as high as 0.34 tons per year for a country, when considering 7.3 kg of ground-baits/angler/year and 3.3 million active anglers, as in Germany (Arlinghaus and Niesar, 2005), but this does largely depend on the activity of anglers in a country and the amounts of baits they apply in their angling. Nevertheless, this estimate is comparable to the annual 0.15 tons of microplastics released through the use of winter de-icing salts applied to roads in some European countries (Rødland *et al.*, 2020). At a larger scale, the microplastic contribution through angling baits can be considered minor compared to the top sources of European riverine microplastics (tyre wear particles, polymer-based textiles and polymers washed in from road dusts) which may each contribute more than 0.3 kilotons of microplastic a year (Siegfried *et al.*, 2017). Nevertheless, angling bait-derived microplastics may make up a large proportion of local microplastic concentrations in particular locations or at particular times of the year when the baits are heavily used. Also, as expected for natural particles, microplastic concentration might increase with decreasing particle size (Filella, 2015), and the total contamination by microplastics would probably be substantially higher, as only particles from 700 μm to 5 mm were considered here. Further investigations are needed to fully understand the potential contribution of this cryptic source of microplastic pollution compared to the global microplastic pollution in freshwater ecosystems. This contribution will likely be extremely variable among ecosystems and countries, depending on both the characteristics of the ecosystems (e.g. size, fish community) and of the fisheries (e.g. amount and type of bait used). We posit that in small lakes with intense fishing pressure targeting coarse fishes angling baits might represent an important source of microplastic pollution compared to other sources. Since angling baits are already known to contribute to freshwater eutrophication (Amaral *et al.*, 2013; Arlinghaus and Niesar, 2005), the additional release of microplastics from contaminated baits may represent another, co-occurring stressor to freshwater systems which requires further investigation. The awareness of hidden sources could contribute to the design of studies investigating consequences and impacts of microplastic to human

and animal health.

The ubiquitous presence of microplastic particles in the environment means that mitigating this angling source of contamination might have a negligible impact. Nevertheless, our results are important in highlighting a previously unknown source of microplastic loadings in freshwater fishes and thus have helped identify a novel source and pathway. Such cryptic sources of microplastic contamination to freshwater ecosystems reveals the ubiquity of plastics within products used in daily human activities and, more specifically, on the relevance of angling activity in European countries in increasing the exposure of fish to these plastics. The manufacturing process of industrialized food, either for human or animal consumption, thus represents a potential source of microplastic contamination that has yet to be fully quantified and therefore further studies are encouraged in order to investigate the sources of these cryptic microplastics, and their fates and impacts in the environment.

6 | Ecological drivers of microplastic consumption by aquatic macroinvertebrates and fish

Adapted from: Garcia, Flavien, Aline R. de Carvalho, Louna Riem-Galliano, Loïc Tudesque, Magali Albignac, Alexandra ter Halle, and Julien Cucherousset. 2021. “Stable Isotope Insights into Microplastic Contamination Within Freshwater Food Webs.” *Environmental Science & Technology*. 55 (2): 1024–35. <https://doi.org/10.1021/acs.est.0c06221>.

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 ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) 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.

6.1 Introduction

Freshwater ecosystems provide a myriad of services to humans but are facing growing impacts from human activities (Vörösmarty et al., 2010) with multiple and interacting perturbations altering biodiversity and ecosystem functioning (Jackson et al., 2016). Microplastic pollution, the presence of small fractions (< 5 mm) of plastic (Arthur et al., 2009) 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 (Collard et al., 2019; Rochman, 2018; Wagner et al., 2014).

Studies on microplastic pollution have typically focused on marine ecosystems, (Eerkes-Medrano et al., 2015; C. Li et al., 2020) but streams and rivers play a crucial role in the global microplastic pollution (Rochman, 2018). Indeed, 70-80% of marine plastics are transported by freshwaters (Cole et al., 2011; Horton, Walton, et al., 2017). Freshwater microplastic pollution is strongly variable within hydrological networks (Mani and Burkhardt-Holm, 2019; Vermaire et al., 2017) and usually higher in urban and industrialized areas (Pinheiro et al., 2017). Microplastics are ingested by freshwater organisms and the consequences of these ingestions are variable (Collard et al., 2019; Eerkes-Medrano and Thompson, 2018; Eerkes-Medrano et al., 2015). High levels of ingestion generally occur in sites with high microplastic pollution in the water (Horton et al., 2018; Scherer et al., 2017) or sediment (Merga et al., 2020), but this relationship does not hold systematically (McNeish et al., 2018; Roch et al., 2020). Microplastic ingestion is also dependent upon organism biological traits. This includes, for instance, body size, whereby ingested microplastic size and abundance typically increase with organism body size (Jâms et al., 2020; McNeish et al., 2018). Microplastic ingestion can differ between functional feeding groups and foraging style (McNeish et al., 2018; Thushari et al., 2017; Walkinshaw et al., 2020), with microplastic abundance ingested by visual foragers increasing with increased microplastic concentration in water (Roch et al., 2020). In addition, microplastic characteristics can also influence their consumption by organisms, with their size being limited by gill raker apparatus in fish (Collard et al., 2017), while food-like and sinking particles were reported to be more often ingested by fish (Roch et al., 2020). 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 (Collard et al., 2019, 2017; López-Rojo et al., 2020). Investigations are therefore needed to better understand the mechanisms of microplastic ingestion by freshwater organisms.

Studies on microplastic ingestion neglected that individuals within species are highly variable ecologically (Des Roches et al., 2018), and that trophic niches are shaped by complex and interacting ecological parameters (Araújo et al., 2011). Intraspecific variability occurs both in terms of functional traits and trophic niches, within and between the life-stage of a species (Violle et al., 2012; Zhao et al., 2019, 2014). Therefore, the use of functional feeding groups might oversimplify individual trophic niches, precluding an integrative understanding of microplastic ingestion. During the last two decades, stable isotope analyses have emerged as an integrative tool used by trophic ecologists to quantify the realised trophic niche (Layman et al., 2012). Compared to traditional methods such as stomach content and faeces 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 analysed, of the diet of individuals (Fry, 2006; Layman et al., 2012). 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), maximising the amount of information obtained from sampled individuals. Specifically, stable isotope analyses of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) 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 (Cucherousset et al., 2012; Fry, 2006; Jackson et al., 2017). 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 ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). 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, colour, size and polymer composition) between microplastic in the environment and those ingested by organisms. Finally, we quantified the relationship between the trophic ecology of organisms and microplastic ingestion.

6.2 Material and methods

Sampling strategy and sample treatment are described at Chapter 2.

6.2.1 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 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 ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) were performed by Cornell University Stable Isotope Laboratory (COIL, USA).

6.2.2 Statistical analyses

To quantify microplastic contamination in macroinvertebrates and fish, we first calculated microplastic abundance as the number (count) of microplastic ingested per individual. For macroinvertebrates, the number of individuals included in each sample was variable. However, the maximum number of microplastic measured in each sample was 1, indicating that it could have been ingested only by a single individual. We therefore counted the total microplastic 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 analysed 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 ($\text{microplastic}\cdot\text{m}^{-3}$) and microplastic pollution in the sediment was calculated as the number of microplastics per surface area sampled ($\text{microplastic}\cdot\text{m}^{-2}$). 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 sam-

ple 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 the sediment and those ingested by macroinvertebrates and fish, χ^2 tests were used for microplastic shape (fragments and fibres) and colour (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 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. $\delta^{13}\text{C}$ values were transformed following Jackson et al. (2017) (Jackson et al., 2017):

$$RO_{\text{SAMPLE}} = \frac{{}^{13}\text{C}_{\text{SAMPLE}} - {}^{13}\text{C}_{\text{ALLO}}}{{}^{13}\text{C}_{\text{AUTO}} - {}^{13}\text{C}_{\text{ALLO}}}$$

where RO_{SAMPLE} is the resource origin value for a given consumer sample, $\delta^{13}\text{C}_{\text{ALLO}}$ is the average value of allochthonous primary producers in a given site (i.e. leaf litter) and $\delta^{13}\text{C}_{\text{AUTO}}$ 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). $\delta^{15}\text{N}$ values were then used to calculate the trophic position (TP) (Post, 2002):

$$TP_{\text{SAMPLE}} = TP_{\text{BASE}} + \frac{{}^{15}\text{N}_{\text{SAMPLE}} - {}^{15}\text{N}_{\text{BASE}}}{\text{TEF}}$$

Using the primary producers as baseline, TP_{BASE} of 1 and the mean trophic fractionation of $\delta^{15}\text{N}$ (TEF) of 3.4. The average $\delta^{15}\text{N}$ value per sampled site of leaf litter was used as a baseline because some $\delta^{15}\text{N}$ values of periphyton and macrophytes were unexpectedly high in the most urbanised sites, likely because of anthropogenic nitrogen inputs, explaining also why the estimated trophic position of consumers were elevated. We specifically compared microplastic ingestion between functional groups and tested the relationship between microplastic ingestion, trophic position estimated using $\delta^{15}\text{N}$ and the origin of the resource consumed quantified using $\delta^{13}\text{C}$. First, we tested the relationship between the body size (log-transformed) of macroinvertebrates and fish and the abundance of ingested microplastic using generalized linear mixed-effects models and sampled site as a random factor. Generalized linear mixed-effects

models with sampled site 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 also used to test the relationship between stable isotope metrics (trophic position and resource origin), microplastic colour (abundance of the dominant colour for macroinvertebrates and fish, respectively) and microplastic shape (abundance of fragments) using individual identity nested in 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 analyses were performed in R (Version 1.3.1056) (Team, 2019) and generalised linear mixed effects models and linear mixed effects models were performed using the package lme4 v.1.1.10 (Bates et al., 2015). Significant levels of mixed effects models were obtained using the ‘Anova’ function in the car package (Fox and Weisberg, 2019).

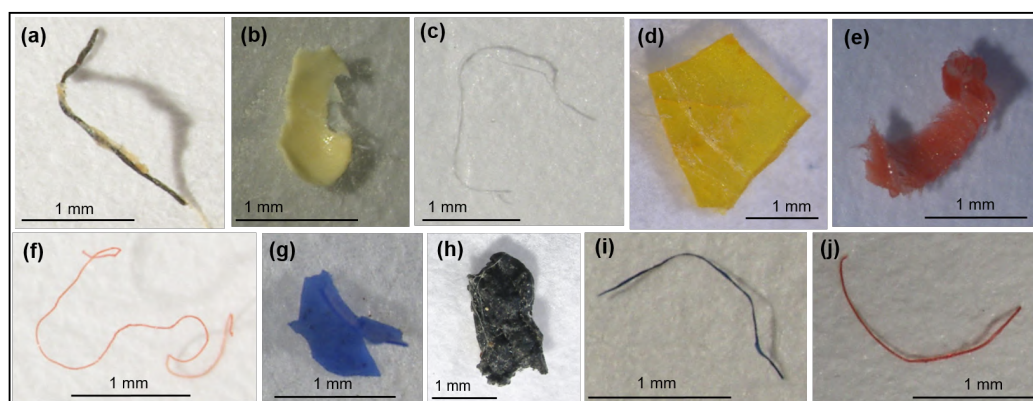


Figure 6.1: Examples of microplastics ingested by organisms and collected in the environment: (a) black fiber – PP (water); (b) yellow fragment – polyvinylester (fish); (c) white fiber – NA (macroinvertebrate); (d) yellow fragment – PE (water); (e) red fragment – polyester (fish); (f) red fiber – polyester (water); (g) blue fragment – PP (sediment); (h) black fragment – PP (water); (i) blue fibre – NA (fish); (j) red fibre – NA (fish).

6.3 Results

Some microplastic particles examples are described in Fig. 6.1 and detailed data about contaminated macroinvertebrates and fish in Tables 6.2 and 6.3.

6.3.1 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 6.2) and 61 microplastics in fish ($n = 492$ individuals belonging to 21 species, Table 6.3), representing an occurrence of 2% and 10 %, respectively. Microplastic abundance in macroinvertebrates (mean = $0.02 \text{ microplastic.ind}^{-1} \pm 0.15 \text{ SD}$) was significantly lower than in fish (mean = $0.13 \text{ microplastic.ind}^{-1} \pm 0.42 \text{ SD}$, glmm: $\chi^2 = 73.26$, $p < 0.001$). Microplastic abundance in macroinvertebrates and fish did not differ significantly among sampled sites (glm: $\chi^2 = 7.4467$, $p = 0.190$ and $\chi^2 = 9.172$, $p = 0.102$, respectively, Fig. 6.2).

Microplastic pollution in the surface water (mean = $0.87 \text{ microplastic.m}^{-3} \pm 1.24 \text{ SD}$) was significantly different among sampled sites (glmm: $\chi^2 = 77.297$, $p < 0.001$), with a significantly higher level of microplastic pollution in the surface water in sites TOU and LAU (Fig. 6.3a). Microplastic pollution in the sediment (mean = 24.84

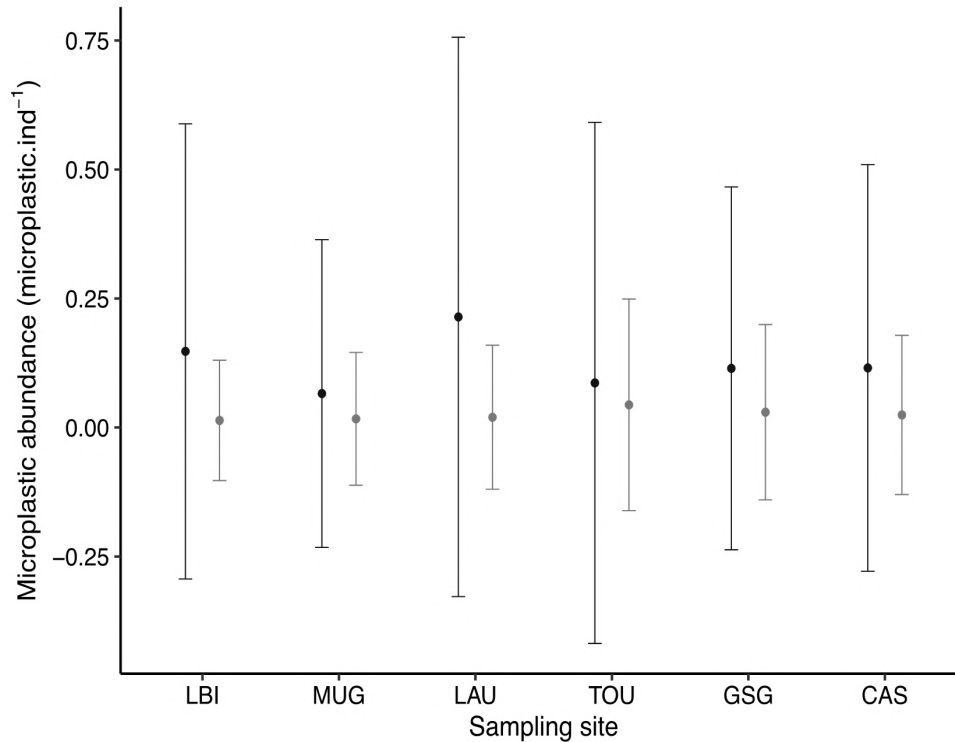


Figure 6.2: Microplastic abundance (mean \pm SD) in macroinvertebrates (grey symbols) and fish (black symbols) in the six studied sites

microplastic.m⁻² \pm 24.38 SD) was not significantly different between sampled sites (glmm: $\chi^2 = 7.770$, $p = 0.169$, Fig. 6.3b). Microplastic pollution in the sediment was strongly positively correlated with microplastic pollution in the surface water (Spearman correlation, $\rho = 0.90$, $p = 0.015$) but there was no significant correlation between environmental microplastic pollution and microplastic contamination of macroinvertebrates and fish (Spearman correlation, $\rho < 0.38$, $p > 0.462$, respectively).

6.3.2 Microplastic characteristics

Fragments represented 51% of all collected microplastics while fibres represented 49%. There was no significant difference in the proportion of particles and fibres between surface water and sediments (χ^2 test: $\chi^2 = 7.359$, $p = 0.289$). Macroinvertebrates and fish ingested a significantly higher proportion of fibres than available in the environment (χ^2 test: $\chi^2 > 8.653$, $p < 0.001$ and $\chi^2 > 22.677$, $p < 0.001$, respectively). There was no significant difference in the proportion of particles and fibres between macroinvertebrates and fish (Fig. 6.4a).

Across all collected microplastics, black and white were the most abundance

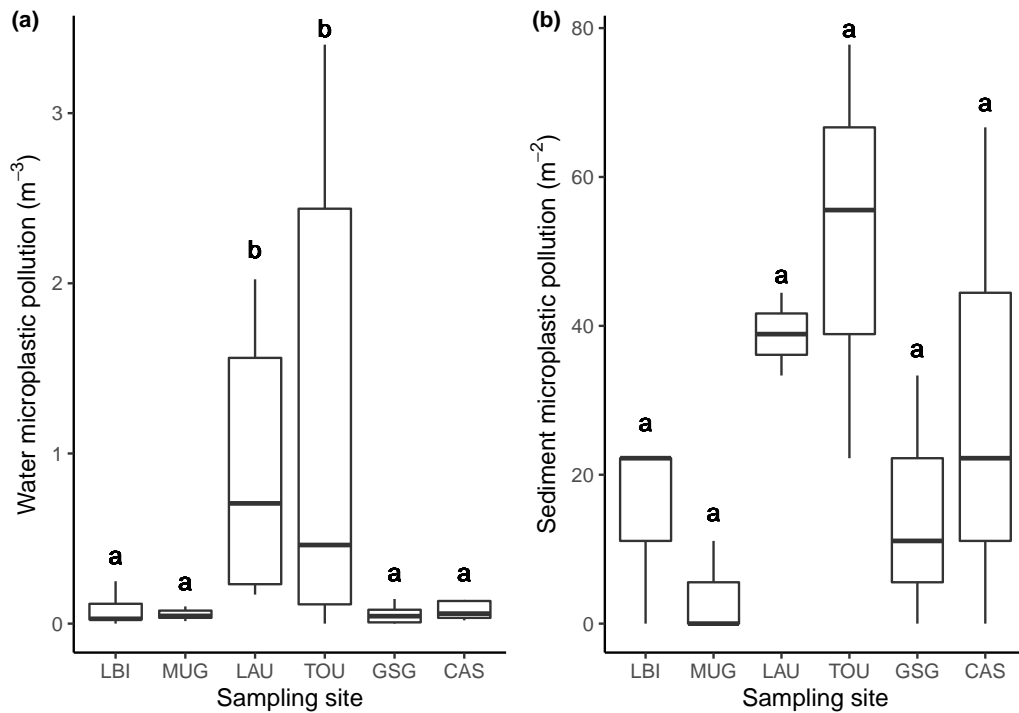


Figure 6.3: Microplastic pollution in (a) surface waters (MP.m⁻³) and (b) sediments (MP.m⁻²) in the studied sites. Different letters indicate significant difference ($p < 0.05$)

colours, representing 34 and 26 %, respectively, followed by red (14 %), blue (14 %), yellow (7 %) and green (5 %). The distribution of microplastic colour did not differ significantly between surface water and sediment (χ^2 test: $\chi^2 = 4.7647$, $p = 0.445$). The distribution of microplastic colours significantly differed between microplastics sampled in the environment (surface water and sediment) and those ingested by macroinvertebrates (χ^2 test: $\chi^2 > 16.089$, $p < 0.007$) 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: $\chi^2 < 10.928$, $p > 0.091$). The colour of microplastics ingested by macroinvertebrates was significantly different from those ingested by fish (χ^2 test: $\chi^2 = 20.371$, $p = 0.001$), with a higher proportion of white microplastics (post-hoc test: $p < 0.001$, Fig. 6.4b).

Microplastic size 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: $\chi^2 = 10.835$, $p = 0.013$, Fig. 6.5a) 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

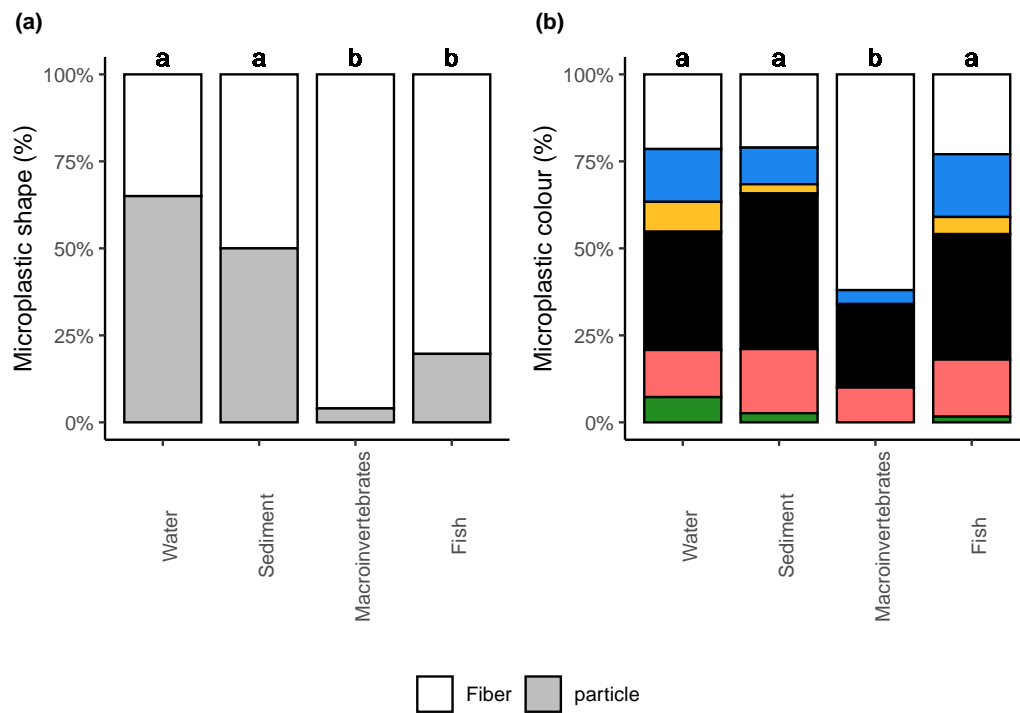


Figure 6.4: Microplastic characteristics in surface waters, sediments, macroinvertebrates and fish: (a) shape (fibres in white and fragments in grey), (b) colour (white, blue, yellow, black, red and green). Different letters indicate significant difference ($p < 0.05$).

ingested microplastics (lmm: $\chi^2 = 5.469$, $p = 0.019$) while this relationship was not significant for fish (lmm: $\chi^2 = 1.785$, $p = 0.182$, Fig. 6.6).

Across all particles, polyethylene (PE) represented 41 % of the total, 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: $\chi^2 = 7.359$, $p = 0.289$, Fig. 6.5d). Polymer composition significantly differed between microplastics found in the environment (water and sediment) and those ingested by organisms (χ^2 test: $\chi^2 > 39.665$, $p < 0.001$ and $p = 0.005$, respectively), with a higher proportion of artificial additives for macroinvertebrates (post-hoc test: $p < 0.001$, Fig. 6.5b) and a higher proportion of polyacrylate and polyester for fish (post-hoc test: $p < 0.018$, Fig. 6.5b). 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 (Fig. 6.5b).

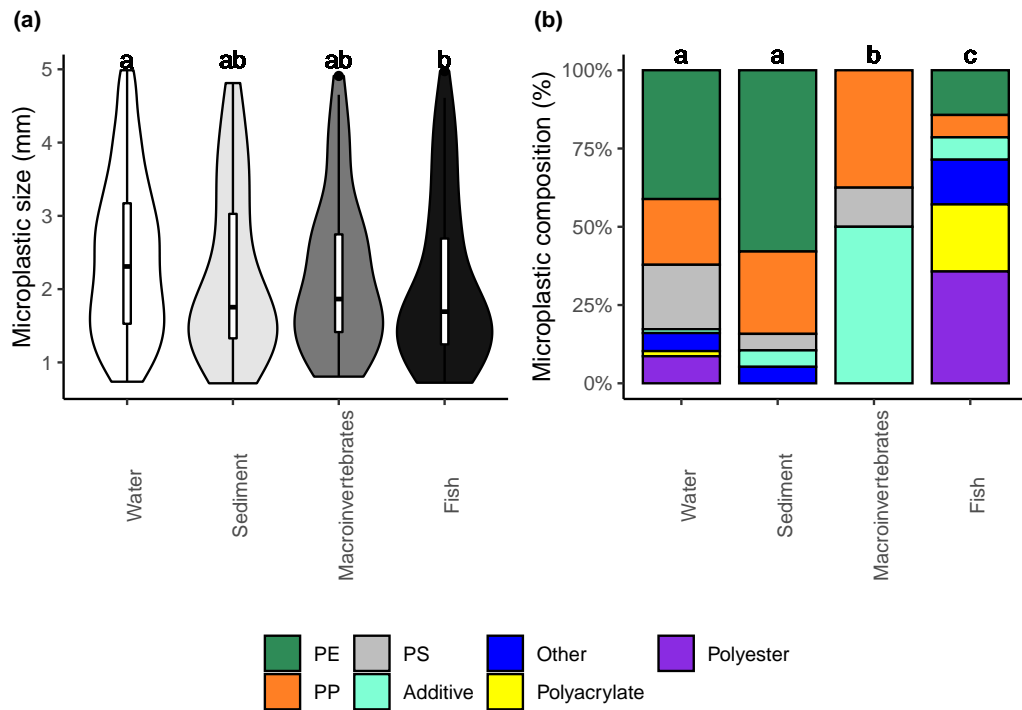


Figure 6.5: Microplastic characteristics in surface waters, sediments, macroinvertebrates and fish: (a) size (mm) and (b) polymer composition (polyethylene in green, polypropylene in orange, polystyrene in grey, artificial additives in cyan, polyacrylate in yellow, polyester in purple and other in dark blue). Different letters indicate significant difference ($p < 0.05$).

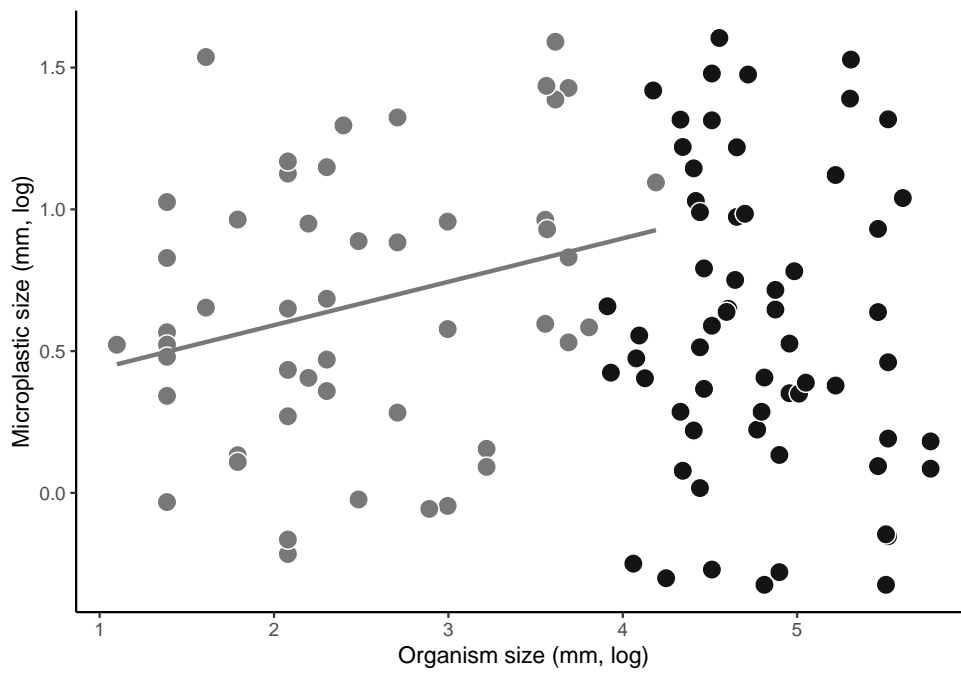


Figure 6.6: Relationship between organism size (mm) and the size of ingested microplastics (mm). Macroinvertebrates are displayed with grey symbols and fish are displayed with black symbols. The grey line represents the significant relationship between the size of the microplastics ingested by macroinvertebrates and their own size.

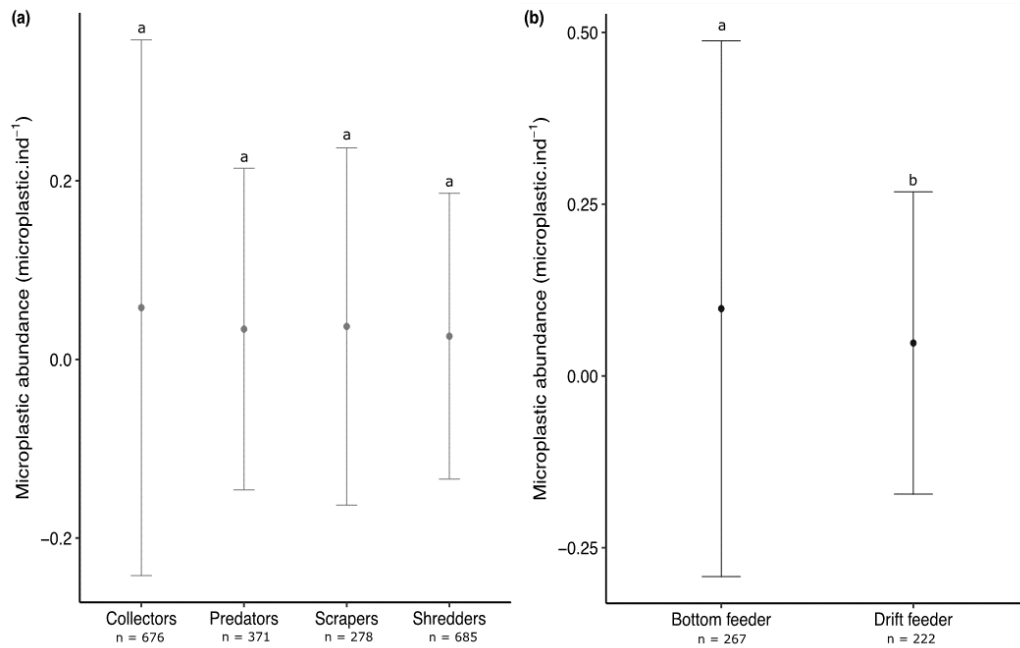


Figure 6.7: Microplastic abundance (mean \pm SD, microplastic.ind⁻¹) in the different feeding groups of (a) macroinvertebrates and (b) fish. Different letters indicate significant difference.

6.3.3 Microplastic contamination and the trophic niche of organisms

Microplastic abundance significantly increased with increasing body size for both macroinvertebrates and fish (glmm: $\chi^2 > 6.494$, $p < 0.011$). Microplastic abundance did not significantly differ between feeding groups of macroinvertebrates (glmm: $\chi^2 = 3.151$, $p = 0.369$, Fig. 6.7a), while the difference was significant in fish (glmm: $\chi^2 = 4.104$, $p = 0.043$, Fig. 6.7b), with bottom-feeders displaying a higher microplastic abundance than column-feeders. Stable isotope analyses (Fig. 6.8) revealed a high level of trophic niche variability within species. In macroinvertebrates, there was a nearly significant relationship between microplastic abundance and their trophic position (glmm: $\chi^2 = 3.029$, $p = 0.082$), and this relationship was not significant with resource origin (glmm: $\chi^2 = 0.071$, $p = 0.790$, Fig. 6.8).

Microplastic abundance in fish was not significantly related to their trophic position (glmm: $\chi^2 = 0.566$, $p = 0.452$) but decreased significantly when resource origin increased (glmm: $\chi^2 = 5.140$, $p = 0.023$, Fig. 6.8), i.e. microplastic abundance was higher in fish consuming resources containing a higher proportion of allochthonous carbon.

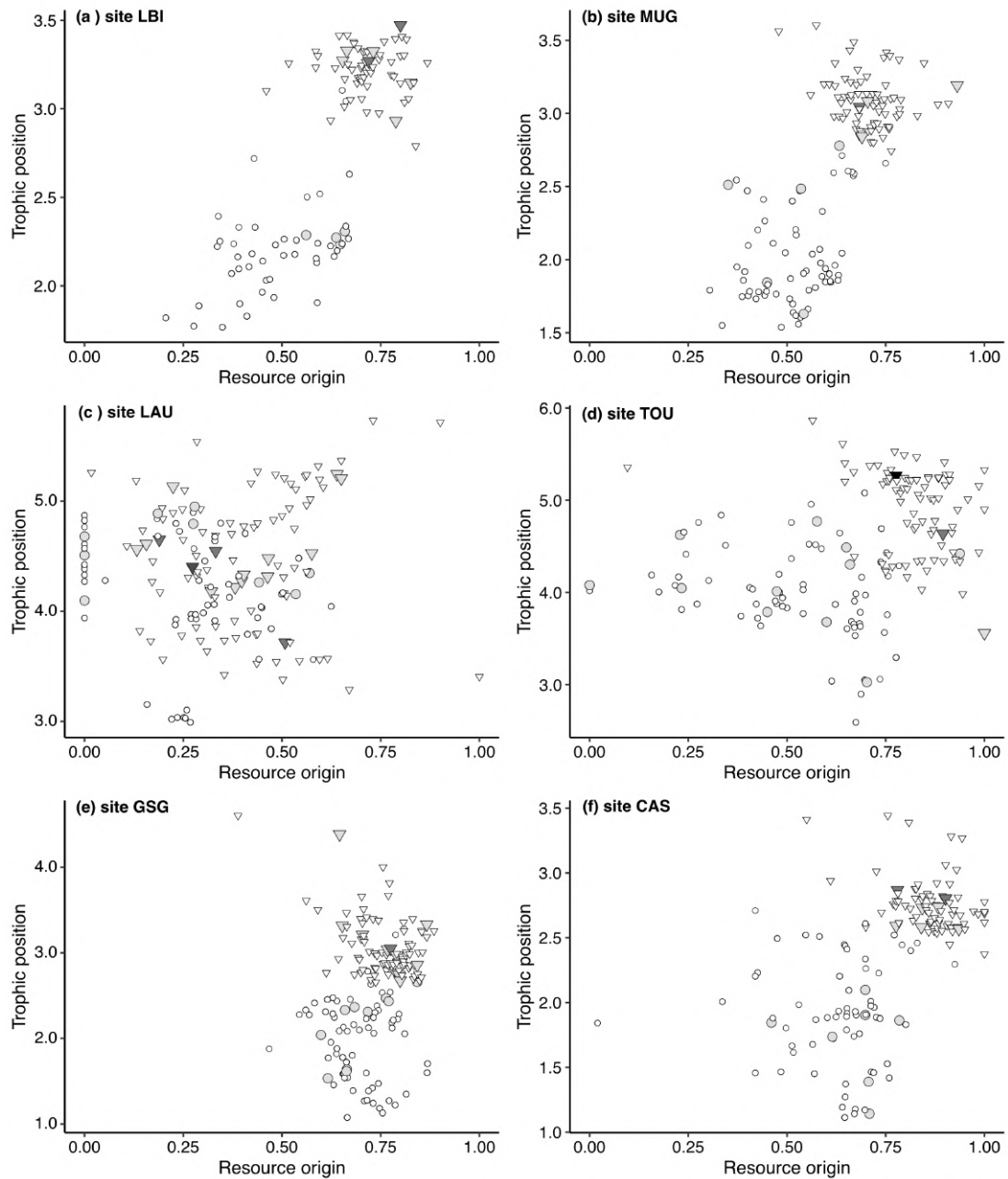


Figure 6.8: Biplot of resource origin and trophic position of each organism measured using stable isotope analyses in each studied site. Macroinvertebrates are displayed with circles and fish are displayed with triangles. Microplastic abundance is displayed using colours: white (no microplastic), clear grey (1 microplastic), medium grey (2 microplastics), dark grey (3 microplastics) and black (4 microplastics).

There was no significant effect of the stable isotope metrics (trophic position and resource origin) on the colour and shape of microplastics ingested by macroinvertebrates. Microplastic size was unrelated to the trophic position of macroinvertebrates (glmm: $\chi^2 = 0.372$, $p = 0.542$), however microplastic size was significantly higher in macroinvertebrates consuming resources with allochthonous carbon (glmm: $\chi^2 = 6.644$, $p = 0.010$, Table 6.1, Fig. 6.9). There was no significant relationship between stable isotope metrics and microplastic characteristics (colour, shape and size) in fish (Table 6.1).

Table 6.1: 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 (colour, shape and size) of microplastics ingested by macroinvertebrates and fish

Macroinvertebrates / Response variables	Predictor	Estimate (SE)	z	p
Colour (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
Size	Intercept	-13.67 (9.87)	-1.385	0.166
	TP	-0.05 (0.08)	-0.610	0.542
	Intercept	0.79 (0.26)	3.064	
Fish / Response variables	RO	0.72 (0.28)	2.578	0.010
	Intercept	-0.21 (0.25)	0.845	0.398
Colour (black)	Predictor	Estimate (SE)	z	p
	TP	-0.16 (1.48)	-0.105	0.916
Shape	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
	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

Size	Intercept	-11.70 (6.71)	-1.744	0.081
	TP	-0.01 (0.12)	-0.120	0.904
	Intercept	0.62 (0.44)	1.405	
	RO	-0.56 (0.46)	-1.215	0.224
	Intercept	0.97 (0.35)	2.796	

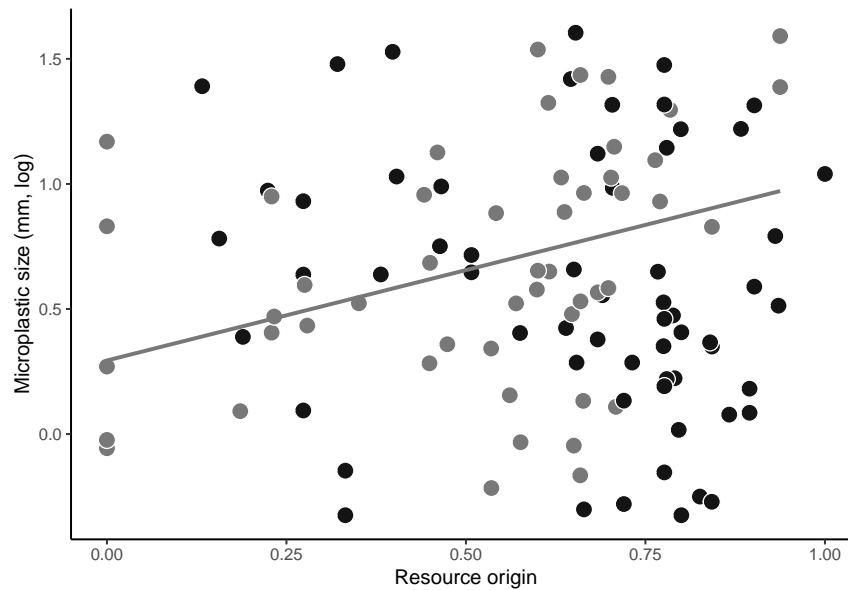


Figure 6.9: Relationship between the resource origin of organisms and microplastic size (mm). Macroinvertebrates are displayed with grey symbols and fish are displayed with black symbols. The grey line represents the significant relationship between the size of the microplastics ingested by macroinvertebrates and the resource origin.

Table 6.2: Number of individuals analysed per macroinvertebrate taxa and per site in the study. The values under brackets represent the number of individual contaminated with microplastics. All contaminated individuals ingested one microplastic.

Taxa	Family	Code	Feeding mode	LBI	MUG	LAU	TOU	GSG	CAS
<i>Asellidae sp</i>	asellidae	ASEL	shredders	0	0	52 (1)	10 (1)	7	8
<i>Echinogammarus sp</i>	gammaridae	GAMM	shredders	107 (3)	58	134 (1)	99 (3)	90 (1)	72 (1)
<i>Corbicula fluminae</i>	corbiculidae	CORB	collectors	12	52	41	34	70 (2)	45 (1)
<i>Radix sp</i>	lymnaeidae	MOL1	collectors	0	7	0	0	16	0
<i>Theodoxus fluviatilis</i>	neritidae	MOL2	collectors	5	15 (1)	0	0	20 (1)	36
<i>Ancylus fluviatilis</i>	planorbidae	MOL3	collectors	7	0	0	0	0	0
<i>Faxonius limosus</i>	cambaridae	FAXO	shredders	0	8 (1)	0	0	8 (2)	5
<i>Procambarus clarkii</i>	cambaridae	PROC	shredders	0	0	1	10 (3)	0	2
<i>Atyaephyra desmarestii</i>	atyidae	CREV	shredders	0	0	0	0	0	14 (2)
<i>Diptera sp</i>	diptera	DIPT	collectors	2	0	0	0	0	0
<i>Ecdyonurus sp</i>	heptageniidae	EPH1	scrapers	0	19	0	0	0	0
<i>Ephemeroptera sp</i>	ephemeroptera	EPH2	scrapers	0	0	0	0	2	0
<i>Baetis sp</i>	baetidae	EPH3	scrapers	0	0	0	0	0	18 (2)
<i>Caenis sp</i>	caenidae	EPH4	scrapers	0	0	0	0	0	1
<i>Ephemerella sp</i>	ephemerellidae	EPH5	scrapers	66	0	0	0	0	0
<i>Ephoron virgo</i>	polymitarcyidae	EPH6	scrapers	0	0	0	53 (2)	0	0
<i>Potamanthus luteus</i>	potamanthidae	EPH7	scrapers	0	40 (1)	0	0	0	0
<i>Ephemera sp</i>	ephemeridae	EPHM	scrapers	12 (2)	0	67	0	0	0
<i>Hydropsyche sp</i>	hydropsychidae	HYDR	collectors	30	30 (1)	66 (2)	17	36	39 (1)
<i>Aphelocheirus aestivalis</i>	aphelochiridae	NOTO	predators	0	16	0	0	12	0
<i>Odonata sp</i>	odonata	ODO1	predators	0	0	0	3	0	0

<i>Anisoptera sp</i>	anisoptera	ODO2	predators	2	2	1	5	3	0
<i>Zygoptera sp</i>	zygoptera	ODO3	predators	4	8	1 (1)	3	0	0
<i>Onychogomphus sp</i>	gomphidae	ODO4	predators	0	0	0 (3)	0	0 (2)	6
<i>Platycnemis sp</i>	platycnemididae	ODO5	predators	0	0	7	0	9	0
<i>Calopteryx sp</i>	calopterygidae	ODO6	predators	0	0	0	0	5 (1)	4
<i>Oligochaeta sp</i>	oligochaeta	OLIG	collectors	0	8	2	47 (1)	0	4
<i>Planariidae sp</i>	planaridae	PLAN	predators	0	35 (1)	82 (1)	32 (3)	48 (1)	27
<i>Chironomidae sp</i>	chironomidae	CHIR	collectors	5	4	0	13	0	4
<i>Achetae sp</i>	achetae	SANG	predators	0	0	0	0	11	0
<i>Simuliidae sp</i>	simulidae	SIMU	collectors	6	0	0	0	0	0
<i>Rhyacophila sp</i>	rhyacophilidae	RHYA	predators	39	3	0	0	0	3
<i>Brachycentrus sp</i>	brachycentridae	TRI1	collectors	0	5	0	0	0	0
<i>Lepidostoma hirtum</i>	lepidostomatidae	TRI2	collectors	0	0	0	4 (2)	0	0
<i>Trichoptera sp</i>	trichoptera	TRI3	collectors	6	0	0	0	0	0
<i>Total collected</i>	-	-	-	291	298	454	342	337	288

Table 6.3: Number of individuals analysed per fish specie and per site in the study. The values under brackets indicate the number of contaminated individuals and the number of asterisks represent the number of microplastics found.

Species	Family	Code	Feeding mode	LBI	MUG	LAU	TOU	GSG	CAS
<i>Alburnus alburnus</i>	cyprinidae	ABL	column feeders	0	1	15 (2* + 1**)	0	18 (1*)	7 (1**)
<i>Barbus barbuis</i>	cyprinidae	BAR	bottom feeders	2	8	20 (4* + 2** + 1***)	30 (1** + 1****)	7 (1*)	17 (1*)
<i>Rhodeus sericeus</i>	cyprinidae	BOU	column feeders	0	0	0	0	1	2
<i>Cyprinus carpio</i>	cyprinidae	CAR	bottom feeders	0	0	3	0	0	0
<i>Squalius cephalus</i>	cyprinidae	CHE	column feeders	0	22	24 (1*)	19 (1*)	42 (5* + 1**)	17 (1*)
<i>Rutilus rutilus</i>	cyprinidae	GAR	column feeders	0	0	0	5	0	0
<i>Gobio occitaniae</i>	cyprinidae	GOU	bottom feeders	20 (3* + 2**)	18 (2*)	28 (1*)	15	18 (1*)	20 (3* + 1**)
<i>Pachychilon pictum</i>	cyprinidae	PAK	column feeders	0	2	0	0	0	0
<i>Pseudorasbora palva</i>	cyprinidae	PSR	column feeders	0	0	0	0	0	6
<i>Alburnoides bipunctatus</i>	cyprinidae	SPI	column feeders	0	2	0	0	1	0

<i>Phoxinus phoxinus</i>	cyprinidae	VAI	column feeders	14 (1*)	11 (1*)	0	0	0	1
<i>Oncorhynchus mykiss</i>	salmonidae	TAC	column feeders	0	0	1	0	0	0
<i>Salmo trutta</i>	salmonidae	TRF	column feeders	7	0	0	0	0	0
<i>Sander lucioperca</i>	percidae	SAN	column feeders	0	0	0	0	0	1
<i>Perca fluviatilis</i>	percidae	PEF	column feeders	0	0	0	0	0	1
<i>Anguila anguila</i>	anguillidae	ANG	bottom feeders	0	4	0	0	4	3
<i>Esox lucius</i>	esocidae	BRO	column feeders	0	2	0	0	0	0
<i>Barbatula barbatula</i>	nemacheili- dae	LOF	bottom feeders	18 (1*)	3	6 (3*)	1	1	0
<i>Ameiurus melas</i>	ictaluridae	PCH	bottom feeders	0	0	0	8	0	0
<i>Lepomis gibosus</i>	centrarchi- dae	PES	column feeders	0	0	1 (1*)	0	0	0
<i>Silurus glanis</i>	siluridae	SIL	bottom feeders	0	3 (1**)	0	3	4 (1*)	3
Total collected	-	-	-	61	76	98	81	96	78
Total contaminated	-	-	-	7	4	16	3	10	7

6.4 Discussion

Understanding the pathways and mechanisms leading to the consumption of microplastic by freshwater organisms is a central research question and the present study reveals that stable isotope analyses can provide novel knowledge. Specifically, we first found that the abundance of microplastic (size range 700 μm – 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, colour, size and composition) observed in the environment differ from those ingested by organisms. For both macroinvertebrates and fish, the abundance of ingested microplastic 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 trophic position tended to be positively associated with the abundance of ingested microplastic 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 (Lusher et al., 2013) and are not passively obtained because microplastic characteristics strongly differed between 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 colours for fish. Fibres were the main microplastic shape consumed by both macroinvertebrates and fish. Microplastic colour and shape are important characteristics responsible for their ingestion by organisms and the existence of such preferences has already been reported in freshwater organisms (Park et al., 2020; Peters and Bratton, 2016; Yuan et al., 2019). Although the mechanisms leading these findings remain to be identified, they could represent a preferential ingestion (Roch et al., 2019) and/or a higher retention time and accumulation in the digestive system (Qiao et al., 2019), increasing the likelihood of microplastics detection in organisms. Fibres were already shown to be dominant in sub-surface water, highlighting the vertical transport of microplastic through the water column (Kanhai et al., 2018) which could potentially affect its availability to aquatic organisms. 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, and although the

number of microplastic 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 (Nuelle et al., 2014). 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 microplastic ingested by aquatic animals (Jâms et al., 2020). Considering the size of studied macroinvertebrates, further investigations are needed to determine the relationship between body size and microplastic size for microplastic smaller than our size limit. At functional feeding group level, there was no significant difference for predators but predatory taxa such crayfish (*Faxonius limosus* and *Procambarus clarkii*), Odonata (dragonfly and damselfly larvae) and Planariidae had the highest occurrence of microplastics (Table. 6.2), highlighting the importance of measuring the realised trophic position using stable isotopes. Assessing the role of gut structure across species on retention time could help to have a better understanding microplastic contamination (German and Horn, 2006). These findings suggest that macroinvertebrates primarily ingest microplastics directly (i.e. primary ingestion) and that the microplastic 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 $\delta^{13}\text{C}$) and feeding modes, a deliberate ingestion by organisms was unlikely to be the main pathway of contamination. We hypothesise that microplastic ingestion was mainly accidental and modulate 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 (Nuelle et al., 2014) 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*, *Silurus glanis*, Table. 6.3), we found no relationship between individual trophic position and abundance of ingested microplastics. Contrary to observations reported elsewhere (Campbell et al., 2017; Collard et al., 2019), predatory fish were not more contaminated (at least in term of abundance) than other trophic levels (Güven et al., 2017; Hurt et al., 2020; Roch et al., 2020), 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 (López-Rojo et al., 2020; Ory et al., 2018; Welden et al., 2018).

Interestingly, $\delta^{13}\text{C}$ analyse reported that 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 (Cucherousset et al., 2020; Larson et al., 2011). 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. 6.3), accidental ingestion of small microplastic in the sediment when consuming prey at the basis of the detritus food chain (e.g. Gammaridae, Asellidae, Table. 6.2) 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 (Collard et al., 2019; Peters and Bratton, 2016). This might be caused, for instance, by three mutually non-exclusive mechanisms. First is the spatial changes in microplastic characteristics across sites (Rodrigues et al., 2018; Skalska et al., 2020) 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 (Araújo et al., 2011), 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 (Ferreira et al., 2019, 2016; Horton et al., 2018), 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, colour, shape), environmental conditions (e.g. turbidity, substrate, 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 (Collard et al., 2019; Sloomakers et al., 2019). When only contaminated individuals were considered, the number of microplastics was always 1 for macroinvertebrates and ranged between 1 to 4 for fish, as observed elsewhere (Collard et al., 2019). 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) (Dris, Gasperi, et al., 2015). The two most urbanised 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 6.2 and 6.3), confirming that urbanisation is a crucial driver of microplastic pollution (Frère et al., 2017) and biotic contamination. Urbanisation can have profound and multiple effects on freshwater organisms and ecosystems (Kern and Langerhans, 2019; Larson et al., 2011; Stranko et al., 2012) and is a ubiquitous driver of microplastic contamination (Frère et al., 2017). It is therefore crucial to decipher the relative importance of microplastic ingestion compared to other environmental stressors on freshwater organisms and to determine whether they act synergistically, additively or antagonistically (Jackson et al., 2016).

In conclusion, this study highlights the importance of quantifying the realised trophic niche when assessing microplastic ingestion by wild organisms and that intraspecific variability in microplastic ingestion within species could be high. Determining how the ecological traits of individuals (e.g. behaviour, metabolism, morphology, trophic specialisation) are driving intraspecific variability in microplastic ingestion represents an important and challenging area of research. Large microplastics, as those studied here (700 µm – 5 mm), represent only a small fraction of the microplastics ingested by freshwater fish (Roch et al., 2019) and stable isotope analyses appear as a robust and insightful method to quantify the distribution and pathways of smaller microplastics in freshwater food webs.

7 | Discussion and perspectives

The recognition of microplastics ubiquity was followed by the urgent need to assess their impacts on wild organisms and ecosystems (C. Li et al., 2020). This included mapping the spatial and temporal dynamics of microplastic pollution in freshwater ecosystems and the consumption of microplastics by aquatic organisms. In this work, we studied microplastic pollution of particles ranging from 700 μm to 5mm, and a resume of the main results is displayed at Figure 7.1. We found that spatial and temporal changes in microplastic pollution in the Garonne catchment was strong and driven by urbanization and hydrological conditions, respectively (**Chapter 3**). Summer periods with low flow exhibited higher microplastic concentrations with smaller microplastic particles. At the opposite, during rapid hydrological changes occurring during flood episodes, microplastic concentration substantially increased in water surface (**Chapter 4**), with a predominance of larger microplastic particles. This increase, however, was modulated by the urbanization context. We have also found that industrially-manufactured angling baits represent a cryptic and potentially important source of microplastics to freshwater ecosystems, notably in ecosystems with high levels of angling pressure (**Chapter 5**). Then, we found that microplastics were consumed by 18 macroinvertebrates taxa and 8 fish species in the Garonne river, out of 35 and 21 investigated, respectively (**Chapter 6**). Microplastic consumption by fish was mainly driven by their foraging behaviour, with bottom feeders exhibiting higher contamination levels. Microplastic contamination in invertebrates was higher in predatory and large taxa, indicating a direct consumption of these particles.

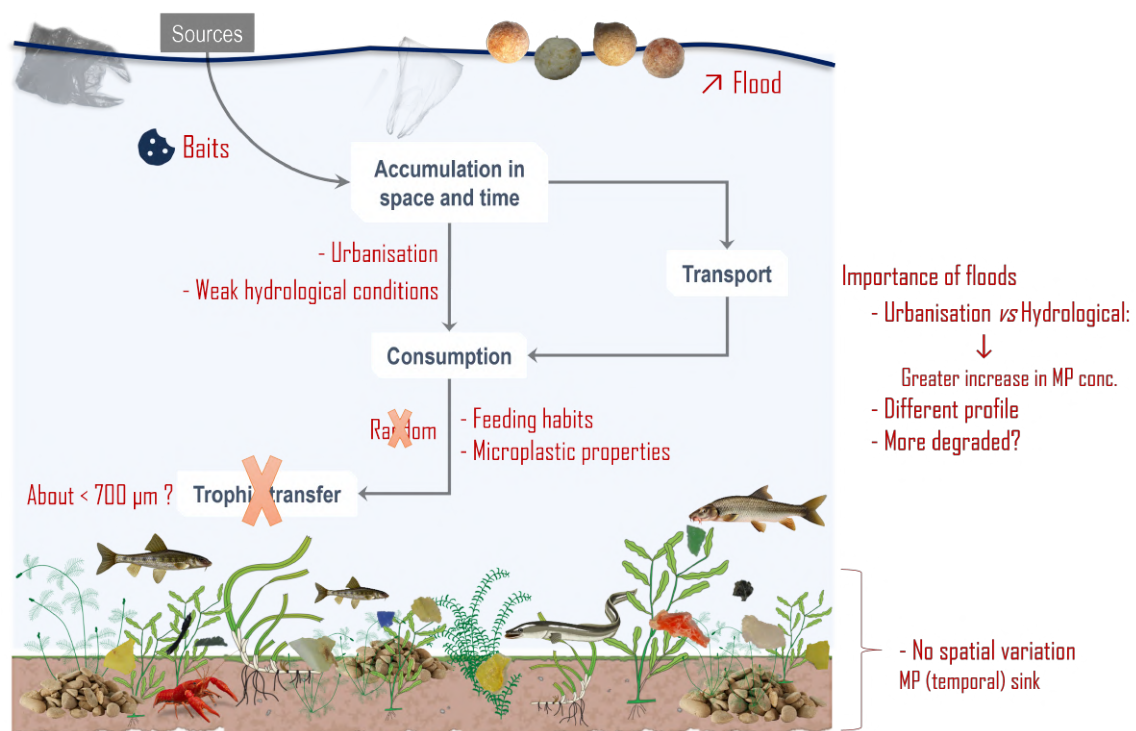


Figure 7.1: Overview of the findings in this thesis regarding each compartment studied and the respective microplastic pollution and contamination

Assessing and mitigating microplastic pollution still faces several challenges and multidisciplinary approaches are essential. In this discussion, four different topics are addressed: i) perception of freshwater microplastic pollution as a co-occurring anthropogenic pressure, mainly related to urbanization;

- ii) interactions between microplastic and the surrounding environment in the context of individual and ecosystem stressors;
- iii) mapping microplastic pollution of smaller particles and of associated chemicals and,
- iv) dealing with environmental plastic pollution, from societal consciousness to policy strategies. Finally, finding the place of plastic pollution among several environmental and ecosystems stressors emerges as an important perspective in the field.

The correlation between these different topics is displayed at Figure 7.2.

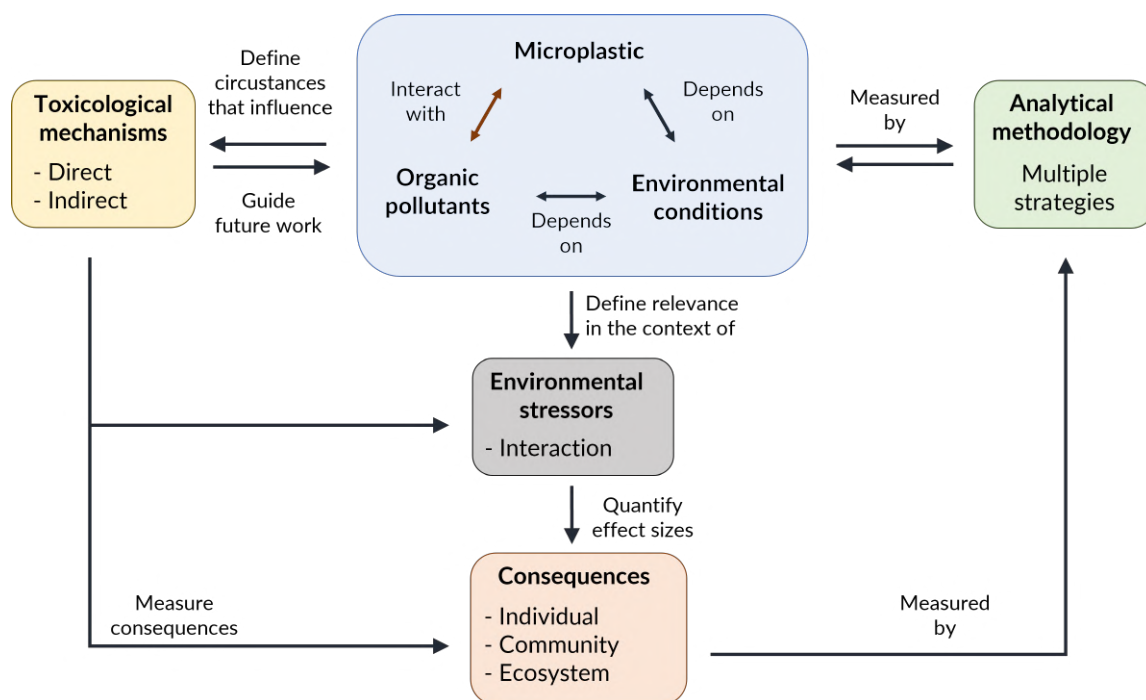


Figure 7.2: Overview of interacting process regarding microplastic, from a particle perspective to the consequences of environmental microplastic pollution.

7.1 Integrating microplastic pollution into the urban stream syndrome

Urbanization is an important pressure on stream ecosystems, negatively affecting water quality and biodiversity (Wen et al., 2016). The so-called ‘urban stream syndrome’ represents the ecological and hydro geomorphic consequences induced by urbanisation, such as changes at a river channel morphology, homogenisation of stream profiles and simplification of ecological biodiversity (Bernhardt and Palmer, 2007; Meyer et al., 2005). Here we highlighted another ecosystem stressor associated to urbanisation, microplastic pollution.

Small but highly urbanized rivers export important mass of plastics, oversized compared with their drainage areas and exceeding the transport from larger catchments (Lebreton et al., 2017; Stubbins et al., 2021). In the present study, the highest microplastic concentrations were recorded in two small, but highly urbanized tributaries of the Garonne river. However, changes within a catchment might also occur, depending on human activities near the streams and the accessibility of river shore (Dris, Gasperi, et al., 2015; Skalska et al., 2020). Microplastic pollution might be alleviated by riparian ecotones, the interface between terrestrial and freshwater

systems (Moore and Palmer, 2005), because microplastic mobilization depends on riparian vegetation type, slope, coverage and roughness, which are highly variable within a watershed (Cowger et al., 2019; Delorme et al., 2021; Windsor, Durance, et al., 2019). In favourable conditions of riparian ecotones, microplastic finally reach streams, distributing differently among transect sections, i.e. line across water surface, and vertically, i.e. through the water column, leading to hot spots of microplastic contamination in surface water (Dris, H. Imhof, et al., 2015; Liu et al., 2020; Niu et al., 2020). Urbanization negatively affects the extent and composition of riparian vegetation (Borisade et al., 2021), facilitating microplastic to reach streams therefore favoring higher levels of microplastic in urban streams.

Urbanization has accentuated the increase in microplastic concentration during a flood with a peak of $1.109 \text{ microplastics.m}^{-3}$, greatly exceeding the annual average of $0.126 \pm 0.128 \text{ microplastics.m}^{-3}$ in the Garonne at Gagnac-sur-Garonne. The increased impervious surface in urban areas contributes to pollution run off during a flood and leads to an increased microplastic concentration in streams (Bernhardt and Palmer, 2007; Walsh et al., 2005). Using the median mass of microplastics at this study, 0.20 mg (average $0.562 \pm 1.38 \text{ mg}$, $n = 2269$), similar to the one recently reported (Weiss et al., 2021), a rough estimate of annual microplastic mass flowing through the Garonne is 1.67 t.year^{-1} (considering an average river discharge of $630 \text{ m}^3.\text{s}^{-1}$). Studies of floating macro- and microplastics in the Rhône (France) reported fluxes in a similar order of magnitude, of 0.71 t.year^{-1} and $0.07\text{--}7.8 \text{ t.year}^{-1}$, respectively (Castro-Jiménez et al., 2019; Schmidt et al., 2018). For the same region, a study investigating plastic additives reported an annual input from river to the ocean of $5\text{--}54 \text{ t.year}^{-1}$, explained by the leaching from floating plastic waste and the released during plastic manufacturing, wastewater treatment plants and atmospheric deposition (Schmidt et al., 2020). If a similar mechanism occurs in the Garonne catchment, amounts of plastic additives might reach mass levels equal or greater than microplastics.

A greater oxidation profile of polyethylene particles was observed during a flood in the urbanized site, with critical consequences to particle fragmentation and sorption and releasing of chemicals (Arp et al., 2021; Garvey et al., 2020; M. Simon et al., 2021). The environmental exposure of polyethylene leads to greater oxidation and potentially increased crystallinity and smaller molecular weight (Garvey et al., 2020; Halle et al., 2016). Consequently, embrittlement and greater chemical degradation are expected, likely generating smaller polyethylene fragments, even at a nano size (Gigault et al., 2016). These smaller microplastics exhibit a higher surface area

to volume than large ones and an increased association process with chemicals is expected. Three main factors concomitantly influence this sorption process. First, inter-correlated properties within a microplastic particle such as polymeric composition and spatial arrangement, additives, roughness and biofilm are known to regulate the sorption mechanisms (Rummel et al., 2017; Wang et al., 2018). The greater the distance between polymer chains, the easier it is for chemicals to diffuse into or through the matrix, explaining the greater sorption capacity of polyethylene compared to polypropylene (Hartmann et al., 2017; Rochman et al., 2013). Second, environmental conditions, such as water pH, temperature, natural organic matter, with lower pH and higher temperature increasing desorption of contaminants from microplastics (Bakir et al., 2014). Third, intrinsic properties of organic contaminants, as pKa and hydrophobicity (Sharma et al., 2021; Teuten et al., 2009), when sorption of most contaminants to microplastics increase with their hydrophobicity and with smaller plastic particles (Wang et al., 2018). The co-occurrence of higher microplastic concentration and increased release of toxic contaminants likely represent greater risks to organisms (Wang et al., 2018). However, reduced microplastic levels under favourable releasing conditions might still represent a critically toxic contaminant. A comprehensive assessment of environmental contaminants, integrating microplastic and environmental conditions, is essential to understand the relevant mechanisms driving microplastic fragmentation and chemicals release from microplastics (Crawford et al., 2022; Garvey et al., 2020; Sharma et al., 2021).

7.2 Individual and ecosystem consequences of microplastic pollution

Organisms interact with all sizes of plastics, with possible effects ranging from physical stress to chemical assimilation. Plastics significantly larger than the organism can represent a substrate for colonization of smaller organisms and invertebrates (Davidson, 2012). Large yet ingestible size classes of plastics represent a risk of gastrointestinal blockages (Gall and Thompson, 2015). After ingestion and digestion, microplastic in the studied size range are generally egested. Microplastic retention time in digestive organs might be similar to natural food items, but direct toxic effects in the digestive tract are expected such as inflammation and gut microbiome disturbance (Cole et al., 2013; Fackelmann and Sommer, 2019; Boqing Li et al., 2020). In the digestive tract microplastics are potentially fragmented into smaller sizes and, when smaller than

10 μm , they might be up taken and translocated through biological membranes and tissues, accumulating and causing toxic effects (Bruinink et al., 2015; Franeker and Law, 2015; Stock et al., 2021; Triebkorn et al., 2019). Rapid stress response in liver, metabolism disorders and oxidative stress caused by microplastic have already been observed, and long-term consequences might also happen, i.e. “toxicity debt” concept (Prata, J. da Costa, Lopes, et al., 2019; Rillig et al., 2021; Yin et al., 2021). Despite increased toxicological research on microplastics, our knowledge to understand and predict the individual and collective impacts of micro and nanoplastics combined with associated chemicals are still limited (Bernhardt et al., 2017; Yin et al., 2021). Furthermore, toxicological studies still fail to assimilate micro and nanoplastics of environmental relevance and to account for the diversity within a microplastic particle (Bucci et al., 2020; Waldman and Rillig, 2020), and one important reason for this is the lack of an adequate analytical methodology (see **sub-section 7.3 below**)

The toxicological effects induced by ingested microplastics happen through two main pathways. The first is a direct pathway induced by the polymeric composition (and its alterations due to aging and weathering), the formulation additives or the organic pollutants (Rochman et al., 2013; Strungaru et al., 2019). Digestive fluids might promote leaching of chemicals adsorbed in microplastic and regulate their potential assimilation by organisms. The relevance of this process compared with the one from naturally occurring particles remains to be assessed (Koelmans et al., 2016; Rodrigues et al., 2019; Triebkorn et al., 2019). A second pathway is an indirect consequence of microplastic ingestion, unbalancing the organisms energy balance due to lower food consumption and/or assimilation (Galloway and Lewis, 2016). This is particularly in accordance with negative effects on fish behaviour, foraging and growth and depend on fish species and life stages (Foley et al., 2018; Salerno et al., 2021). Due to difference in food consumption between sex, where mature females commonly have higher food consumption and/or metabolic costs, females might be more affected through this indirect consequence of microplastic ingestion, as showed by a visible growth reduction (Cormier et al., 2021). Subsequent impacts on reproduction and changes in the trophic structure of ecological communities are expected, with potential effects on ecosystem functioning that remain to be investigated (López-Rojo et al., 2020; Santos et al., 2021).

The co-occurrence of multiple environmental stressors induced by human activities (i.e. multi-stressors), is increasingly recognised. Historically, ecosystem management has focused on individual stressors, disregarding the potential effects of multiple stressors to interact and produce additive, synergic or antagonistic effects (Spears et al.,

2021). However, microplastic should be considered as a multi-stressor itself, motivating studies to assess how they alter ecological process and affect global changes (Bernhardt et al., 2017; Grantham et al., 2019). Ecosystem health depends on its resilience and encompass the evolution of an adaptive response of organisms to plastic pollution, and the ability of freshwater organisms to adapt to pressures caused by microplastic pollution (Santos et al., 2021) remains to be determined.

7.3 Towards a comprehensive analysis of microplastic chemicals and smaller microplastic sizes

Developing good experimental protocols is the heart of environmental analysis and one of the greatest perspective in the field of microplastic pollution lies in the development of methodologies for sample analyses. The choice of an analytical methodology for the identification and quantification of microplastic pollution is mainly driven by particle size (Filella, 2015; Renner et al., 2018). In this work, we applied stereo microscopy and ATR-FTIR spectroscopy to obtain information about the size, morphology, color, and composition of particles ranging from 700 μm – 5 mm. For a smaller size of microplastics different analytical technologies are applied. Among these techniques, imaging and microscopy-FTIR are commonly used (Pan et al., 2021), though an emerging alternative lies in the field of spectrometry (**Appendix B**). Given the particulate, insoluble and non-volatile properties of microplastics, the analysis using gas chromatography-mass spectrometry (GC-MS), for instance, must be preceded by thermal-degradation techniques (**Appendix B**). The generated volatile pyrolytic products can be directly transfer to the chromatographic column (Py-GC-MS) (Fabri, 2001; Funck et al., 2020; Hermabessiere et al., 2018) or trapped and then thermodesorbed (TED-GC-MS) (Dümichen et al., 2015, 2017). Both techniques allow detecting the chemical cocktail within a microplastic and identification of polymeric composition of smaller particles (González-Pérez et al., 2014; Peñalver et al., 2020). A double-shot pyrolysis-gas chromatography-mass spectrometry grants the investigation of chemicals and sorption behaviours, applying a milder temperature in the range of 100°C-300°C, and polymeric analysis by pyrolysis at 650°C (Burrows et al., 2020; Okoffo et al., 2020). Changes in the molecular structure of a polymer, such as the presence of functional group or reduced molecular chain, are among the perspectives of thermo-degradation analysis of environmental plastics (Ainali et al., 2021; Ter Halle

et al., 2017).

For the determination of traces of plastics in environmental samples, i.e. when its proportion is lower than 0.01 % (equivalent to 100 ppm) (Valcárcel Cases et al., 2018), the main challenge is to decrease the limit of detection and quantification in the analytical process. In general, it is necessary to differentiate the target analyte from the background noise from the matrix, and this is especially important when dealing with environmental samples. Strategies to reduce the noise by increasing the selectivity ranges from sample preparation, such as the inclusion of a pre-concentration step (Steinmetz et al., 2019), to mass spectrometry level. Tandem mass spectrometry is known to offer increased selectivity, simplified clean-up procedures, and faster analysis. The selected reaction monitoring (**SRM**) mode is an approach to monitor a metastable decomposition reaction, mainly achieved through triple quadrupoles mass spectrometry arrays (Fig. 7.3I). It allows to acquire a spectrum selectivity, revealing the fragmentation of one specific ion generated of the target compound, with a clear background and signal-to-noise enhancement (Gross, 2017). In SRM analysis, analyte ions are selected based on their mass-to-charge (m/z) at two different stages. In the first one, m/z of the intact analyte (precursor ion) is selected in the first quadrupole (Fig. 7.3I - **Q1**). After fragmentation, typically by collision-induced dissociation (Fig. 7.3I - **Q2**), the resulting product ions are isolated on the Q3 based on their m/z (Fig. 7.3I - **Q3**) (Angel et al., 2012).

Three different mass spectrometric experiments can be considered in triple quadrupole mass-spectrometry instrumentation. The first one is the full scan analysis, where no m/z is selected and all generated ions are monitored. At single-ion-monitoring analysis (**SIM**), only one quadrupole is used (**Q1**), allowing the selection of a single m/z at a time. The use of the SIM analysis instead of full scan analysis was already proposed as a strategy to decrease both limits of detection and quantification (Duemichen et al., 2019; Hermabessiere et al., 2018). However, the majority of studies still applies the full scan method (Dümichen et al., 2017; Eisentraut et al., 2018; Funck et al., 2020) and, to date, no **SRM** methods was applied to the detection and quantification of microplastics particles in environmental samples. Preliminary essays have revealed an increased selectivity when applying the SRM analysis of polystyrene dimer, obtained after pyr-GC of polystyrene in an environmental matrix. The dashed line indicates the retention time window, while the scanned m/z is displayed on the top of each chromatogram (**SRM**: m/z 208>91 and m/z 208>104, **SIM**: m/z 91, m/z 104, m/z 208) (Fig. 7.3II). The reduced background signal in SRM analysis consequently increases the signal-to-noise

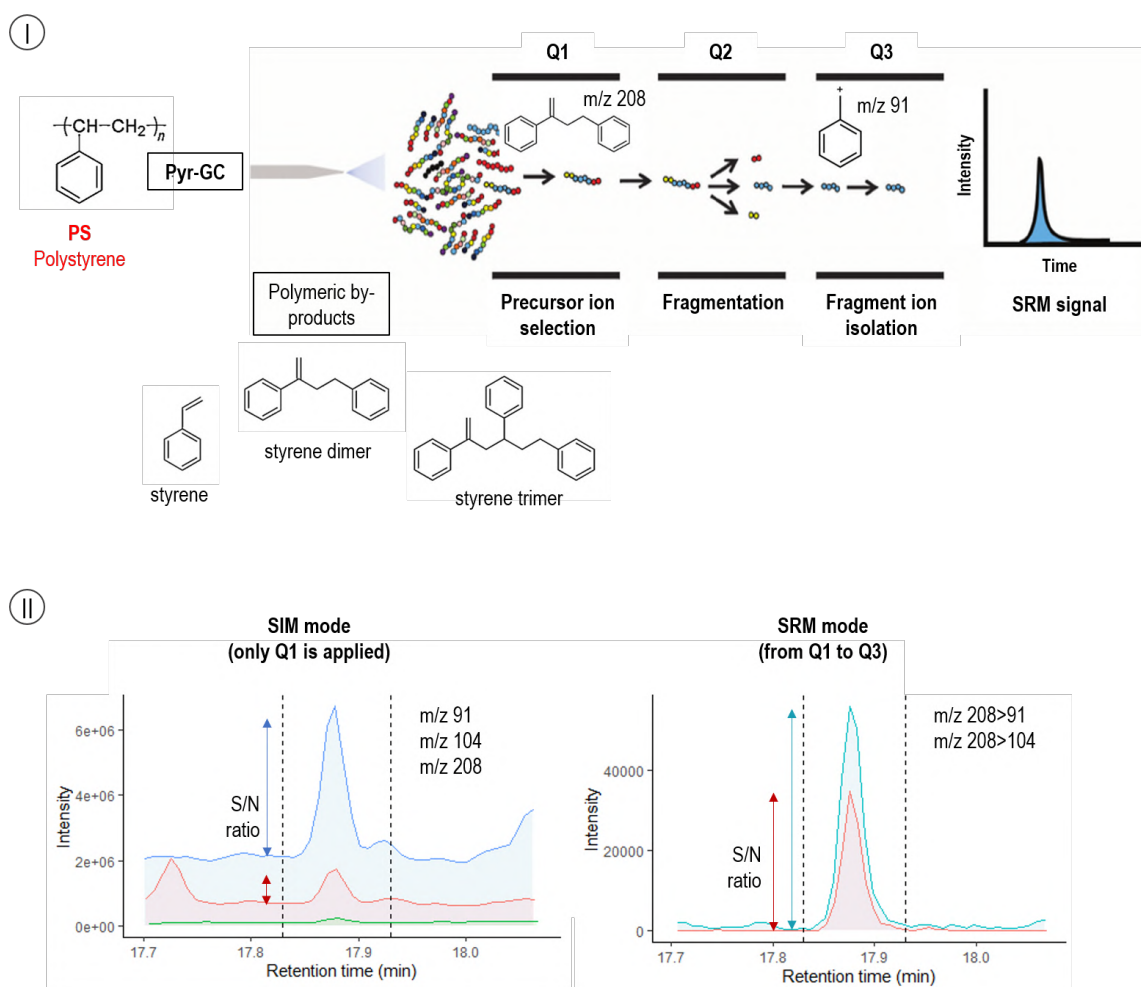


Figure 7.3: Overview of selected reaction monitoring (SRM) mass spectrometry (I), with multiples round of isolation greatly reduce the background signal resulting in greatly improved signal to noise (adapted from Angel et al. (2012)), and chromatogram of polystyrene dimer (II) in SIM analysis (left) and SRM analysis (right), with significant increase in S/N ratio in SRM analysis while reducing signal intensity.

ratio (S/N ratio), an important quality parameter to the definition of an analyte chromatographic peak. Even in environmental samples that commonly exhibits a considerable matrix effect, ion selectivity is improved. However, as expected, the sensitivity of the equipment to detect the analyte is strongly affected, with the total ion abundance reducing from 6.0×10^6 in SIM analysis to 4.0×10^4 in SRM analysis (Fig. 7.3II). Unambiguous identification and quantification of microplastics and their associated chemicals in complex environmental matrices is therefore compromised by the detection limit of the chosen analytical technique.

Although implementation of current mass spectrometry technologies might improve chemical data acquiring, the coupling with thermo-analytical techniques results

in loosen information regarding microplastic size, morphology and color (Saitoh (2021) and **Appendix B**). Given the particulate property of microplastics, these data are particularly useful for understanding the drivers and risks of microplastic consumption (Collard et al., 2019). There is currently a compromise when obtaining data about microplastic pollution features. Multidimensional strategies in the field of analytical chemistry already revolutionized the study of environmental contamination (Duarte and Duarte, 2020) and should now embrace the challenges of micro and nano plastic analyses.

7.4 Environmental plastic pollution: from societal consciousness to policy strategies

Plastics have allowed an undeniable societal evolution and facing its harmful side requires rethinking the way our society and economy use synthetic polymers (Noventa et al., 2021). From individual to societal consciousness, questioning our consumption needs and opting for less use whenever possible seem to be a good way through. Unfortunately, changes in habits does not represent the same effort throughout the society, and less wealthy countries or poorest people within countries could simply not afford these changes. The public awareness of plastic pollution as a serious environmental and public health issue is an important aspect to pressure policy makers and demand solutions (Catarino et al., 2021; Cornwall, 2021; Soares et al., 2021).

In the eagerness of finding a solution for the global threat from plastic pollution, bioplastics re-emerge as an illusory answer (Altmann, 2021). Firstly, because of the confusion of the term, encompassing both bio-based and bio-degradable plastics. Bio-based plastics are made from a renewable source and they safe fossil fuels, though being chemically identical to their fossil-based counterpart (Kakadellis and Rosetto, 2021). However, deforestation and occupational hazards during harvesting are among serious consequences of the bioplastic production chain (Altmann, 2021). On the other hand, bio-degradable plastics are derived from fossil-fuel rather than bio-based sources but are more prone to biological degradation through the actions of microorganisms (Lambert and Wagner, 2017). Thus, bio-degradable plastics should be conceived to last two different lives: the first one presenting the desired functions for which they were conceived for and the second one to serve as resource for microbes. Improvements to transform bioplastics into a realistic alternative should grants their affordability and access their environmental threats upstream their large-scale use. Bioplastic can

easily represent a pitfall and a green-washing strategy, enjoying a favourable public sentiment and supportive governmental policies (Zhu and Wang, 2020). Finding ways to certificate a bioplastic and ensure their sustainable and bio-sourced manufacturing, together with a proved biodegradable mechanism, seem a good way through. Improving plastics biodegradation through enzymatic process and recycling strategies through a depolymerisation process or a dissolution-precipitation one are among great perspectives (Cornwall, 2021; Korley et al., 2021). Besides the reuse of plastics, the chemical recycling would allow to maintain chemicals and materials in the value chain, finally reaching a circularity of the material.

There is no silver bullet for such a complex issue. For the damage that is done, extending producer responsibility represent an important measure to subsidize plastic waste management and cleanup of disposed plastic waste before reaching streams (N. Simon et al., 2021). Doubtless, the best plastic is the one that we do not use. This seems implausible given the dramatic consumption system we are currently experiencing.

7.5 Summary points

- 1) Microplastic pollution interact with several other anthropogenic pressures that are known to contribute to global changes, notably urbanization. To understand whether microplastics are a risk to freshwater ecosystems, risk assessment studies should firstly establish exposure scenarios, which are hampered by changes in microplastic concentration, environmental conditions and co-stressors interaction (Fig. 7.2). Ecological studies should embrace microplastics as a potential agent of global and ecological changes, such as biodiversity loss.
- 2) Assessing the risks of microplastics to organisms health requires the design of environmentally-relevant toxicological essays (Fig. 7.2). Several uncertainties remain about toxicological mechanisms and the relevance of them.
- 3) The development of analytical chemistry strategies is at the heart of the above issues (Fig. 7.2) and the main challenges are the detection and quantification of smaller microplastics and nanoplastics, the mapping of chemical, physical and biological complexity within a particle and determining levels of associated-chemicals.
- 4) Management strategies should be designed taking into account changes in microplastic concentration and profile among space and time. Floods and other

occasional inputs of pollutants, such as microplastic-contaminated angling baits, are important moments of stream contamination subjected to preventive and regulatory actions.

- 5) Plastic pollution issued from our society's consumption system and balancing the benefits and negative consequences of using plastic is fundamental. Strategies of circularity implementation are underway, but given the increasing production and use of plastics, these impacts may never be mitigated.

7.6 Perspectives

There are two main research questions that academics still fail to properly address on the topic of plastics pollution, as was the case within the recent special section on plastic pollution in the journal *Science* (Smith and Vignieri, 2021):

- 1) Are microplastic pollution levels exceeding freshwater boundaries? Freshwaters are always in flux. Novel and exceptionally large changes, including microplastics, should be investigated within the framework of ecologically and environmentally relevant endpoints - given that the natural environment is not particle-free and has never been (Backhaus and Wagner, 2020). Appropriate technologies, policy strategies and economic incentives are needed to constrain microplastic within freshwater boundaries.
- 2) Considering a management perspective with limited resources, where microplastic pollution stands against other environmental and ecosystem pressures? To answer this question, effect sizes of other human-driven changes, such as as land use, invasive species, hydrological modification (e.g. dam construction) and harvest (Carpenter et al., 2011) must be compared (Fig. 7.2). All drivers play a role, some are well established and others, such as microplastic pollution, require further investigation, but mainly by contextualizing the outputs in the multiple-stressors scenario.

8 | Résumé en français

Suivi spatio-temporel de la pollution de microplastique dans la Garonne et potentiel transfert dans le réseau trophique | *Déterminants de la variabilité spatio-temporelle de la pollution et consommation par les organismes aquatiques*

Les écosystèmes d'eau douce fournissent d'innombrables services aux humains et abritent une grande biodiversité ; ils sont également les plus diversifiés sur le plan fonctionnel (Apostolaki et al., 2020; Suring, 2020). Paradoxalement, les écosystèmes d'eau douce sont parmi les plus menacés de la planète, confrontés à de multiples perturbations induites par les changements globaux et l'impact croissant des activités humaines (Vörösmarty et al., 2010). Les changements climatiques (Magnuson et al., 1997), les invasions biologiques (Gallardo et al., 2016), la fragmentation des habitats (Morita et al., 2009) et la pollution de l'eau (Couceiro et al., 2007) font partie des multiples facteurs menaçant les écosystèmes d'eau douce et leur riche biodiversité. Ces perturbations se produisent rarement seuls et mesurer leur variabilité temporelle et spatiale ainsi que leur effet interactif est un défi important dans les sciences environnementales (Perujo et al., 2021).

Les progrès apportés par les produits en plastique ont révolutionné la vie moderne, par exemple les emballages alimentaires, matériaux de construction et dispositifs médicaux (Agarwal and Gupta, 2017; Sastri, 2010). Comme tout autre produit synthétique, la fin de son cycle de vie est devenue une préoccupation sociétale. La pollution plastique est récemment apparue comme une nouvelle source de perturbation. Les plastiques sont issus d'un processus industriel qui combine des polymères synthétiques avec plusieurs adjuvants de formulation, tels que des plastifiants (pour améliorer la flexibilité), des retardateurs de flamme et des antioxydants (aussi connus comme additifs plastiques) (Sastri, 2014). Les plastiques mal gérés pénètrent dans les écosystèmes aquatiques directement par les eaux de ruissellement ou via les stations d'épuration des eaux pluviales et des eaux usées (Dris, H. Imhof, et al., 2015). Dans l'environnement, les plastiques subissent un processus de dégradation par abrasion mécanique, altération photochimique et autres mécanismes (Andrady, 2011; Gewert et al., 2015; Halle et al., 2017). Ces dégradations conduisent à la production de microplastiques, c'est-à-dire de fragments de plastique inférieurs à 5 mm (ECHA, 2020; Thompson, 2004) (Fig. 8.1. De plus, les microplastiques primaires (c'est-à-dire ceux qui ne proviennent pas de la fragmentation de débris plus gros) peuvent entrer directement dans les écosystèmes, car ils sont souvent employés comme additifs cosmétiques et comme vecteurs médicamenteux (Cole et al., 2011; Sun et al., 2020; Yu et al., 2021).

Les taux de dégradation des plastiques dans l'environnement sont très incertains

et dépendent de la composition du plastique, du type de polymère, ou de la présence d'antioxydants et de stabilisants (MacLeod et al., 2021). Les conditions environnementales influencent aussi fortement la dégradation des particules. Par exemple, la pénétration plus élevée de la lumière, par conséquent des niveaux plus élevés d'UV, des températures plus élevées et des contraintes mécaniques peuvent augmenter l'altération des particules de plastique (Free et al., 2014; Law and Thompson, 2014). Le rapport surface/volume plus élevé dans ces plastiques de petite taille et leur structure polymérique probablement altérée facilitent les interactions intermoléculaires avec leur environnement, ce qui entraîne une plus grande capacité de sorption pour les contaminants. Considérant aussi le caractère hydrophobe des microplastiques, l'accumulation de contaminants organiques tels que les hydrocarbures aromatiques polycycliques (HAP), les polychlorobiphényles (PCB), perfluoroalkyles (PFA), diéthers polybromés (PBCD), les produits pharmaceutiques et les métaux lourds sont attendus (Atugoda et al., 2021; Hartmann et al., 2019; Naik, 2019) (Fig. @ref(fig:resfigintro - Microplastique). Dans l'environnement, une « éco-couronne » de matière organique et de micro-organismes se forme rapidement autour des particules de plastique en quelques jours (Harrison et al., 2014; MacLeod et al., 2021; Rummel et al., 2017). Ce biofilm complexe, appelé « plastisphère », a des implications importantes, tels que le développement de communautés microbiennes spécifiques et le potentiel de transport de surface de pathogènes et gènes de résistance aux antibiotiques (Bond et al., 2018; Hossain et al., 2019; Kirstein, 2016; Zettler et al., 2013) (Fig. 8.1 - Microplastique). Au total, ces propriétés conduisent à l'idée d'un « effet cheval de Troie » de ce « cocktail de produits chimiques » pour les particules de microplastique (Bucci et al., 2021; Trevisan et al., 2020; Vethaak and Legler, 2021).

Les études sur la pollution par les microplastiques se sont principalement et historiquement concentrées sur les écosystèmes marins (Eerkes-Medrano et al., 2015; C. Li et al., 2020), mais les rivières sont au cœur de la dynamique de la pollution plastique (Rochman, 2018). En effet, 70 à 80 % des plastiques marins sont transportés par les eaux douces (Horton, Walton, et al., 2017). La pollution microplastique d'eau douce est un phénomène omniprésent (Lusher et al., 2015; Rochman, 2018; Woodall et al., 2014) et fortement variable au sein des réseaux hydrologiques (Mani and Burkhardt-Holm, 2019; Vermaire et al., 2017). Généralement, les zones urbaines et industrialisées présentent une concentration plus élevée en microplastiques (Pinheiro et al., 2017). Cependant, notre connaissance des effets des différentes pratiques d'utilisation des terres sur les caractéristiques de la pollution des microplastiques reste limitée (Chen et al., 2020).

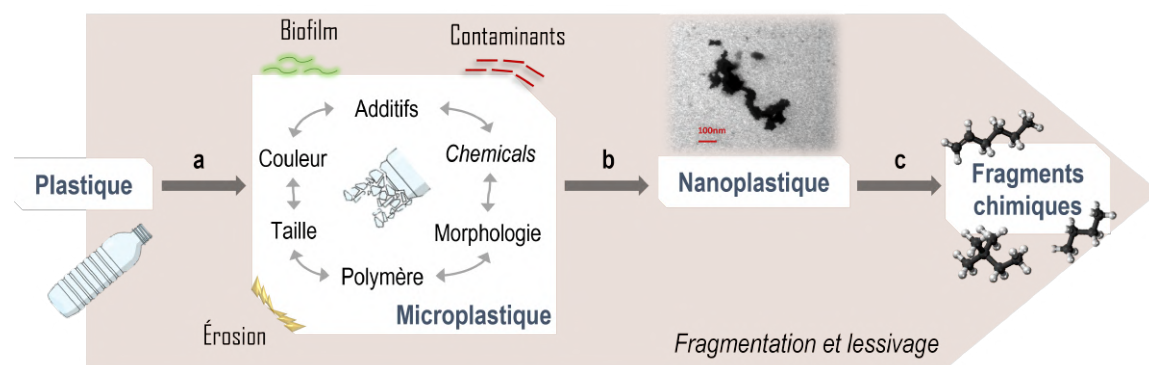


Figure 8.1: Processus de fragmentation du plastique (I) qui produit (a) des microplastiques, (b) des nanoplastiques et (c) des fragments chimiques. Le microplastique peut encore être produit directement. Les caractéristiques des microplastiques sont décrites ainsi que le processus environnemental d'altération, de sorption de contaminants et de formation de biofilm. Image nanoplastique issue de Gigault et al. (2018).

La pollution par les microplastiques dans l'eau douce peut également varier dans le temps en raison de changements dans les conditions hydrologiques et météorologiques (Fig. 8.2 2.b). En effet, les crues et les précipitations peuvent réguler la mobilisation de particules préalablement déposées dans les sédiments ou dans les sols (Zhang et al., 2017). Par exemple, la pollution peut être affectée par les conditions météorologiques et augmenter après les événements de précipitations (Eo et al., 2019). Plusieurs études ont démontré une corrélation positive entre les taux de précipitations et la pollution en microplastique (Cheung et al., 2019; Dris, Gasperi, et al., 2015; G. Wong et al., 2020; Yonkos et al., 2014). Les effets de la variabilité saisonnière sont plus ambigus, avec des études montrant soit la présence (Han et al., 2020; Wu et al., 2019) ou l'absence (Mani and Burkhardt-Holm, 2019; Rodrigues et al., 2018) de tendances saisonnières. Malgré leur importance pour le développement de stratégies de gestion efficaces de la pollution, la quantification et la caractérisation intégratives de la pollution par les microplastiques et les analyses complètes de ses moteurs spatiaux et temporels font défaut (Lebreton et al., 2017; C. Li et al., 2020).

L'étude de la présence de microplastiques dans des sources encore imperceptibles et inconnues (Fig. 8.2 2.a), spécialement liées à l'activité anthropique, et leurs voies d'accès dans les écosystèmes d'eau douce est donc important pour réduire leurs impacts potentiels (Dris et al., 2018; Horton, Svendsen, et al., 2017; Rochman and Hoellein, 2020). La pêche est une activité récréative répandue et pratiquée par plus de 10 % de la population mondiale (Cooke and Cowx, 2004) où l'utilisation d'amorces de pêche est une pratique courante dans de nombreux pays européens (Arlinghaus, 2004; Arlinghaus and Mehner, 2003). Les amorces représentent des sub-

ventions trophiques importantes pour les écosystèmes d'eau douce qui peuvent en outre contribuer à l'eutrophisation par l'ajout de phosphore, un facteur de stress reconnu (Amaral et al., 2013; Arlinghaus and Niesar, 2005). En plus, étant donné que ces amorces disponibles dans le commerce sont principalement produites industriellement, il est possible qu'elles contiennent également des microplastiques, soit présents dans les matières premières, soit introduits lors de la fabrication (Rødland et al., 2020). Par conséquent, les amorces de pêche pourraient représenter une voie inconnue de contamination microplastique dans les écosystèmes d'eau douce qui nécessite une quantification.

Cet ajout et cette accumulation des microplastiques dans les écosystèmes représentent un risque toxicologique important pour les organismes aquatiques (Prata, Paço, et al., 2020; Smith et al., 2018). Les microplastiques sont ingérés par les organismes d'eau douce et les conséquences de ces ingestions sont variables et encore mal connues (Collard et al., 2019; Eerkes-Medrano and Thompson, 2018; Eerkes-Medrano et al., 2015). Les organismes d'eau douce peuvent ingérer directement des microplastiques, ce qui est défini comme une ingestion primaire (Fig. 8.2 2.d). L'ingestion primaire peut être intentionnelle (active) ou accidentelle. L'ingestion secondaire se produit lorsque les microplastiques sont consommés via la consommation de proies qui ont consommé des microplastiques, c'est-à-dire indirectement ingérés. L'ingestion secondaire peut aussi représenter une forme de bioaccumulation (Collard et al., 2019, 2017; López-Rojo et al., 2020) (Fig. 8.2 2.d et 2.e). Des niveaux élevés d'ingestion se produisent généralement dans des sites à forte pollution microplastique dans l'eau (Horton et al., 2018; Scherer et al., 2017) ou des sédiments (Merga et al., 2020), mais cette relation ne tient pas systématiquement (McNeish et al., 2018; Roch et al., 2020). L'ingestion de microplastiques dépend également des caractéristiques biologiques de l'organisme. Cela inclut, par exemple, la taille des organismes. La taille et l'abondance des microplastiques ingérés augmentant généralement avec la taille du corps de l'organisme (Jâms et al., 2020; McNeish et al., 2018). L'étude des propriétés des microplastiques, telles que sa composition, sa densité, sa taille et sa couleur, peut non seulement contribuer à identifier leurs origines, mais aussi fournir des informations sur les mécanismes de consommation par les organismes aquatiques (Collard et al., 2019; Wenfeng Wang et al., 2019).

L'ingestion de microplastiques peut différer entre groupes fonctionnels (guildes trophiques) et le comportement de recherche de nourriture (McNeish et al., 2018; Thushari et al., 2017; Walkinshaw et al., 2020), avec une abondance de microplastiques ingérés avec l'augmentation de la concentration de microplastiques dans l'eau

(Roch et al., 2020). Les études sur l'ingestion de microplastiques ont négligé le fait que les individus au sein des espèces sont très variables sur le plan écologique (Des Roches et al., 2018), et que les niches trophiques des individus sont variables (Araújo et al., 2011). Par conséquent, l'utilisation de groupes fonctionnels pourrait simplifier à l'excès les niches trophiques individuelles, empêchant une compréhension intégrative de l'ingestion de microplastiques. Au cours des deux dernières décennies, les analyses d'isotopes stables sont apparues comme un outil intégratif utilisé par les écologistes trophiques pour quantifier la niche trophique réalisée (Layman et al., 2012). Par rapport aux méthodes traditionnelles telles que les analyses des contenus stomacaux, qui ne représentent qu'une image instantanée du régime alimentaire des organismes, les analyses d'isotopes stables permettent une quantification intégrative, sur plusieurs semaines voire plusieurs mois selon le tissu analysé, du régime alimentaire des individus (Fry, 2006; Layman et al., 2012). Des investigations sont donc nécessaires pour mieux comprendre les mécanismes d'ingestion des microplastiques par les organismes d'eau douce et les effets potentiels sur la biodiversité d'eau douce (Fig. 8.2 2.f) (Eerkes-Medrano and Thompson, 2018; Eerkes-Medrano et al., 2015).

L'objectif principal de cette thèse était de caractériser la pollution en microplastique et la contamination des organismes dans un écosystème d'eau douce. La pollution microplastique et la contamination de particules allant de 700 μm à 5 mm ont été évaluées dans le bassin versant de la Garonne, situé dans le sud-ouest de la France. Cette évaluation a été à la fois quantitative (nombre ou masse de la pollution en microplastiques) et qualitative (caractéristiques des microplastiques). Compte tenu des enjeux liés au traitement d'une grande quantité d'échantillons environnementaux, riches en matière organique naturelle, nous avons dans un premier temps développé un protocole de traitement d'échantillons à haut débit centré sur les échantillons d'eau (**Chapter 2**). Ensuite, nous avons étudié la variabilité spatiale et temporelle de la pollution microplastique dans les eaux de surface et identifié ses déterminants environnementaux (**Chapter 3**). Spécifiquement, des investigations ont été menées sur quatorze sites répartis tout au long du bassin versant de la Garonne durant quatre saisons pour capturer une variabilité spatiale et temporelle forte des conditions environnementales. Le chapitre suivant (**Chapter 4**) s'est concentré sur le rôle des inondations dans la dynamique de la pollution microplastique. Nous avons ensuite quantifié la contamination par les microplastiques dans les amorces de pêche, une source sous-estimée et potentiellement importante de microplastiques dans l'écosystème d'eau douce (**Chapter 5**). Enfin, le dernier chapitre (**Chapter 6**) visait à quantifier la consommation de microplastiques par les macroinvertébrés et les poissons et

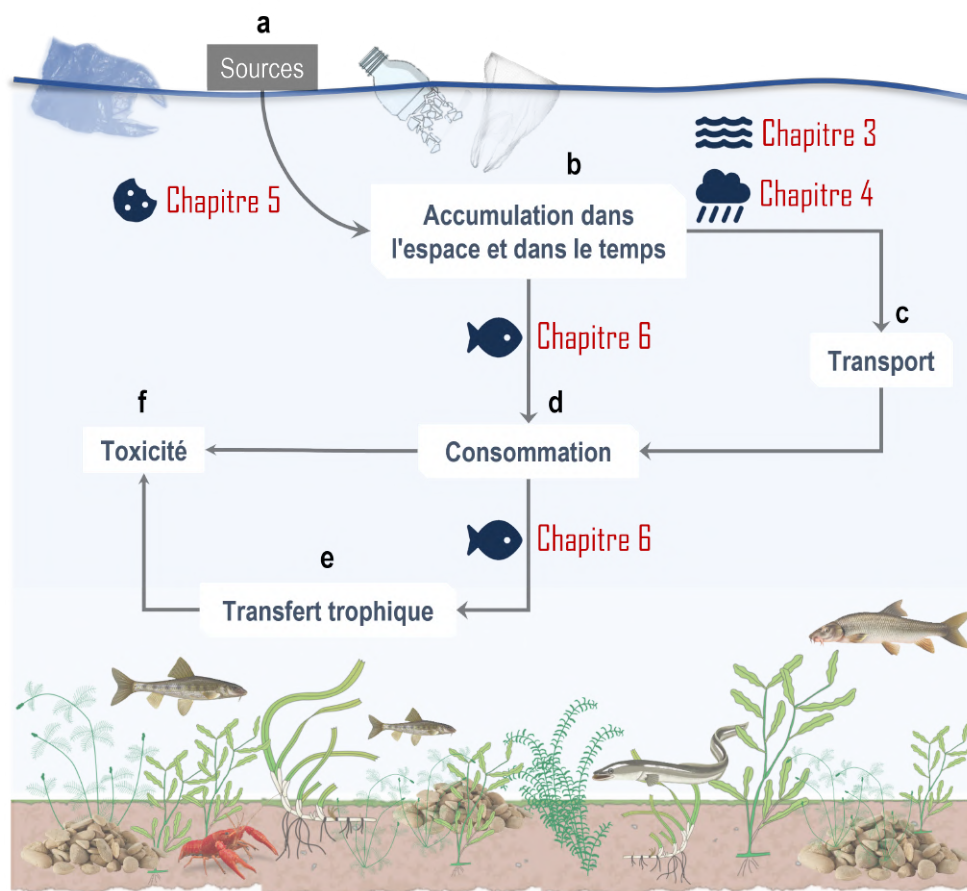


Figure 8.2: Aperçu de la pollution et de la contamination par les microplastiques dans les écosystèmes d'eau douce. La position de chaque chapitre est affichée.

à tester l'existence d'un transfert trophique potentiel à l'aide d'analyses d'isotopes stables.

La Garonne est le troisième plus large fleuve français avec un débit annuel moyen de $630\text{m}^3.\text{s}^{-1}$. Le fleuve principal coule vers le nord depuis sa source dans les Pyrénées centrales, en Espagne, traverse la grande ville de Toulouse et arrive à l'océan Atlantique à proximité de Bordeaux, en France. Pour le traitement des échantillons d'eau visant à extraire le microplastique et en éliminant les interférences (c'est-à-dire le nettoyage), un protocole a été adapté en considérant les composants de la matrice environnementale (contenus inorganiques et organiques) et de l'instrument analytique sélectionné. La digestion chimique de la matière organique naturelle a été effectuée par un procédé chimique en deux étapes, avec l'hydroxyde de potassium (KOH) 10% (m/m) et le peroxyde d'hydrogène (H_2O_2) 30% (m/m), respectivement. Ces sont les deux principales réactifs utilisés dans l'analyse de la contamination environnementale

par les microplastiques, avec impact minimal sur l'intégralité des particules (Nguyen et al., 2019; Prata, J. da Costa, A. C. Duarte, et al., 2019).

Ensuite, une étape commune à tous les échantillons a été l'analyse sous loupe binoculaire pour la sélection des potentielles particules plastiques, réalisées au totale de deux fois par des opérateurs distincts. Les particules sélectionnées ont été photographiées, mesurées, et pesées. Sachant que l'analyse des microplastiques repose fortement sur leur identification chimique, les microplastiques dans cette étude ont été identifiés par spectroscopie infrarouge, la technique d'analyse instrumentale la plus largement acceptée pour caractériser les plastiques (Andrade et al., 2020). Plus précisément, la spectroscopie infrarouge à transformation de Fourier (FTIR), couplée à un dispositif de réflectance totale atténuée (ATR), a été appliquée à chaque particule (Nicolet 6700 ThermoScientific). Le spectre obtenu a été comparé avec ceux disponibles dans des bibliothèques spectrales commerciales et des bibliothèques 'maison' pour la détermination de la composition polymérique des particules. Un minimum de 60% de correspondance entre le spectre investigué et la bibliothèque spectrale a été établi pour que la composition polymérique d'une particule soit assignée.

Nous avons quantifié dans le **Chapitre 3** la variabilité spatiale et temporelle de la pollution en microplastiques dans les eaux de surface sur 14 sites situés à travers le bassin versant de la Garonne (6 dans le fleuve principal et 8 affluents, de l'amont à l'aval : les rivières Neste, Salat, Louge, Ariège, Hers, Touch, Save et Gers) pendant 4 campagnes d'échantillonnage (Février, Avril, Juillet et Octobre 2019). L'échantillonnage de chaque site a été effectué à l'aide d'un filet Manta avec une maille de 500 μm (ouverture de 32 cm x 82 cm), immergé pendant 10 minutes et répliqué trois fois successives à chaque événement d'échantillonnage, totalisant 168 échantillons (4 événements \times 14 sites \times 3 répliques). Le volume totale d'eau filtré à chaque événement a été mesuré à l'aide d'un débitmètre fixé à l'entrée du filet.

La concentration en microplastiques était, en moyenne, de 0.15 (\pm 0.46 SD) particules. m^{-3} et variait fortement à la fois dans l'espace et dans le temps. En utilisant une approche multivariée, les conditions environnementales ont été résumées et nous avons pu constater que la variation spatiale a été induite par l'urbanisation (c'est-à-dire la population humaine et une couverture urbaine des sols) et n'a été pas influencée par la taille des rivières. La concentration en microplastiques augmente avec l'urbanisation. Une forte variabilité temporelle de la concentration en microplastiques était entraînée par des changements hydrologiques saisonniers (c'est-à-dire débit de la rivière et turbidité de l'eau), avec une concentration plus élevée observée dans les conditions de faible débit. Le polyéthylène (PE) (44.5 %), le polystyrène (PS) (30.1

%) et le polypropylène (PP) (18.2 %) étaient les principaux polymères composant les microplastiques retrouvés dans l'environnement, et leur proportion ne variait pas de manière significative entre les sites échantillonnés. Cette répartition entre types des polymères diffère de la demande totale de plastique européenne, dans laquelle ces trois types de polymères ne représentent que 55.4% (PlasticsEurope, 2020), suggérant une différence entre la production et cette fraction de pollution microplastique d'eau douce. La distribution en couleur des microplastiques différait par type de polymère, avec des proportions plus élevées de PS qui était blanc et de PE noir. La distribution de taille variait entre chaque polymère, avec les particules en PS plus grosses que celles en PE et PP. Comme la plupart du PS expansé présentait une forme sphérique, leur présence à la surface de la colonne d'eau était attendue (Van Melkebeke et al., 2020). De plus, comme ces particules n'ont été altérées qu'à leur surface, une hypothèse d'émission relativement récente pourrait être faite, car on s'attend à ce qu'elles se fragmentent facilement sous l'effet de facteurs mécaniques (Mani and Burkhardt-Holm, 2019; Song et al., 2017).

Enfin, nous avons constaté que la variabilité temporelle de la taille des microplastiques était due aux changements hydrologiques saisonniers, avec des particules plus petites retrouvées dans des conditions de faible débit. Cela pourrait être dû aux processus hydrodynamiques avec des microplastiques plus gros nécessitant des débits plus élevés pour être remis en suspension et transportés (Cheung et al., 2019). Indépendamment de la composition du microplastique et de l'événement d'échantillonnage, une corrélation négative entre la taille des particules et la distance à la source de la Garonne a été observée. Considérant les sites d'échantillonnage dans la Garonne, la taille des particules diminuait avec l'augmentation de la distance à la source. Deux hypothèses non-mutuellement exclusives pourraient expliquer ce résultat. D'abord, une éventuelle fragmentation des particules de microplastique pourrait se produire le long du cours d'eau (Garvey et al., 2020; Kataoka et al., 2019). Ensuite, et bien qu'elle n'ait pas été mesurée systématiquement dans cette étude, la profondeur des cours d'eau diffère entre les sites d'échantillonnage. Cela peut avoir affecté la taille moyenne des particules microplastiques échantillonnées dans les eaux de surface, car elles ne semblent pas être uniformément réparties dans la colonne d'eau (Kooi et al., 2017; Kukulka et al., 2012; Law, 2017). Nous soulignons ainsi que la concentration et le type de polymère doivent être quantifiés dans l'analyse de la pollution microplastiques. La variation des propriétés des microplastiques telles que la taille, la densité et la couleur, peut fournir une meilleure compréhension des sources et de la dynamique de cette pollution. La pollution dynamique des microplastiques à travers les bassins

versants et à son puits final, l'environnement marin, est complexe et multiforme, et des efforts doivent encore être faits pour améliorer la résolution spatiale et temporelle de notre compréhension de la pollution microplastique dans les écosystèmes aquatiques pour la gestion de cette pollution (Cable et al., 2017; Rochman, 2018; Skalska et al., 2020).

Dans le **Chapitre 4**, deux sites localisés dans la Garonne et situés en amont (Muret) et en aval (Gagnac) de Toulouse ont été échantillonnées lors de deux épisodes de crues (Octobre 2018 et Mai 2019). Dans la première période, l'échantillonnage a été réalisé quatre fois pendant l'épisode de crue (crue A). Lors de la deuxième période, le suivi temporel a été réalisé à une plus grande échelle, avec six événements d'échantillonnage classés pendant et en dehors de la crue (crue B). L'échantillonnage a suivi le même protocole que dans le Chapitre 3.

Au total, 456 et 1580 microplastiques ont été collectés lors des crues A et B, respectivement. La concentration de microplastiques était, en moyenne, de $0.032 (\pm 0.035 \text{ ET})$ particules.m⁻³ en Muret et $0.401 (\pm 0.330 \text{ ET})$ particules.m⁻³ en Gagnac, lors de la crue A, et de $0.031 (\pm 0.058 \text{ ET})$ particules.m⁻³ en Muret et $0.252 (\pm 0.307 \text{ ET})$ particules.m⁻³ en Gagnac au cours de la crue B. Plus précisément, nos résultats ont mis en évidence que l'augmentation de la concentration en microplastiques lors des épisodes de crue était plus forte dans le site touché par l'urbanisation (en aval de Toulouse) et modulée par le débit. Considérant la forte corrélation entre les propriétés des microplastiques (couleur, composition, taille, etc.), une approche statistique multivariée (analyse factorielle de données mixtes) a été appliquée. Deux composantes ont été sélectionnées, représentant la taille et la qualité des particules microplastiques. La taille des microplastiques variait considérablement au cours des épisodes de crue, à la fois en amont et en aval de la ville, avec des particules plus grosses en période de période de débit plus élevé. Ce résultat corrobore l'hypothèse hydrodynamique de microplastiques plus gros qui nécessitent une énergie plus élevée du débit de la rivière pour être remobilisés (Cheung et al., 2019). Cependant, la qualité des microplastiques pendant la crue a significativement varié que dans le site en aval, avec une proportion plus élevée de PE colorés lors d'une inondation. Cela pourrait représenter des sources plus diversifiées de microplastiques originaires du milieu urbain, considérant la prédominance des plastiques de PE dans les emballages et dans l'usage unique (PlasticsEurope, 2020). Le débit de la rivière était positivement corrélé avec la qualité des microplastiques en amont et négativement corrélé sur le site en aval.

Finalement, les spectres infrarouges du principal type de polymère trouvé dans le

site aval, le PE, ont été analysés pour quantifier les différences de profil chimique des microplastiques pendant ou hors crue. Leurs spectres ont été soumis à une approche statistique multivariée (analyse en composantes principales) et les deux composantes principales représentaient respectivement la variation de la bande spectrale en teneur en hydroxyle et en teneur en carbonyle. Des bandes carbonyles, entre 1700 et 1760 cm^{-1} , apparaissent classiquement lorsque le polymère vieillit et, bien que dépendantes de son milieu environnant, pourraient fournir des informations précieuses sur le profil de dégradation (Andrady, 2011; Karlsson et al., 2018; Kedzierski et al., 2019). La teneur en carbonyle était significativement plus élevée dans les microplastiques en PE échantillonnés pendant l'épisode de crue et due à l'augmentation du débit d'eau. Combiné à la polarité et à la capacité de sorption plus élevées des produits chimiques pour les particules dégradées (Tang et al., 2019; Wang et al., 2021), un épisode de crue pourrait représenter un moment particulier de plus grand risque pour les organismes aquatiques (Cormier et al., 2021).

Concernant la contamination microplastique des amorces de pêche (**Chapitre 5**), nous avons acheté les produits industriels les plus populaires et disponibles dans le commerce, c'est-à-dire des farines, des bouillettes et des pellets. Au total, 16 amorces différentes ont été achetées (6 farines, 6 bouillettes et 4 pellets), fabriqués par 6 fabricants différents, comprenant parfois plusieurs catégories d'amorces pour le même fabricant. Chaque produit a eu son contenu en carbone et azote mesuré (calcul du ratio C:N) qui a ensuite été utilisé pour déterminer la quantité relative d'ingrédients d'origine animale et végétale (rapport C:N plus faible avec une forte proportion d'ingrédients d'origine animale) (Gibb et al., 2015; Martin, 2007). Pour chaque produit, dix répliqués ont été sélectionnés, totalisant 160 échantillons. Au total, dans ces échantillons, 28 microplastiques ont été identifiés dans les farines et les bouillettes, avec une concentration moyenne de $17.4 (\pm 48.1 \text{ ET}) \text{ particules.kg}^{-1}$ et $6.78 (\pm 29.8 \text{ ET}) \text{ mg.kg}^{-1}$, mais aucun microplastique de cette taille n'a été enregistré dans les pellets. Les concentrations de microplastiques différaient significativement entre les catégories d'amorces et les fabricants, mais les caractéristiques des microplastiques ne variaient pas. Les microplastiques étaient principalement composés de PE et d'additifs artificiels tels que les résines alkydes et les additifs pour peinture, et étaient principalement blancs, rouges et bleus. Il n'y avait aucune corrélation entre le nombre d'ingrédients des amorces et leur concentration en microplastiques. Cependant, le rapport C:N des amorces était positivement corrélé avec le niveau de contamination, indiquant une contamination plus élevée dans les amorces avec une proportion plus élevée d'ingrédients à base de plantes.

Les microplastiques identifiés dans les amorces étaient d'une composition différente de celle de leur emballage, suggérant qu'ils peuvent être introduits accidentellement lors de la fabrication et/ou provenant de matières premières contaminées, comme a été déjà montré pour plusieurs aliments industrialisés (Hanachi et al., 2019; Karbalaei et al., 2020; Rødland et al., 2020). L'absence de particules considérées dans cette étude (700 μm - 5 mm) dans les pellets pourrait être expliquée par le niveau plus élevé de fabrication industrielle, conduisant à des particules plus petites dans ce type d'amorces.

Compte tenu de l'incidence et du nombre de microplastiques par unité de masse d'amorce, ils pourraient représenter une source importante de microplastiques pour les poissons d'eau douce lorsque la pression de pêche est élevée. Une fois dans l'eau, les poissons peuvent consommer les microplastiques dérivés des amorces soit directement, c'est-à-dire les microplastiques libérés des amorces, soit indirectement par l'ingestion d'amorces contaminées ou d'autres organismes qui ont eux-mêmes consommé des amorces. Les espèces de la famille des Cyprinidae sont la cible principale des amorces étudiées et des microplastiques ont déjà été détectés dans la carpe commune (*Cyprinus carpio*) de plusieurs rivières (Jabeen et al., 2017; Merga et al., 2020; Park et al., 2020; Warrack et al., 2018). De plus, les effets négatifs de la consommation des microplastiques sur le comportement, l'alimentation et la croissance des poissons se sont montrés significativement plus élevés dans le *C. carpio* (Salerno et al., 2021). Plus d'études sont donc désormais nécessaires pour quantifier l'importance relative de cette source cryptique de contamination et son influence sur la contamination de microplastiques des poissons d'eau douce.

Comprendre les voies et les mécanismes conduisant à la consommation de microplastiques par les organismes d'eau douce est une question de recherche centrale. Plus précisément, dans le **Chapitre 6**, nous avons échantillonné 6 sites d'étude qui représentaient des conditions environnementales contrastées : deux sites situés sur la Garonne en amont de Toulouse, deux sites situés sur des affluents au sein de l'agglomération toulousaine (Hers et Touch), et 2 sites situés sur la Garonne en aval de Toulouse. La campagne d'échantillonnage a été réalisée en Juillet 2019 pour collecter l'eau, sédiments, macroinvertébrés et poissons. L'eau de surface et les sédiments grossiers ont été échantillonnés à l'aide du filet Manta et du filet Surber de 500 μm , respectivement, totalisant 18 échantillons de chaque matrice. En moyenne, 65.8 (\pm 10.1 ET) échantillons de macroinvertébrés ont été collectés dans chaque site. En raison de la petite taille de certains taxons de macroinvertébrés et du niveau potentiellement faible d'ingestion de microplastiques dans la gamme de taille étudiée, des

individus ont été regroupés pour composer un échantillon de macroinvertébrés. Ainsi, chaque échantillon était, en moyenne, composé de 5.4 (± 2.9 ET) individus. Les poissons ont été échantillonnés par pêche électrique et sélectionnés (moyenne de 82 (± 14 ET) individus par site) pour représenter la diversité taxonomique, de classe de taille et fonctionnelle (alimentateurs de fond et d'alimentation en colonne) de chaque communauté.

Un totale de 50 microplastiques ont été trouvés dans les échantillons de macroinvertébrés (396 échantillons composées par 2010 individus appartenant à 36 taxons) et 61 microplastiques dans les échantillons de poissons (492 individus appartenant à 21 espèces), représentant une occurrence de 2% et 10%, respectivement. L'abondance des microplastiques chez les macroinvertébrés a été significativement inférieure que celle chez poissons (moyenne de 0.02 microplastique.ind⁻¹ ± 0.15 ET et de 0.13 microplastique.ind⁻¹ ± 0.42 ET, respectivement). L'abondance des microplastiques chez les macroinvertébrés et chez les poissons n'a pas été différent entre les sites d'échantillonnage. La pollution par les microplastiques dans l'eau (moyenne de 0.87 microplastique.m⁻³ ± 1.24 ET) était significativement différente entre les sites échantillonnés, avec une concentration plus élevée dans les tributaires Hers et Touch. La pollution par les microplastiques dans les sédiments (moyenne de 24.84 microplastique.m² ± 24.38 ET) n'a pas été différent entre les sites échantillonnés.

Nous avons d'abord constaté que l'abondance de microplastiques ingérés par les macroinvertébrés et les poissons n'était pas liée au niveau de pollution dans les eaux de surface et les sédiments. Nous avons ensuite démontré que les caractéristiques des microplastiques (forme, couleur, taille et composition) observées dans l'environnement différaient de ces ingérées. Cela soutient l'hypothèse que les particules de microplastiques sont ingérées activement par les organismes (Lusher et al., 2013) et ne sont pas obtenues passivement. Tant pour les macroinvertébrés que pour les poissons, l'abondance des microplastiques ingérés augmentait avec la taille des organismes. Compte tenu de la taille des macroinvertébrés étudiés, des investigations supplémentaires sont nécessaires pour déterminer la relation entre la taille du corps et la taille des microplastiques pour les microplastiques plus petits que notre limite inférieure de taille (700 μ m). Enfin, les analyses isotopiques stables du carbone ($\delta^{13}\text{C}$) et de l'azote ($\delta^{15}\text{N}$) ont permis l'évaluation de l'origine des ressources consommées et la position trophique des individus dans la chaîne alimentaire, respectivement. Notre étude a démontré que les groupes de recherche de nourriture et la niche trophique ont affecté différemment l'ingestion de microplastiques pour les macroinvertébrés et les poissons. Chez les macroinvertébrés, il n'y avait pas de différence significative entre les

groupes avec habitudes de recherche de nourriture différentes, tandis que la position trophique avait tendance à être positivement associée à l'abondance de microplastique ingéré. L'origine de la ressource consommée n'affectait pas l'abondance de microplastique consommés. Ces résultats suggèrent que les macroinvertébrés ingèrent principalement des microplastiques directement (c'est-à-dire une ingestion primaire) et que les microplastiques présents plus haut dans la chaîne alimentaire n'étaient probablement pas le résultat d'un transfert trophique. Nous émettons donc l'hypothèse que l'ingestion de microplastiques était principalement accidentelle et modulée par des caractéristiques des microplastiques qui influencent leur disponibilité, telles que la forme, la taille ou la densité. Chez les poissons, l'ingestion de microplastiques était plus élevée chez les individus se nourrissant au fond des cours d'eau que chez ceux se nourrissant dans la colonne. Elle était significativement associée à l'origine de la ressource alors qu'il n'y avait pas de relation significative avec la position trophique des poissons. La consommation directe par les poissons était donc la plus probable, comme plusieurs études l'ont déjà démontré (López-Rojo et al., 2020; Ory et al., 2018; Welden et al., 2018).

Une concentration plus élevée de microplastiques dans l'eau n'induit pas nécessairement une ingestion plus élevée de microplastiques (Collard et al., 2019; Peters and Bratton, 2016). Cela peut être causé, par exemple, par trois mécanismes mutuellement non-exclusifs. D'abord, les changements spatiaux des caractéristiques des microplastiques à travers les sites (Rodrigues et al., 2018; Skalska et al., 2020) qui pourraient moduler leur ingestion par les organismes. Ensuite, la variabilité des conditions environnementales d'un site à l'autre. Plusieurs paramètres abiotiques tels que la turbidité de l'eau, les caractéristiques du substrat et la température sont connus pour moduler la capacité des organismes d'eau douce à détecter et/ou manipuler leurs proies, et ils sont donc susceptibles d'affecter l'ingestion de microplastiques. Les conditions biotiques telles que la densité de population, la prédation et les conditions intraspécifiques, en modulant la niche trophique individuelle (Araújo et al., 2011), sont également susceptibles d'affecter l'ingestion de microplastiques. En troisième, la structure des communautés de macroinvertébrés et de poissons, qui varie selon les sites. Parce que les traits des individus et des espèces influencent l'ingestion de microplastiques (Ferreira et al., 2019, 2016; Horton et al., 2018), les changements dans la structure de la communauté peuvent fortement moduler l'ingestion globale de microplastiques au niveau du réseau trophique. Des approches expérimentales qui manipulent les caractéristiques des microplastiques (par ex., composition, couleur, forme), les conditions environnementales (par ex., la turbidité, le substrat, la température) et la compo-

sition de la communauté sont donc nécessaires pour évaluer pleinement la relation entre pollution microplastique dans l'environnement et contamination des organismes d'eau douce.

La reconnaissance de l'omniprésence des microplastiques dans l'environnement a été suivie par l'urgence d'évaluer leurs risques pour les organismes et le fonctionnement de l'écosystème. La cartographie de la pollution microplastique est devenue primordiale, et doit inclure la dynamique spatiale et temporelle en eau douce et la consommation par les organismes aquatiques. Cette thèse a analysé la pollution en microplastiques de taille comprise entre 700 μm et 5 mm et a mis en évidence la forte variabilité spatiale et temporelle de cette pollution dans le bassin versant de la Garonne induite respectivement par l'urbanisation et les conditions hydrologiques (**Chapitre 3**). Les périodes de faible débit, estivales notamment, présentaient une concentration microplastique plus élevée de particules plus petites. A l'inverse, lors d'une variation hydrologique courte et forte, comme lors des épisodes de crue, la concentration en microplastiques augmente substantiellement à la surface de l'eau (**Chapitre 4**), avec des particules microplastiques plus grosses. Cette augmentation a cependant été plus forte en aval des zones urbaines. Nous avons également identifié que les amorces de pêche représentent une source cryptique et potentiellement importante de microplastique dans les écosystèmes d'eau douce (**Chapitre 5**). Par conséquent, un pêche récréative intense comme observé dans certains écosystèmes pourrait représenter une forte augmentation des apports de microplastiques et des risques pour les organismes aquatiques. En effet, des microplastiques ont été retrouvés chez 18 taxons de macroinvertébrés d'eau douce et 8 espèces de poissons représentatifs du bassin versant de la Garonne, dans un totale de 35 et 21 étudiés, respectivement (**Chapitre 6**). Les poissons consomment du microplastique principalement en raison de leurs habitudes de recherche de nourriture, les poissons se nourrissant au fond présentant des niveaux de contamination les plus élevés. La contamination microplastique chez les invertébrés était quant à elle plus forte chez les taxons prédateurs et les grands individus, comme les larves de libellules et les écrevisses, indiquant une consommation directe de ce polluant.

L'urbanisation est une pression importante sur les écosystèmes des cours d'eau, affectant négativement la qualité de l'eau et la biodiversité (Wen et al., 2016). Le « *syndrome des fleuves urbains* » représente les conséquences écologiques et hydrogéomorphiques induites par l'urbanisation, telles que les modifications de la morphologie des cours d'eau, l'homogénéisation des profils des cours d'eau et la simplification de la biodiversité écologique (Bernhardt and Palmer, 2007; Meyer et al., 2005). Dans cette

thèse, nous avons mis en évidence un autre facteur associé au stress écosystémique causé par l'urbanisation, la pollution microplastique.

Les rivières petites mais très urbanisées exportent une quantité importante de plastiques, surdimensionnée par rapport à leurs bassins versants et dépassant le transport des plus grands bassins versants (Lebreton et al., 2017; Stubbins et al., 2021). Dans cette étude, les concentrations les plus élevées de microplastiques ont été enregistrées dans deux petits affluents très urbanisés de la Garonne. Cependant, des changements au sein d'un bassin versant peuvent également se produire, en fonction des activités humaines à proximité des cours d'eau et de l'accessibilité des rives de la rivière (Dris, Gasperi, et al., 2015; Skalska et al., 2020). La pollution microplastique pourrait être atténuée par les écotones riverains, l'interface entre les systèmes terrestres et d'eau douce (Moore and Palmer, 2005), car la mobilisation des microplastiques dépend du type de végétation riveraine, de la pente, de la couverture et de la rugosité, qui sont très variables au sein d'un bassin versant (Cowger et al., 2019; Delorme et al., 2021; Windsor, Durance, et al., 2019). Dans des conditions favorables d'écotones riverains, comme une faible végétation, les microplastiques atteignent finalement les cours d'eau. Une fois dans l'eau, les microplastiques se répartissent différemment à la fois entre les sections de transect, c'est-à-dire la ligne à travers la surface de l'eau, et verticalement, c'est-à-dire à travers la colonne d'eau. De cette façon, des points de contamination plus élevés en microplastique sont attendus dans les eaux de surface (Dris, H. Imhof, et al., 2015; Liu et al., 2020; Niu et al., 2020). L'urbanisation affecte négativement l'étendue et la composition de la végétation riveraine (Borisade et al., 2021), facilitant l'accès plus direct des microplastiques aux cours d'eau, favorisant ainsi des niveaux plus élevés de microplastiques dans les cours d'eau urbains.

L'urbanisation a accentué l'augmentation de la concentration en microplastiques lors d'une crue avec un pic de $1.11 \text{ microplastiques.m}^{-3}$, dépassant largement la moyenne annuelle de $0.126 \pm 0.128 \text{ microplastiques.m}^{-3}$ dans la Garonne à Gagnac-sur-Garonne. L'augmentation de la surface imperméable dans les zones urbaines contribue au ruissellement de la pollution lors d'une inondation et conduit à une concentration accrue de microplastiques dans les cours d'eau (Bernhardt and Palmer, 2007; Walsh et al., 2005). En utilisant la masse médiane des microplastiques dans cette étude, soit 0.20 mg (moyenne $0.562 \pm 1.38 \text{ mg}$, $n = 2269$) qui est similaire à celle récemment rapportée (Weiss et al., 2021), une estimation approximative de la masse annuelle de microplastiques traversant la Garonne est de 1.67 t. an^{-1} (en considérant un débit moyen de $630 \text{ m}^3.\text{s}^{-1}$). Des études de macro- et microplastiques flottants dans le Rhône (France) ont rapporté des flux d'un ordre de grandeur similaire, de

0.71 t.an⁻¹ et 0.07–7.8 t.an⁻¹, respectivement (Castro-Jiménez et al., 2019; Schmidt et al., 2018). Pour la même région, une étude portant sur les additifs plastiques a rapporté un apport annuel de la rivière à l’océan de 5 à 54 t.an⁻¹, expliqué par le lessivage des déchets plastiques flottants et le rejet lors de la fabrication du plastique, des usines de traitement des eaux usées et dépôt atmosphérique (Schmidt et al., 2020). Si un mécanisme similaire se produit dans le bassin versant de la Garonne, les quantités d’additifs plastiques pourraient atteindre des niveaux de masse égaux ou supérieurs aux microplastiques.

Un profil d’oxydation plus important des particules de polyéthylène a été observé lors d’une inondation dans le site urbanisé, avec des conséquences importantes sur la fragmentation et la sorption des particules et la libération de produits chimiques (Arp et al., 2021; Garvey et al., 2020; M. Simon et al., 2021). L’exposition environnementale du polyéthylène entraîne une oxydation plus importante et une cristallinité potentiellement accrue ainsi qu’un poids moléculaire plus faible (Garvey et al., 2020; Halle et al., 2016). Par conséquent, une fragilisation et une dégradation chimique plus importante sont attendues, générant probablement des fragments de polyéthylène plus petits, même à une taille nanométrique (Gigault et al., 2016). Ces microplastiques plus petits présentent un ratio surface/volume plus élevé que les grands microplastiques et un processus d’association accru avec les produits chimiques est attendu. Trois facteurs principaux influencent concomitamment ce processus de sorption. D’abord, les propriétés inter-corrélées au sein d’une particule microplastique telles que la composition polymère et l’agencement spatial, les additifs, la rugosité et le biofilm sont connus pour réguler les mécanismes de sorption (Rummel et al., 2017; Wang et al., 2018). Plus la distance entre les chaînes polymères est grande, plus il est facile pour les produits chimiques de se diffuser dans ou à travers la matrice, ce qui explique la plus grande capacité de sorption du polyéthylène par rapport au polypropylène (Hartmann et al., 2017; Rochman et al., 2013). Deuxièmement, les conditions environnementales, telles que le pH de l’eau, la température, la matière organique naturelle, avec un pH plus bas et une température plus élevée augmentant la désorption des contaminants des microplastiques (Bakir et al., 2014). En troisième, les propriétés intrinsèques des contaminants organiques, comme leur potentiel d’ionisation et l’hydrophobie (Sharma et al., 2021; Teuten et al., 2009), lorsque la sorption de la plupart des contaminants sur les microplastiques augmente avec leur hydrophobie et avec des particules de plastique plus petites (Wang et al., 2018). La co-occurrence d’une concentration plus élevée de microplastiques et d’une libération plus importante de contaminants toxiques représente probablement des risques plus importants pour

les organismes (Wang et al., 2018). Cependant, une concentration en microplastique réduite avec conditions favorables de libération des composants chimiques pourraient toujours représenter un contaminant extrêmement toxique. Une évaluation complète des contaminants environnementaux, intégrant les conditions microplastiques et environnementales, est essentielle pour comprendre les mécanismes pertinents à l'origine de la fragmentation des microplastiques et de la libération de produits chimiques par les microplastiques (Crawford et al., 2022; Garvey et al., 2020; Sharma et al., 2021).

Les organismes interagissent avec toutes les tailles de plastiques, avec des effets possibles allant du stress physique à l'assimilation chimique. Les débris de plastiques d'une plus grosse taille peuvent représenter un substrat pour la colonisation d'organismes et d'invertébrés plus petits (Davidson, 2012). Les classes de tailles de plastiques de grande taille mais ingérables représentent un risque de blocage gastro-intestinal (Gall and Thompson, 2015). Après ingestion et digestion, les microplastiques de la gamme de taille étudiée sont généralement ingérés. Le temps de rétention des microplastiques dans les organes digestifs peut être similaire à celui des aliments naturels, mais des effets toxiques directs dans le tube digestif sont attendus, tels qu'une inflammation et une perturbation du microbiome intestinal (Cole et al., 2013; Fackelmann and Sommer, 2019; Boqing Li et al., 2020). Dans le tube digestif, les microplastiques sont potentiellement fragmentés en plus petites tailles et, lorsqu'ils sont inférieurs à 10 μm , ils peuvent être absorbés et transférés à travers les membranes et les tissus biologiques, s'accumulant et provoquant des effets toxiques (Bruinink et al., 2015; Franeker and Law, 2015; Stock et al., 2021; Triebkorn et al., 2019). Une réponse rapide au stress dans le foie, des troubles du métabolisme et un stress oxydatif causés par les microplastiques ont déjà été observés, et des conséquences à long terme pourraient également se produire, à savoir le concept de « dette de toxicité » (Prata, J. da Costa, Lopes, et al., 2019; Rillig et al., 2021; Yin et al., 2021). Malgré une recherche toxicologique accrue sur les microplastiques, nos connaissances pour comprendre et prédire les impacts individuels et collectifs des micro et nanoplastiques combinés aux produits chimiques associés sont encore limitées (Bernhardt et al., 2017; Yin et al., 2021). De plus, les études toxicologiques ne parviennent toujours pas à inclure les micro et nanoplastiques pertinents pour l'environnement et à rendre compte de la diversité au sein d'une particule microplastique (Bucci et al., 2020; Waldman and Rillig, 2020), et l'une des raisons importantes à cela est le manque de méthodologie analytique adéquate.

Le développement de protocoles expérimentaux est au cœur de la chimie environnementale et l'une des plus grandes perspectives dans le domaine de la pollu-

tion microplastique réside dans le développement de méthodologies pour les analyses d'échantillons. Le choix d'une méthodologie analytique pour l'identification et la quantification de la pollution microplastique est principalement guidé par la taille des particules (Filella, 2015; Renner et al., 2018). Dans ce travail, nous avons appliqué la stéréo-microscopie et la spectroscopie ATR-FTIR pour obtenir des informations sur la taille, la morphologie, la couleur et la composition des particules allant de 700 μm à 5 mm. Pour une plus petite taille de microplastiques, différentes technologies analytiques sont appliquées. Parmi ces techniques, l'imagerie et la microscopie-FTIR sont couramment utilisées (Pan et al., 2021), bien qu'une alternative émergente réside dans le domaine de la spectrométrie. Compte tenu des propriétés particulières, insolubles et non volatiles des microplastiques, l'analyse par chromatographie en phase gazeuse-spectrométrie de masse (GC-MS), par exemple, doit être précédée de techniques de dégradation thermique. Les produits pyrolytiques volatils générés peuvent être directement transférés vers la colonne chromatographique (Py-GC-MS) (Fabbri, 2001; Funck et al., 2020; Hermabessiere et al., 2018) ou piégés puis thermo-désorbés (TED-GC-MS) (Dümichen et al., 2015, 2017). Les deux techniques permettent de détecter le cocktail chimique au sein d'un microplastique et d'identifier la composition polymérique de particules plus petites (González-Pérez et al., 2014; Peñalver et al., 2020). Une pyrolyse à double injection couplé à chromatographie en phase gazeuse-spectrométrie de masse permet d'étudier les comportements chimiques et de sorption, en appliquant une température plus douce dans la plage de 100°C à 300°C, et une analyse des polymères par pyrolyse à 650°C (Burrows et al., 2020; Okoffo et al., 2020). Les modifications de la structure moléculaire d'un polymère, telles que la présence de groupement fonctionnel ou de chaîne moléculaire réduite, font partie des perspectives d'analyse de la thermo-dégradation des plastiques environnementaux (Ainali et al., 2021; Ter Halle et al., 2017).

Pour la détermination de traces de plastiques dans des échantillons environnementaux, c'est-à-dire lorsque sa proportion est inférieure à 0,01 % (équivalent à 100 ppm) (Valcárcel Cases et al., 2018), le principal défi est de diminuer la limite de détection et de quantification dans le processus analytique. En général, il est nécessaire de différencier l'analyse cible du bruit de fond de la matrice, ce qui est particulièrement important lorsqu'il s'agit d'échantillons environnementaux. Stratégies pour réduire le bruit en augmentant les plages de sélectivité ont été mis en place au niveau de la préparation des échantillons, comme l'inclusion d'une étape de pré-concentration (Steinmetz et al., 2019). Au niveau de la spectrométrie de masse, le mode en tandem est connu pour offrir une sélectivité accrue, des procédures de nettoyage simplifiées et

une analyse plus rapide. Bien que la mise en œuvre des technologies actuelles de spectrométrie de masse puisse améliorer l'acquisition de données chimiques, le couplage avec des techniques thermo-analytiques entraîne un desserrement des informations concernant la taille, la morphologie et la couleur des microplastiques (Saitoh, 2021). Compte tenu de la propriété particulière des microplastiques, ces données sont particulièrement utiles pour comprendre les moteurs et les risques de consommation de microplastiques (Collard et al., 2019). Il existe actuellement un compromis lors de l'obtention de données sur les caractéristiques de la pollution par les microplastiques. Les stratégies multidimensionnelles dans le domaine de la chimie analytique ont déjà révolutionné l'étude de la contamination environnementale (Duarte and Duarte, 2020) et devraient désormais relever les défis des analyses des micro- et nano-plastiques.

Les effets toxicologiques induits par les microplastiques ingérés se produisent par deux voies principales. La première est une voie directe induite par la composition polymérique (et ses altérations dues au vieillissement et aux intempéries), les additifs de formulation ou les polluants organiques (Rochman et al., 2013; Strungaru et al., 2019). Les fluides digestifs pourraient favoriser la lixiviation des produits chimiques adsorbés dans les microplastiques et réguler leur assimilation potentielle par les organismes. La pertinence de ce processus par rapport à celui des particules naturelles reste néanmoins à évaluer (Koelmans et al., 2016; Rodrigues et al., 2019; Triebkorn et al., 2019). Une deuxième voie est une conséquence indirecte de l'ingestion de microplastiques, déséquilibrant le bilan énergétique de l'organisme en raison d'une consommation alimentaire et/ou d'une assimilation diminuées (Galloway and Lewis, 2016). Ceci est particulièrement d'accord avec les effets négatifs sur le comportement, la recherche de nourriture et la croissance des poissons et dépend des espèces de poissons et des stades de leur vie (Foley et al., 2018; Salerno et al., 2021). En raison de la différence de consommation alimentaire entre les sexes, où les femelles matures ont généralement une consommation alimentaire et/ou des coûts métaboliques plus élevés, les femelles pourraient être plus affectées par cette conséquence indirecte de l'ingestion de microplastiques, comme le montre une réduction visible de sa croissance (Cormier et al., 2021). Des impacts ultérieurs sur la reproduction et des changements dans la structure trophique des communautés écologiques sont attendus, avec des effets potentiels sur le fonctionnement des écosystèmes qui restent à étudier (López-Rojo et al., 2020; Santos et al., 2021).

La co-occurrence de nombreux facteurs de stress environnementaux induits par les activités humaines (c'est-à-dire des facteurs de stress multiples) est de plus en plus reconnue. Historiquement, la gestion des écosystèmes s'est concentrée sur ces facteurs

de stress individuellement, sans tenir compte des effets potentiels de plusieurs facteurs de stress pour interagir et produire des effets additifs, synergiques ou antagonistes (Spears et al., 2021). Cependant, le microplastique doit être considéré comme un multi-stress lui-même, motivant des études pour évaluer comment ils modifient les processus écologiques et affectent les changements globaux (Bernhardt et al., 2017; Grantham et al., 2019). La santé de l'écosystème dépend de sa résilience et englobe l'évolution d'une réponse adaptative des organismes à la pollution plastique, et la capacité des organismes d'eau douce à s'adapter aux pressions causées par la pollution microplastique (Santos et al., 2021) reste à déterminer.

Il y a deux questions de recherche principales que les scientifiques ne parviennent toujours pas à traiter correctement sur le sujet de la pollution plastique, comme ce fut le cas dans la récente section spéciale sur la pollution plastique dans la revue *Science* (Smith and Vignieri, 2021):

- 1) Les niveaux de pollution par les microplastiques dépassent-ils les limites que l'eau douce peut résister ? Considérant que les eaux douces sont toujours en mouvement, avec une énorme capacité de se renouveler, les nouveaux et exceptionnellement importants changements, y compris la pollution par les microplastiques, devraient être étudiés dans le cadre de critères de capacité de résilience de ces écosystèmes, sur le plan écologique et environnemental. Des technologies appropriées, des stratégies politiques et des incitations économiques sont nécessaires pour restreindre les microplastiques dans les limites considérés comme « de sécurité » pour l'eau douce.
- 2) Considérant une perspective de gestion avec des ressources limitées, où est-ce que la pollution par les microplastiques se place par rapport à d'autres pressions environnementales et écosystémiques ? Pour répondre à cette question, l'intensité des effets écologiques induits par d'autres changements environnementaux causés par l'homme, tels que l'utilisation des terres, les invasions biologiques, la modification hydrologique (par exemple la construction de barrages) et la récolte (Carpenter et al., 2011) doivent être comparé à celle induite par les microplastiques (Fig. 8.3). Tous les facteurs jouent un rôle, certains sont bien établis et d'autres, comme la pollution par les microplastiques, nécessitent une enquête plus approfondie, mais principalement en contextualisant les résultats dans le scénario à facteurs de stress multiples.

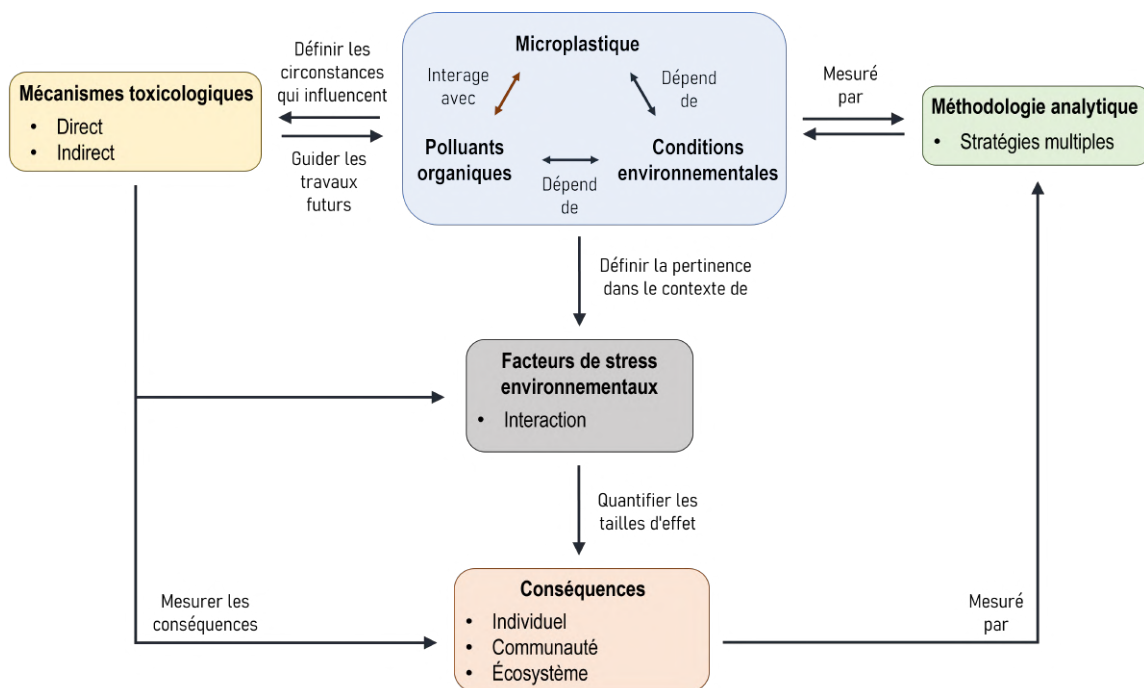


Figure 8.3: Processus d'interaction des microplastiques, y compris dans la perspective intra-particule, avec la pollution environnementale. Les potentiels conséquences toxicologiques et dans les écosystèmes sont encore à être étudiés.

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A - Article “Protocol for microplastic pollution monitoring in freshwater ecosystems: Towards a high-throughput sample processing - MICRO PLASTREAM”



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Method Article

Protocol for microplastic pollution monitoring in freshwater ecosystems: Towards a high-throughput sample processing - MICROPLASTREAM



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ABSTRACT

Robust and reproducible quantification of microplastic pollution in freshwater ecosystems requires the processing of a large amount of samples collected in varying environmental conditions. Such samples are characterized by a high amount of organic matter compared to microplastics and are highly variable in terms of the quantity and the composition of matrices, requiring a standardized analytical protocol for sample treatment and analysis. However, two important and time-consuming steps for microplastic recovery are the elimination of organic matter and microscopic inspection of samples. Here, we developed and validated a protocol, targeting particles with length ranging from 700 μm to 5 mm, that includes a double-step digestion of organic matter, consisting of incubation with potassium hydroxide followed by hydrogen peroxide solutions, and two stereomicroscopic analyses. In addition, we developed several technical improvements allowing reducing the time needed to process samples, such as the design of an adapted filter-cap to improve the content transfer. The absence of physical and chemical alterations in the investigated microplastic pellets and the average reduction of 65.8% (\pm 9.59 SD) of organic matter in real samples demonstrated that our protocol is fit for purpose. We recommend a second stereomicroscopic analysis to avoid underestimating microplastic concentration and particle size distribution biased towards larger particles. When used for a large-scale monitoring of microplastic pollution, this protocol resulted in an estimated time of 38 h for one person for the treatment of a batch of 24 samples, allowing a higher throughput sample processing and reproducible quantification.

- Protocol customization towards high-throughput sample processing
- Double step digestion to improve organic matter elimination
- Importance of stereomicroscopic analysis for microplastic recovery

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Method name: MICROPLASTREAM

Keywords: Organic matter, Digestion, Polymers, FTIR

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Specifications Table

Subject Area:	Environmental Science			
More specific subject area:	<i>Microplastic pollution</i>			
Method name:	MICROPLASTREAM			
Name and reference of original method:				
Resource availability:	Material	Specifications	Quantity	Observations
	96-well plates	–	–	–
	Aluminum tray	–	–	–
	ATR-FTIR spectroscope	Thermo Nicolet 6700, Thermo Fisher Scientific	–	–
	Balance	AT21 Comparator, <i>d</i> = 0.001 mg, Mettler Toledo	–	For particles
	Bottle	250 mL, GL-45	24	–
	Filter paper	–	–	–
	Heating plate	–	–	–
	Hydrogen peroxide	30% (w/w) solution	–	CAS: 7722–84–1
	Nitex tissue	500 µm	0.5 m ²	–
	Open screw cap	GL-45	24	–
	Petri dish	8 cm diameter	–	Similar quantity as samples
	Potassium hydroxide	> 85% purity, pellets	–	CAS: 1310–58–3
	Sieve	500 µm	1	Stainless
	Stereomicroscope with camera	Leica MZ 75 and Nikon SMZ 800	–	Equipped with a digital camera
	Thermometer	–	–	–
	Tweezers	–	2	Straight and curved ones

Method details

General context

Environmental microplastic pollution, i.e. plastic particles smaller than 5 mm [1], is an emerging concern due to their potential impacts on organism health, biological diversity and ecosystems [6,16]. Microplastic pollution has primarily been quantified and characterized in marine ecosystems, considered as a final sink of these particles [8,26]. Freshwater ecosystems (streams, rivers and lakes) are also extremely important in the dynamic of microplastic pollution because they act as a main source and are responsible for its transport and retention [26]. Accordingly, an increasing number of studies have focused on microplastic pollution in freshwater ecosystems [12,13]. For a robust assessment of microplastic pollution, studies performed at large spatial (e.g. across watersheds) and temporal (e.g. across months and seasons) scales, resulting in high amount of samples, are needed. Therefore, the development of a simplified and reproducible protocol for sample processing is crucial. The detection of microplastics in environmental matrices faces two crucial issues: reduction of matrices effects without altering the target particle, and the unequivocal identification of the targets [29]. However, the quantity and content of freshwater matrices, notably in terms of organic matter and level of microplastic pollution, are highly variable, limiting our ability to settle long-term monitoring of microplastic pollution. The establishment of a standard and high throughput protocol for the quantification and characterization of microplastic in freshwater ecosystems should therefore consider these aspects [17].

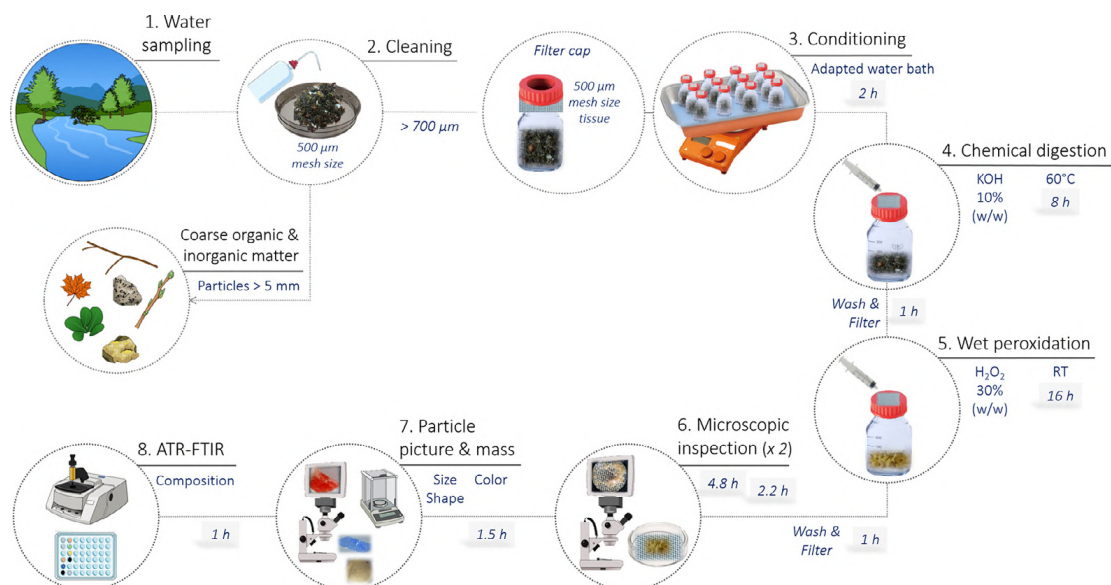


Fig. 1. Global overview of the protocol and its different steps. The time displayed represent the analyses of a batch of 24 samples.

Protocol for sample processing

Current processing of environmental samples for microplastic detection consists of sample collection followed by sample treatment to reduce organic matter content and sample analysis for particles identification [17,22]. The diversity of organic matter composition has led to the development of distinct protocols for sample digestion, either for marine water (e.g. NOAA), sediments or aquatic organisms samples [2,18–20]. Importantly, protocol selection or the adaptation of an existing protocols should take into account the purposes of the study and the studied matrix. Organic matter elimination through digestion might be achieved by incubating the sample with an acidic or alkaline solutions, such as potassium hydroxide (KOH), with peroxides solutions, such as hydrogen peroxide (H₂O₂) or through an enzymatic reaction. Opposite findings regarding the efficacy of organic matter digestion through different protocols and matrices have already motivated the use of a multiple-step digestion, with different reagents [7,21], although a single reagent is still used in many studies [22].

In this study, a double-step digestion consisting of two different reagents, potassium hydroxide (KOH) (pellets, Sigma-Aldrich, USA) 10% (w/w) and hydrogen peroxide (H₂O₂) 30% (w/w) (Merck KGaA, Germany) solutions was used to optimize the digestion of the rich and diverse organic matter content in freshwater samples (Fig. 1). KOH and H₂O₂ are the two main reagents used for digestion purposes in microplastic monitoring studies [25] and were therefore tested in this protocol. Because a multi-step digestion protocol would require the inclusion of washes and filtrations steps, a customized filter-cap was designed to facilitate content drain-out. The glass bottle was covered with a Nitex tissue (500 µm, similar to the water sampling net), and a commercially available screw open-cap (Fig. 3). A syringe was used to facilitate liquid addition through the tissue. Finally, the critical step of microscopic analysis of samples was verified and we concluded that two stereomicroscope analyses, by two different operators, represent a good compromise between analyses time and particles recovery, both in terms of quantity and characteristics of microplastics. In this protocol, microplastic was defined as particle with a major axis larger than 700 µm (i.e. diagonal of the 500 µm mesh net of sampling device) and smaller than 5 mm, and with composition defined as plastic, comprising synthetic polymers, petroleum-based waxes, tire and wear particles and, paint resins [11]. Fibers were not considered here. Considering the instrumental size limitation associated with the detection and quantification of particles by visual inspection using a microscope [9], the selected size range (700 µm



Fig. 2. Sample collection in the field with (a) Manta trawl equipped with (b) a removal cod-end. Samples are filtered in the field (c) using a 500 μm sieve and stored in a plastic bag before processing in the laboratory.

– 5 mm) favors an optimal chemical identification by attenuated total-reflectance Fourier-transformed infra-red (ATR-FTIR) spectroscopy, in which a minimum score of 60% of library match was applied.

1. Sample collection (field sampling)

1.1. Field sample with a Manta trawl of 500 μm mesh (Fig. 2a) and collected the in the cod-end (Fig. 2b).

1.2. Filter the sample through a 500 μm metal sieve (Fig. 2c).

1.3. Transfer the retained sample into labelled and sealable plastic bags (Fig. 2c).

1.4. Store in the fridge (4 $^{\circ}\text{C}$) until analyses.

2. Sample treatment (laboratory analysis)

2.1. Measure the wet mass of the sample.

2.2. Over a 500 μm sieve, remove coarse organic and inorganic debris, such as branches, pebbles, leaves and gravels, and particles larger than 5 mm, rinsing with distilled water.

2.3. Transfer the retained content into labelled glass bottles of 250 mL.

2.4. Add KOH 10% (w/w) solution in a proportion of 4 units of volume (mL) for 1 unit of mass of sample (g).

If sample wet mass > 40 g, leading to > 160 mL of reagent, it is recommended to split the sample into several glass bottles to avoid overflow.

2.5. Place pre-cut fabrics of Nitex tissue in square format (5 cm x 5 cm) on the top of bottles and use open screw caps to close (Fig. 3).

2.6. Cover the bottles with aluminum (foil or tray).

2.7. Incubate in a water bath at 60 $^{\circ}\text{C}$ for 8 h.

– Heating at 60 $^{\circ}\text{C}$ was proposed to reduce incubation period [5].

– An adapted aluminum tray bath was employed (Fig. 1, step 3), although an inox tray is recommended due to its higher resistance to oxidation.

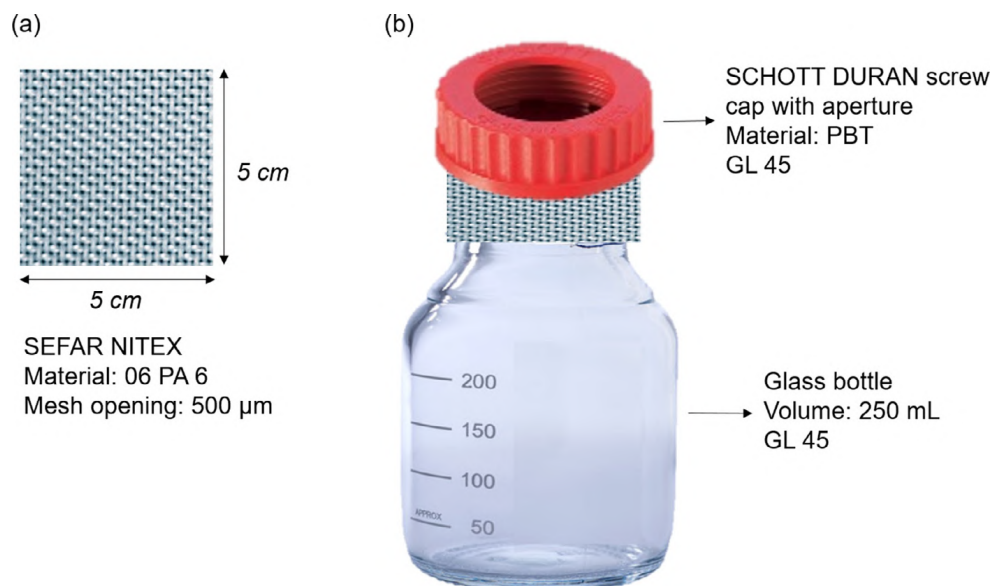


Fig. 3. Customized bottles used for sample digestion. (a) A piece of tissue is placed (b) between the screw open and the glass bottle.

- Monitor temperature with a thermometer immersed in a similar glass bottle filled with water only.
 - Verify water level in the bath at every 2 h and refill when needed.
- 2.8. Remove the liquid in the bottles by pouring through the tissue.
 - 2.9. Add 40 mL of distilled water with a syringe through the tissue (Fig. 1, step 4).
 - 2.10. Shake and stir the bottle to enhance the washing (Fig. 4a).
 - 2.11. Remove the liquid in the bottles by pouring them.
 - 2.12. Repeat steps 2.8 to 2.11 three times minimum or until obtaining a clear rinsing liquid.
 - 2.13. Add H_2O_2 30% (w/w) solution until fully covering the whole sample (Fig. 1, step 5).
 - This step should be performed with caution once this process may result in a highly reactive mixture.
 - 2.14. Incubate overnight at room temperature (16 h equivalent).
 - Due to the reactive mixture, samples were not heated. Then, the incubation period was slightly longer.
 - 2.15. Repeat steps 2.8 to 2.11 (Fig. 4b).
 - 2.16. Remove the filter-cap, place it upside-down and filter the sample through the tissue, adding water to remove all remaining content in the bottle (Fig. 5a).
 - 2.17. Place the tissue with the retained sample in labelled Petri-dish (8 cm diameter) and store at room temperature.
3. Sample analysis (laboratory analysis)
 - 3.1. Analyze the petri-dish under a stereomicroscope (14-fold magnification suggested) (Fig. 1, step 6, Fig. 5b and 5c) and select potential plastic particles, placing them temporarily in a new identified petri-dish (Fig. 5b).
 - The time of analysis may strongly vary depending on the amount of remaining organic matter and microplastic concentration.

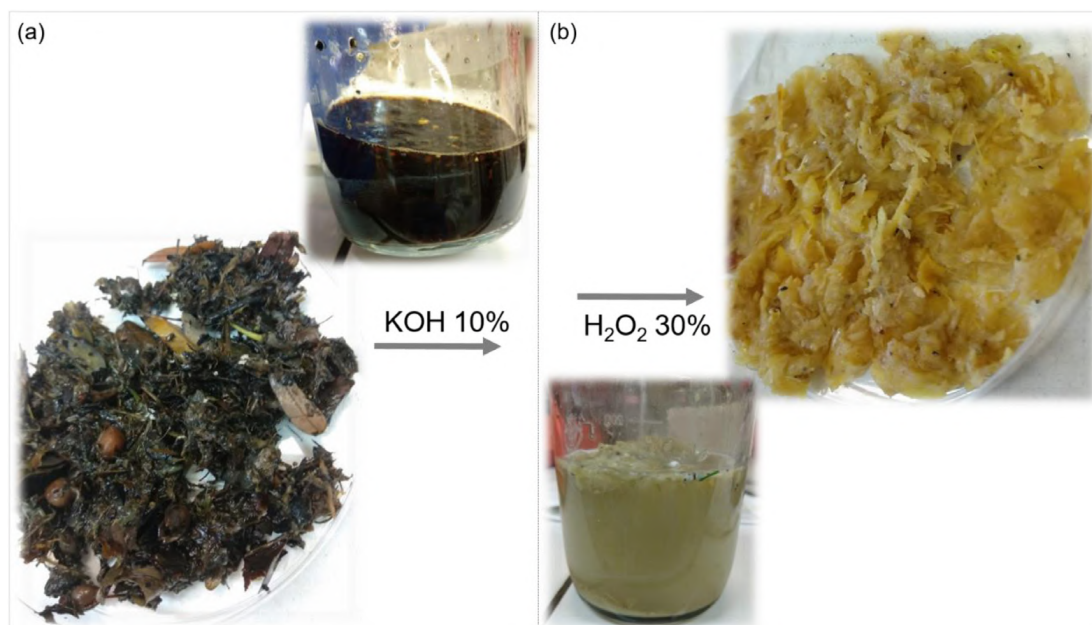


Fig. 4. Changes in sample characteristics induced by the double digestion protocol: (a) before and (b) after incubation with potassium hydroxide solution followed by hydrogen peroxide solution.

3.2. Repeat step 3.1.

- To reduce the risk of missing microplastic through manual selection and to avoid potential bias in detection (e.g. particle color, visual appearance), we recommend a second stereomicroscope analysis by a different operator.

- We also recommend to randomize the order of processed samples.

3.3. Picture each particle together with a ruler or size reference and store them individually in a pre-identified petri-dish (Fig. 1, step 7).

- A 96 well-plate is recommended for storing, at room temperature, individually all particles until further analyses.

3.4. Categorize the shape of each particle into one of five predefined categories (Fig. 6) adapted from Zobkov [31]:

- (a) line: thin elongated items with one dimension significantly greater than the other two;
- (b) film: sheets, with their thickness significantly lower than other two dimensions;
- (c) fragment: pieces of thick plastics of irregular shape with all three dimensions comparable;
- (d) pellet: pieces of regular and non-rounded shape or primary produced particles;
- (e) sphere: three dimensional items of spherical shape.

3.5. Using a picture software such as ImageJ [24], measure the two main orthogonal axes in the picture of each particle (i.e. maximal length and height).

- The particle width may be estimated considering each particle shape category [15].

3.6. Measure the mass (nearest 0.001 mg) of each particle individually and store them back in the same location within the well plate (Fig. 1, step 7).

3.7. Analyze each particle by ATR-FTIR spectroscopy (Fig. 1, step 8).

- Compare the spectra found for each particle with a spectrum library (open source program available, [3]) to assign a composition to each particle.

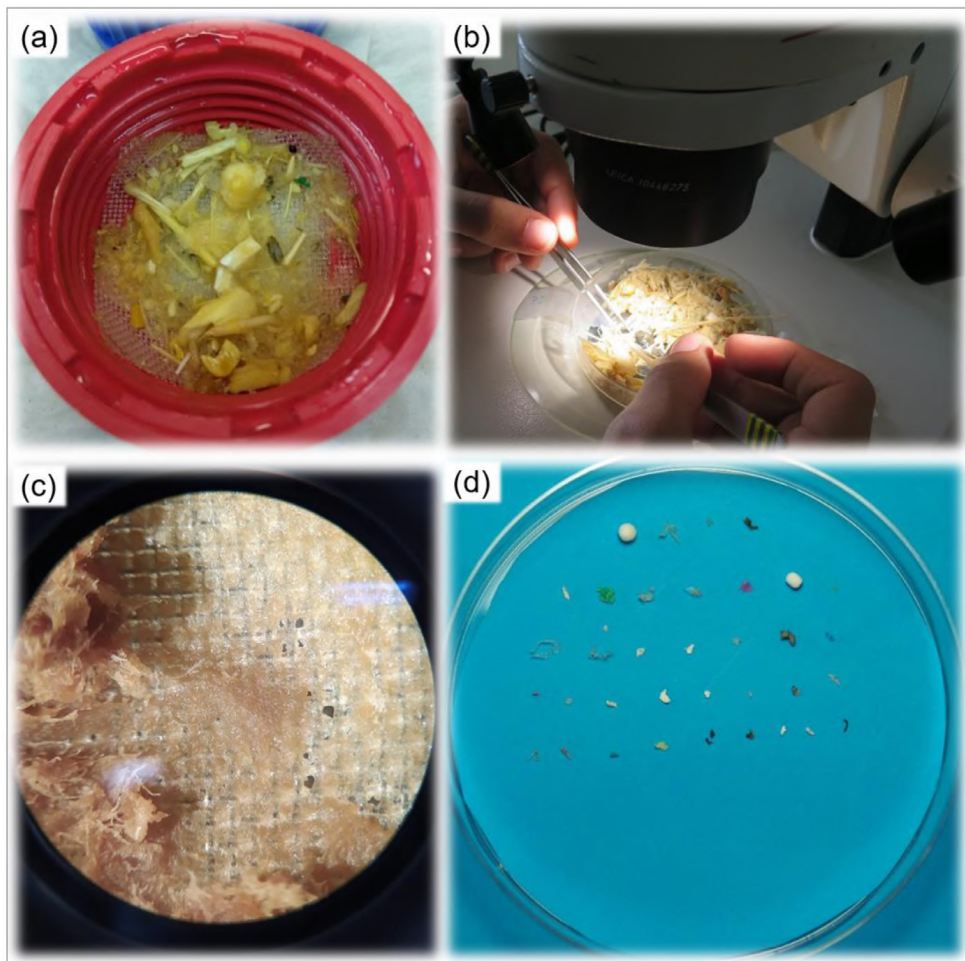


Fig. 5. Sample processing after organic matter digestion: (a) transfer to the open cap, (b) microscopic inspection and (c) magnified view and (d) recovered particles.

Customization and verification essay

In this study, the efficacy of organic matter digestion was quantified using samples collected from the same catchment ($n = 35$) and randomly submitted to three different digestion protocols: two single-reagent digestion (single step; chemical digestion: KOH 10% 60 °C, 24 h and wet peroxidation: H₂O₂ 30% room temperature - RT, 24 h) and one double-step digestion (KOH 10% 60 °C followed by H₂O₂ 30% RT, totalizing 24 h). We measured sample wet mass before and after digestions and calculated digestion efficiency as the percentage of wet mass loss. We found that the double digestion protocol ($n = 6$) allowed the elimination of, on average, 65.8% (± 9.59 SD) of mass, significantly more efficient than the single ones, with 43.5% (± 15.2 SD) digested for KOH ($n = 19$) and 39.4% (± 7.29 SD) for H₂O₂ protocol ($n = 9$) (Kruskal test, $\chi^2 = 10.845$, $p = 0.004$). No difference was found between the two single protocols (post-hoc comparison, $p = 0.212$) (Fig. 7). The reduction of the organic matter content together with the bleaching effect caused by the wet peroxidation step greatly facilitate the subsequent visual inspection of samples (Fig. 4).

Although it has been reported that virgin microplastic pellets are not affected by these single protocols [5,14], we quantified potential physical damages through mass changes and chemical modification (assessed by ATR-FTIR) arising from each step of the double digestion protocol.

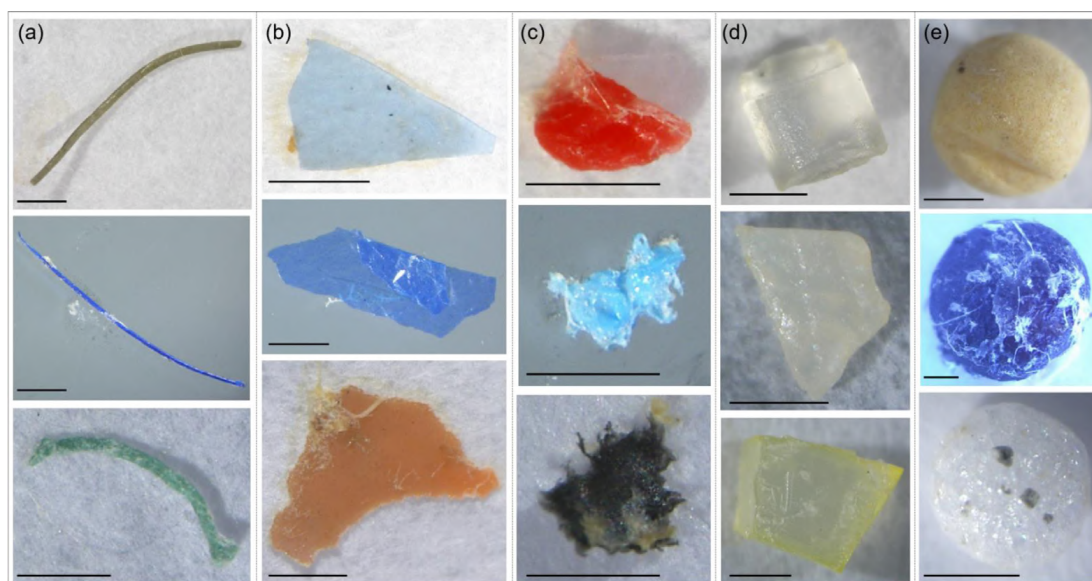


Fig. 6. Illustrations of the five categories of particle shape used: (a) line, (b) film, (c) fragment, (d) pellet and (e) sphere. The black line represents 1 mm.

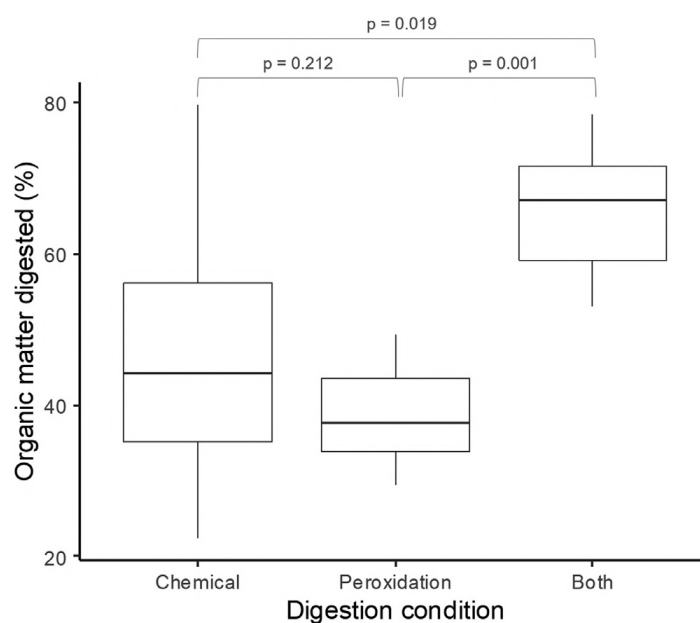


Fig. 7. Organic matter digested (%) by the digestion protocols.

Three to five virgin pellets (1–5 mm) from 12 different synthetic polymers were tested in triplicates: polyethylene (PE) with three different densities, polystyrene (PS), expanded polystyrene, polypropylene (PP), polyethylene terephthalate (PET – from two different manufacturers, Sigma and GoodFellow), polyamide 6 and 12, ethylene vinyl acetate, polycarbonate and polyetherimide (**Supplementary Table S1**). The polymers tested represented the main microplastic composition found in environmental samples [28]. No significant alteration that could lead to misidentification was observed in the infrared spectra of particles submitted to digestion protocol when compared with

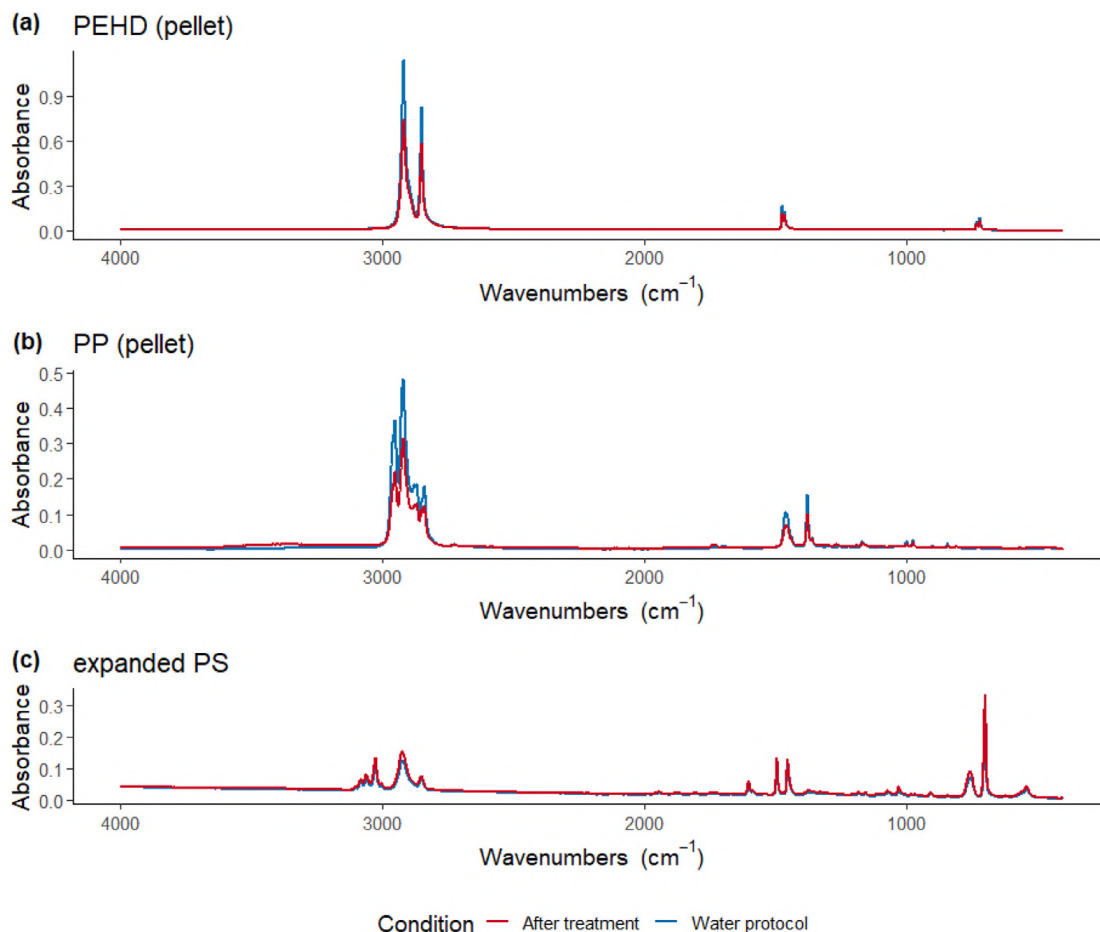


Fig. 8. Examples of ATR-FTIR spectra in control condition (blue line) and after digestion protocol (red line) for (a) PE high density (HD, $d = 0.952$ g/mL), (b) expanded PS, (c) PP.

two control conditions, the virgin particle and the treatment with distilled water (Fig. 8). Despite the FTIR spectra of PET after digestion protocol showed a distinct peak at wavenumber 3320 cm^{-1} (Fig. 9, only for pellets from Sigma Aldrich), indicating carboxylic acid and alcohol functional groups (R-OH stretching, $3000\text{--}3500\text{ cm}^{-1}$) [27], all particles were unequivocally identified (Fig. 8) [4]. Similarly, no significant mass changes occurred (Kruskal test, $\chi^2 = 1.495$, $p = 0.474$), excepted for the two PET batches from Sigma Aldrich (Supplementary Table S1), where a significant mass loss of 17.0% (± 5.18 SD) was observed (Kruskal test, $\chi^2 = 15.699$, $p = 0.003$). Tests with PET pellet from a different manufacture - GoodFellow (Supplementary Table S1) showed no significant mass variation following the treatment ($98.2\% \pm 1.81$ SD). We highlight that the diversity among plastic formulation might interfere in their chemical stability and further studies regarding potential impacts of this treatment on smaller and/or chemically-altered microplastics are needed.

Microscopic analysis of samples is a critical step for particles detection and we tested the gains obtained with a second and third inspections by different operators. We found that, on average, 23 min (± 10.4 SD) were needed for the first inspection of a sample and that it allowed to recover 91.1% of particles found in the sample. The second and third inspections lasted 5.6 min (± 1.9 SD) and 6.4 min (± 2.0 SD), respectively, and allowed to recover 6.7% and 2.3% of detected particles, respectively

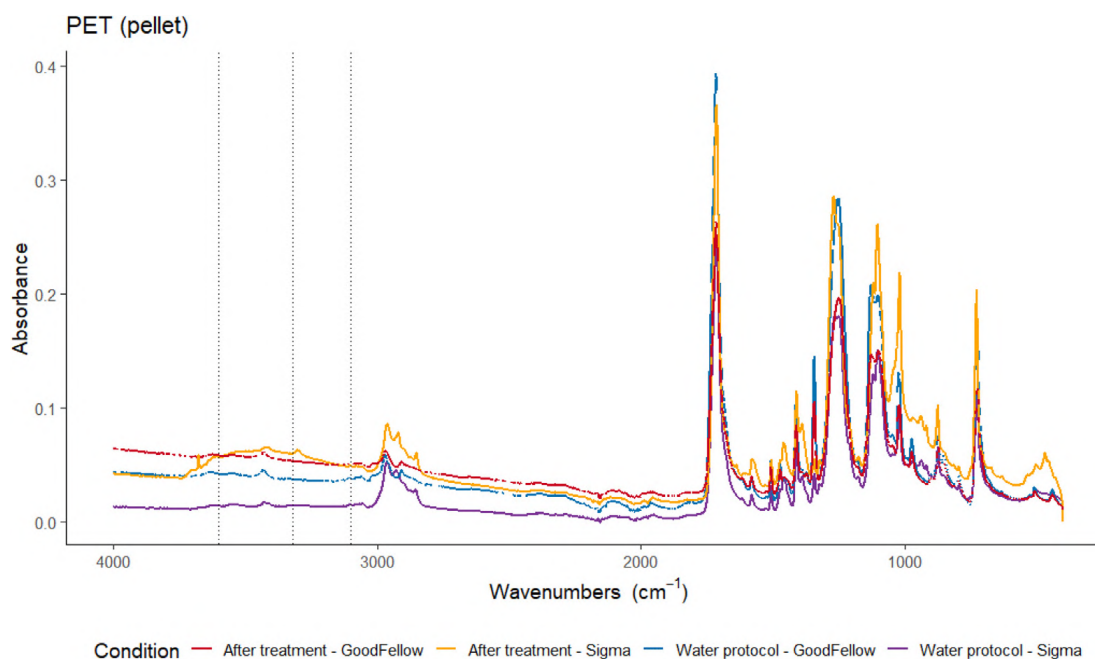


Fig. 9. Examples of ATR-FTIR spectra in control condition (blue and purple lines) and after digestion protocol (red and orange lines) for PET from GoodFellow and Sigma manufacturers. Dotted lines in PET spectra (d) represent wavenumbers 3600, 3320 and 3100 cm^{-1} .

Applying the protocol to microplastic pollution monitoring

The protocol was applied to a total of 204 samples collected in fourteen sites, in triplicates, in the Garonne catchment from February to October 2019. Important temporal (Fig. 10a) and spatial (Fig. 10b) variations of organic matter were observed, both in terms of quantity and composition. On average, sample wet mass was $45.1 \text{ g} \pm 76.4 \text{ SD}$. Samples containing a large amount of organic matter were divided (see step 2.4) to obtain a similar mass, resulting in a total of 290 samples in the end. Batches of 24 samples were processed, and the entire processing of a given batch lasted, on average, 38 h (Fig. 1). We found that the digestion protocol finally removed $56.3\% \pm 25.8 \text{ SD}$ of organic matter.

The first stereomicroscopic inspection lasted, on average, 13.2 min ($\pm 7.91 \text{ SD}$) and recovered 87% of particles. The second inspection, by a different operator, lasted 5.71 min ($\pm 2.79 \text{ SD}$), representing 5.8% of the total time spent with one sample and 13% of the recovered particles. There was no significant difference in particle color and shape between the two inspections. However, a significant difference was observed regarding particle composition, i.e. plastic or not plastic (χ^2 -test, $\chi^2 = 4.091$, $p = 0.043$), with higher percentage of non-plastic recovered in the second inspection (14.67% against 19.99%). No difference was found regarding microplastic composition (Fisher test, $p = 0.894$). Independently of particle composition, particles recovered during the second inspection were significantly smaller than those recovered during the first inspection (lmm, $\chi^2 = 5.288$, $p = 0.021$) (Fig. 11).

Based on these results, we recommend a double-step digestion and a double stereomicroscope inspection by a different operator in order to facilitate sample inspection and avoid bias in concentration and characteristics while quantifying microplastic pollution. This protocol was optimized for our objectives and the environmental matrix found in river surface water. Further adaptations comprising other matrices and/or microplastic smaller than $700 \mu\text{m}$ are in perspective. In the case of smaller microplastics and because of the instrumental size limitation of ATR-FTIR, other analytical techniques might be applied to guarantee the unequivocal identification of particle composition, such as micro-FTIR (FTIR combined with an optical microscope), Raman or

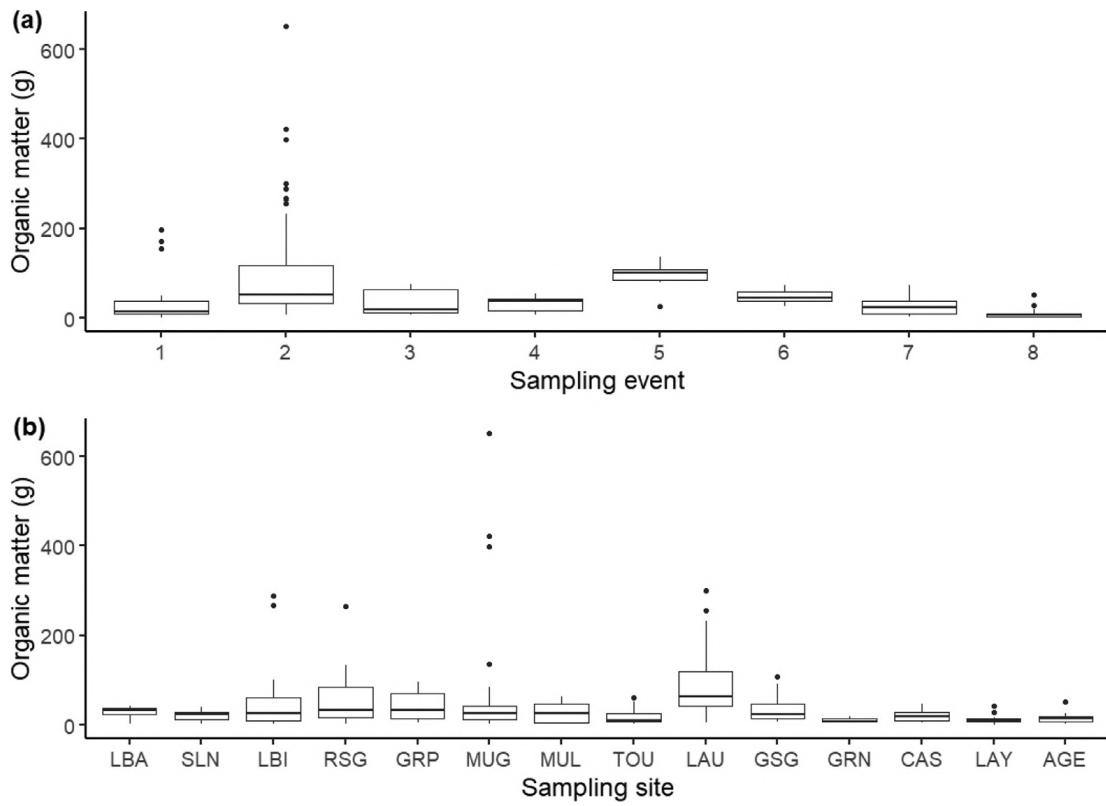


Fig. 10. Organic matter mass (g) collected in the samples across (a) sampling events and (b) sampling sites.

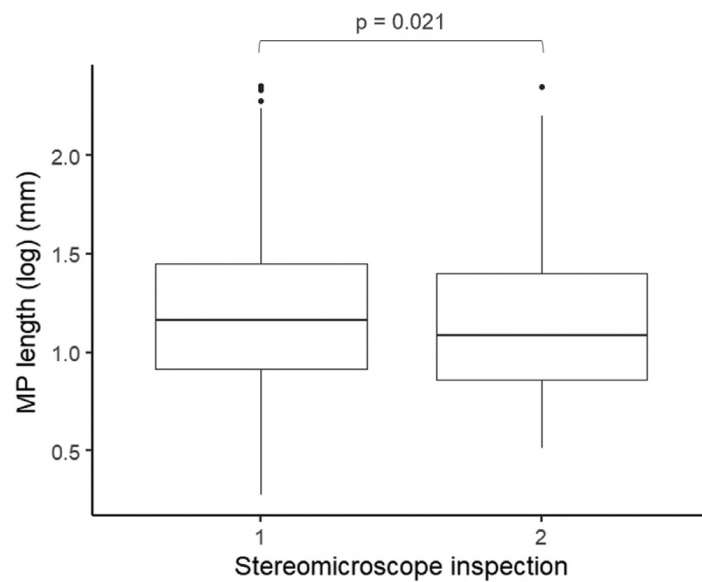


Fig. 11. Length (log-transformed) of microplastics recovered during the first and the second stereomicroscope inspections.

thermoanalytical methods, e.g. pyrolysis coupled to gas chromatography and mass spectrometry (pyr-GC-MS) [9,30]. In the case of smaller microplastics, it is also important to be careful with mesh size in sampling devices as they can lead to net clogging and underestimation of microplastic pollution [4]. Finally, to ensure the robustness of future microplastic pollution monitoring, we also identify a need to improve our knowledge related to the initial step of the process, i.e. field collection and to fully understand the role of small spatial (i.e. lateral and vertical variability) and temporal (e.g. diurnal changes) variations on our estimate of microplastic pollution.

Statistical analysis

In the verification essay, we used Kruskal-Wallis test to verify if the digestion of organic matter (percentage) differed between digestion protocols and pairwise comparisons were performed with Wilcoxon test. In the microplastic resistance essay, we used the same test to verify differences in microplastic mass due to digestion protocols. To compare the composition of particles, i.e. plastic or not, among the two stereomicroscope inspections, χ^2 tests were performed. Fisher Exact tests were applied to compare particle color (seven categories), particle shape (five categories) and composition (eleven categories) among the two stereomicroscope inspections due to limited amount of particles in some categories. The relationship between particle size (log-transformed) with stereomicroscope inspections were tested using a linear mixed-effect model (lmm) with particle color and polymer type as random factor. All statistical analyses were performed using R v.4.0.2 (R [23]). Significant levels of mixed effects model were obtained using the 'Anova' function in the car package [10]. Assumptions of linearity and homogeneity of variances on residuals from all models were checked visually.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.mex.2021.101396](https://doi.org/10.1016/j.mex.2021.101396).

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B - Article “Emerging use thermo-analytical method coupled with mass spectrometry for the quantification of micro and nano plastics in environmental samples”



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Emerging use thermo-analytical method coupled with mass spectrometry for the quantification of micro(nano)plastics in environmental samples



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ABSTRACT

Plastic pollution is of major environmental concern. The impact of this pollution on the ecosystem is not fully understood mainly due to the lack of analytical methods to detect and quantify micro(nano)plastic. The use of mass spectrometry was initially not considered as technique of choice but a steady flow of recent publications show its promises. This review is giving a thorough state of the art on the use of thermal degradation-mass spectroscopy for the detection of micro(nano)plastics and points at technological issues that remain to be resolved. It is discussed the possibility to perform minimum sample purification which is a substantial advantage compare to existing methods. Matrix interferences are discussed with regards to the indicator compounds selected. Mass spectrometry development, like the use of high resolution, opens up promising perspectives to improve the method performances. Mass spectrometry will be a major tool in this domain in the near future.

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1. Introduction

Research reporting the presence of plastic pollution throughout the planet is constantly progressing. Plastic is present even in the most remote regions of the planet, perhaps as a result of atmospheric deposition [1]. The risks plastic pollution poses are debated and largely unknown. To evaluate the potential impact of plastic pollution and to develop appropriate management and remediation strategies, we must have reliable and consistent analytical techniques for measuring plastics in environmental matrices. Microplastic analysis is difficult to perform, and standardized methods do not exist [2–5]. Recent developments in mass spectrometry have presented interesting perspectives [6,7].

In recent years, questions concerning very small plastic particles, nanoplastics (NPs, 1–1000 nm) [8,9], have emerged. The current analytical methods developed for microplastics have a particle size limitation and cannot reach the submicrometer range [10]. Nanoplastics were detected very recently for the first time in marine surface waters [11] and continental waters [12] using thermo-analytical method coupled with mass spectrometry. As new methods have to be developed to cover the nanometer range [10],

mass spectrometry appears to be a promising technique to meet these challenges [11,12].

Thermal degradation allows the conversion of macromolecules into low molecular weight chemicals, which are then chromatographed and ionized for mass spectrometry analysis. “Pyrolysis” means the decomposition under inert atmosphere; it is the same notion as “thermal decomposition”. Thermo-analytical methods use the thermal treatment process for analysis. Both pyrolysis and thermoextraction and desorption rely on the same chemical processes; both were coupled to mass spectrometry [13,14]. We use the term “thermo-analytical method” to refer to both approaches and we will focus on this approach in this review. We acknowledge the very recent use of matrix-assisted laser desorption/ionization (MALDI) coupled with mass spectrometry for microplastic analysis [15,16], but this technique is not discussed further here.

In this review article, we discuss the effectiveness and limitations of sample preparation for thermal degradation-mass spectrometry analysis. We critically discuss the choice of indicator compounds for each polymer and the evaluation of matrix interferences. The approaches developed for the external calibration or the use of an internal standard are presented. We also consider the steps for mitigating contamination and the efforts made for quality control. In our opinion, the main issue in microplastic monitoring is quantification; a large part of the discussion focuses

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Abbreviations			
ABS	acrylonitrile butadiene styrene	NP	Nanoplastic
ATR-FTIR	attenuated total reflection- Fourier transform infrared	PA6	polyamide 6
FTIR	Fourier transform infrared	PC	polycarbonate
IS	internal standard	PE	polyethylene polypropylene (PP)
LC-HRMS	liquid chromatography to high-resolution mass spectrometry	PET	polyethylene terephthalate
LMP	large microplastics (1–5 mm)	PMMA	poly(methyl methacrylate)
LOQ/LOD	limit of quantification/limit of detection	PS	polystyrene
MALDI	matrix-assisted laser desorption/ionization	PU	poly(urethane)
MP	microplastics (1–1000 μm)	PVC	polyvinyl chloride
NOM	natural organic matter	Py-GC-MS	pyrolysis-gas chromatography-mass spectrometry
		RSD	relative standard deviation
		TED-GC-MS	thermoextraction and desorption - gas chromatography-mass spectrometry
		TMAH	tetramethylammonium hydroxide

on these developments. The perspectives in terms of simplification of the sample treatment and development of high-throughput analysis are discussed.

2. Materials and methods

We examined studies reporting the use of mass spectrometry coupled with thermal degradation for the analysis of micro(nano) plastics in environmental samples. We selected articles written in the English language in peer-reviewed scientific literature from the 1970s to December 2019. We identified literature referring to “microplastic” and “thermal degradation” or “pyrolysis” and “mass spectrometry” using Science Direct. We also mined the journals Environmental Science and Technology, Analytical Chemistry, Environmental Pollution, Trends in Analytical Chemistry and Marine Pollution Bulletin because they regularly publish material relevant to this review article. The historical literature about structural polymer characterization by pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) [17], polymer quality/control in production [18] and forensic sciences [19] are not presented. These studies provide important information for understanding the thermal degradation of polymers but are out of the scope of this article. In total, we selected 24 publications that presented in Table 1, and discussed in detail in this review article.

3. Analysis of micro(nano)plastics

3.1. Mass spectrometry vs spectroscopy

The characterization of microplastics by thermal degradation-mass spectrometry is an emerging approach. Fourier transform infrared (FTIR) spectroscopy is the most common technique used so far for microplastic analysis. This technique is followed in order of importance by Raman spectroscopy [10]. The use of mass spectrometry instead of spectroscopy implies a very distinct approach to sample preparation and provides distinct information. Large microplastics, in the range of millimeters (LMP, 1–5 mm), are manually sorted from environmental samples. LMPs are typically analyzed in succession by attenuated total reflection- FTIR (ATR-FTIR). Microplastics in the micrometer range (microplastics, MPs, 1–1000 μm) are typically characterized by microspectroscopy. Microspectroscopy requires several steps of sample purification to isolate MPs. After purification, the sample is deposited on an appropriate filter for mapping by microspectroscopy. The particles are characterized in succession. The plastic particles have to be extracted from the samples with a high degree of efficacy because i) as the particles are analyzed in succession, if numerous natural

particles are present in the sample, the time of analysis and volume of data treatment are increased; ii) the amount of sample deposited on the filter has to be limited to avoid particle overlap, which prevents particle detection and analysis. Microspectroscopy, in addition to structural identification of the polymer, provides information about the total number of particles (and two dimensions of the particle, length and width).

In comparison, thermal degradation-mass spectrometry is more adapted to MP analysis than LMP. Thermal degradation-mass spectrometry allows the simultaneous analysis of MP in mixture for the polymers targeted. In the case of a quantification development, the analysis provides a mass concentration. The sample treatment does not necessarily require intensive purification and some authors have even opted for no purification at all. Thermal degradation-mass spectrometry has no size limitation and it is compatible with NP analysis [11,12]. Thermal mass spectrometry main strengths could be summarized as it allows: 1) the analysis of microplastic with minimum sample purification 2) the determination of several polymers (typically 7 to 8) in one run 3) to reach levels of detection in the nanogram range 4) nanoplastics analysis.

3.2. Pyrolysis vs thermoextraction and desorption

As specified earlier, pyrolysis consists in the decomposition under inert atmosphere (and is synonymous to thermal decomposition). In contrast to this, the thermo-oxidative treatment occurs under oxygen containing atmosphere. Because only the decomposition under inert atmosphere leads to reproducible results, thermo-analytical methods are commonly done in inert atmosphere. Upon pyrolysis, the chemical decomposition occurring at high temperature breaks down large molecules into smaller fragments. The fragments of higher abundance are generally monomers, dimers or trimers generated from the decomposition of the polymer backbone. In analytical pyrolysis, the fragments generated are readily separated by gas chromatography and identified by a mass detector (Py-GC-MS). The thermoextraction and desorption coupled with gas chromatography-mass spectrometry method (TED-GC-MS) relies on quite similar processes. The thermal decomposition, another term for pyrolysis, is carried out in a thermal balance. This provides additional information: mass losses upon heating. Usually, the crucible for TED-GC-MS accommodates a higher volume than that for Py-GC-MS. This allows the introduction of up to a few tens of milligrams, while Py-GC-MS is generally limited to a few milligrams. In the next step of TED-GC-MS, the products are collected onto an adsorber. When thermal extraction is complete, the adsorber is transferred for thermal desorption and gas chromatography-mass detection. All the steps can be

Table 1

Microplastic analysis by thermo-analytical method coupled with mass spectrometry ordered chronologically.

First author, year, Publication	Type of sample	Sample preparation	Sample introduction	Analytical method	Quantification
Fabbri 1998 [30]	Sediment	No treatment	Mixture	Py-GC-MS	Yes
Fabbri 2000 [31]	Suspended particulate matter	No treatment	Mixture	Py-GC-MS	Yes
Fabbri 2001 [32]	Sediment	Soxhlet extraction	Mixture	Py-GC-MS	No
Unice 2012 [21]	Soil samples and air samples	No treatment	Mixture	Py-GC-MS	Yes
Fries 2013 [33]	Sediment	Density separation	Isolated particles	Py-GC-MS	No
Dekiff 2014 [34]	Sediment	Density separation	Isolated particles	Py-GC-MS	No
Duemichen 2015 [20]	Biological tissues, suspended particulate matter and soils	No treatment, homogenization by cryomilling	Mixture	TED-GC-MS	Yes
Fischer 2017 [35]	Artificial soil	Enzymatic and chemical digestion	Mixture	Py-GC-MS	Yes
Duemichen 2017 [13]	Ferment and suspended particulate matter	No extraction, homogenization by cryomilling	Mixture	TED-GC-MS	Yes
Ravit 2017 [36]	Suspended particulate matter	Chemical digestion	Isolated particles	Py-GC-MS	No
ter Halle 2017 [11]	Suspended particulate matter	Chemical digestion and Ultrafiltration ^a	Mixture	Py-GC-MS	No
Eisentraut 2018 [22]	Street runoff	No treatment	Mixture	TED-GC-MS	Yes
Hermabessiere 2018 [24]	Biological tissues and Suspended particulate matter	No treatment	Isolated particles	Py-GC-MS	No
Peters 2018 [37]	Biological tissues	No treatment	Isolated particles	Py-GC-MS	No
David 2018 [38]	Spike soils	No treatment	Mixture	TGA-MS	Yes
Kappler 2018 [39]	Suspended particulate matter	Density separation	Isolated particles	Py-GC-MS	No
Mintenig 2018 [12]	Drinking water and suspended particulate matter	Ultrafiltration ^a	Mixture	Py-GC-MS	No
Hendrickson 2018 [40]	Sediment	Chemical digestion	Mixture		
	Suspended particulate matter	Chemical digestion and density separation	Isolated particles	Py-GC-MS	No
Duemichen [20]	Suspended particulate matter	No treatment	Mixture	TED-GC-MS	Yes
Gomiero [27]	Sediment	Chemical, enzymatic digestion + density separation	Both	Py-GC-MS	Yes
Dierkes [28]	Sediment, suspended particulate matter and sewage sludge	Pressurized liquid extraction	Mixture	Py-GC-MS	Yes
Doyen 2019 [41]	Sediment	Density separation	Isolated particles	Py-GC-MS	No
Fischer [42]	Sea salt and suspended particulate matter	Density separation	Mixture	Py-GC-MS	Yes
Zhou 2019 [43]	Suspended particulate matter	Cloud point extraction ^a	Mixture	Py-GC-MS	Yes

^a Studies dedicated to nanoplastics.

automated [20]. With TED-GC-MS, the thermal decomposition profile is slightly different from that of Py-GC-MS. The larger fragments are more represented with pyrolysis. This is because the thermal desorption programs typically occur at maximum temperatures of 200°C, and the larger compounds are not desorbed and transferred into the GC-MS. TED-GC-MS presents the advantage of introducing fewer substances in the spectrometer, and there is supposedly less fouling. Bypassing the larger molecules with TED-GC-MS does not present a problem in terms of the detection of polymers because most indicator compounds are ultimately chosen among rather low molecular weight molecules. Both techniques allow pyrolysis to proceed under controlled conditions and to generate highly reproducible and consistent compositions of degradation products. It was demonstrated with both techniques that the quantification of microplastics is possible.

3.3. Polymer targeted

Thermo-analytical method coupled with mass spectrometry allows the simultaneous determination of various polymers within a single GC-MS run. Typically, the most common plastics that are

included in analytical development are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC), poly(methyl methacrylate) (PMMA), polycarbonate (PC), polyamide 6 (PA6) and poly(urethane) (PU). Some studies were aimed at tire particle analysis [21]. One study combines tire particles analysis with common thermoplastic analysis [22]. Thermo-analytical method coupled with mass spectrometry is particularly interesting for tire particle detection because spectroscopic methods are not suitable for these kinds of particles [23].

3.4. Type of matrix

Thermo-analytical method coupled with mass spectrometry were developed for all kinds of matrices, such as suspended particulate matter, sediment, fish tissues and airborne particles (see Fig. 2). Table 1 lists the type of matrices studied with the corresponding references. Approximately 50% of the references were applied to suspended particulate matter (Fig. 1). As the amount of natural organic matter (NOM) is higher in continental waters, the matrix interference should be more important. Generally, this aspect is not discussed in the development of the analytical

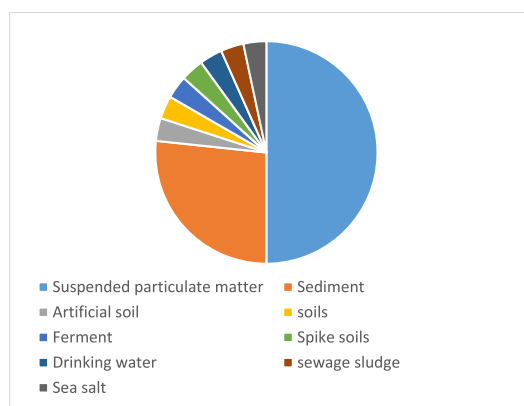


Fig. 1. Repartition of the type of matrices for micro(nano)plastic analysis with thermo-analytical method coupled with mass spectrometry in studies reported in this review article.

methods, and we do not specify in Table 1 the origin of the sample (whether from marine or continental waters). Ter Halle et al. indicated that the volume of seawater sampled in the open ocean was limited (1 L), which prevented the detection of the signature of the oceanic NOM in the extracted colloidal fraction [11].

3.5. Plastic particle size

Two references (marked with a star in Table 1) focus on nanoplastic analysis, while the other studies analyze MP and/or LMP. The LMPs are analyzed individually after manual extraction from the sample (Fig. 3). It is recommended to introduce only a small fragment of LMP in the instrument to prevent saturation of the signal [24]. Thermo-analytical method coupled with mass spectrometry perspectives concern mostly MP and NP analyses in mixtures. For MP analysis, there is also a size limit recommendation of 500 μm [25]. Above 500 μm , there are problems with introducing the particles in the crucibles. Because of the heterogeneity of the sample and the obligation to subsample a few milligrams for analysis, it is recommended to cryomill the sample prior to analysis [13,26].

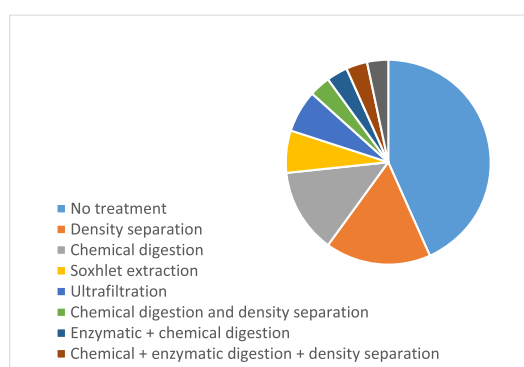


Fig. 2. Repartition of the sample treatment procedures for micro(nano)plastic analysis with thermo-analytical method coupled with mass spectrometry in studies reported in this review article.

Thermo-analytical method coupled with mass spectrometry does not provide the size distribution of the plastic particles detected, unlike microspectroscopy. To obtain this information, some authors have performed sieving [27], wet sieving [28] and cascade filtration [29] to fractionate the sample and proceeded to analyze the fractions.

3.6. Sample preparation

The focus here is on MP and NP analysis sample preparation. Some protocols consist of intensive sample purification based on a multistep procedure combining enzymatic and oxidizing treatments followed by density separation [27]. Other approaches were based on multistep purification [35,42]. Interestingly, the use of pressurized liquid extraction for PE, PS and PP determination was proposed [28]. This is an efficient extraction method based on the differential solubility of polymers and NOM in common solvents. Fabbri and colleagues applied a similar approach using Soxhlet extraction [31,32]. Several authors opted for no purification even with complex matrices such as soil and sediment [20–22,26,29,38]. The interference of NOM is discussed in detail in the section Indicator compound selection. In addition to removing matrix interference, sample purification is a way to concentrate plastic particles. For example, for sediment, soil or suspended particulate matter, inorganic particles could represent a very important fraction of the sample. The removal of the inorganic fraction typically allows concentration factors of 10. This aspect was not often discussed in the literature data gathered here.

Within the last years an increasing amount of methods were proposed using a chemical extraction process and then a subsequent analysis of the extract (GC/MS, but also FTIR or NMR ...). Here we are reporting a totally different concept that could be achieved in a single run. Indeed, during a multi-step temperature programs pyrolysis allow to eliminate part of the NOM during sample analysis. This is possible because some natural organic molecules or natural macromolecules decompose at lower temperatures than polymers. For example, for wood composite analysis, a multistep program was proposed to eliminate the wood. Approximately 50% of the weight of the sample decomposed between 250 and 380°C, while PP decomposed later in the temperature cycle (380–492°C) [20]. This quite easy-to-handle purification step has not been considered often thus far.

4. Quantification of micro(nano)plastics

The most expected breakthrough is in quantification development. Half of the references gathered developed this aspect (Fig. 3). Owing to the importance of this issue, it is discussed in detail (Table 2).

4.1. Indicator compound selection

Generally, once the polymers that need to be analyzed are selected, the first step consists of obtaining an overall picture of the compounds produced during the thermal decomposition of each polymer. Sometimes, a polymer can produce a very large number of volatile substances upon thermal decomposition, with many isomers or substances that are quite chemically similar. The signal is then a complex chromatogram with overlapping peaks. The first step consists of examining the pyrolytic signature of the polymer to select outstanding decomposition products, if possible. Selecting the main decomposition products offers the perspective of better sensitivity. However, some of these main decomposition products are not specific, i.e., they can be formed by a large variety of natural substances present in the sample or are common to many

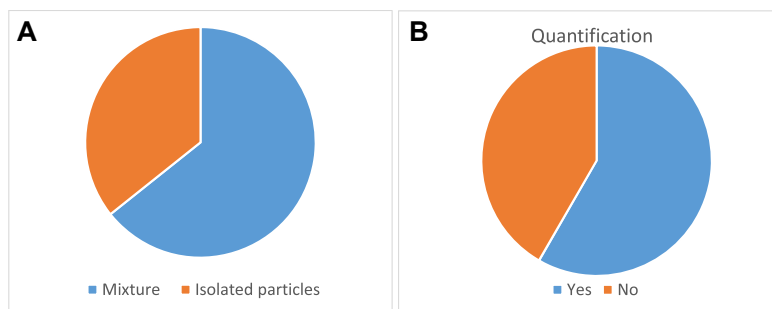


Fig. 3. Repartition A) of analyses of microplastic mixture and isolated microplastic and B) development of quantification methods for micro(nano)plastic analysis with thermo-analytical method coupled with mass spectrometry in studies reported in this review article.

polymers. For each polymer that needs to be monitored, the choice of the indicator compound(s) is a subtle balance between a high sensitivity and high specificity. This aspect is the core issue of mass spectrometry development for microplastic analysis in environmental samples. As an illustration, we discuss in detail these choices for some important polymers. We show that very distinct options were taken by scientists. For some polymers, the question is still open for future development.

The indicator compound selection and sample purification development must be considered together. If the analytical method relies on the choice of unspecific indicators, then the sample purification has to remove any trace of interfering constituents. An analytical method based on the selection of specific indicator compounds is compatible with less extended sample purification and even offers the perspective of no purification.

4.1.1. Polyethylene

The thermal decomposition of PE is well-described [44,45]. PE pyrograms show a suite of equally spaced multiplets. The multiplets [45] are often referred to as triplets. The three most intense peaks correspond to the response of hydrocarbons with an equal number of carbon atoms. Under classic chromatographic conditions, the pyrograms include hydrocarbons containing 7 to 35 carbon atoms. The triplet is composed, usually in the following order, of a terminal n-alkadiene followed by a terminal n-alkene and an n-alkane. The second peak, the alkene, shows the highest response. The fragment $m/z = 55$ was often chosen to monitor PE pyrolysis because it is common to aliphatic compounds and is one of the most intense signals [26].

Biogenic materials usually contain fats or waxes that release n-alkanes and n-alkenes upon pyrolysis [26,35,46]. Ultimately, the NOM pyrolytic fingerprint in terms of the formation of alkane could appear quite similar to that of PE, showing a Gaussian distribution in the range of C_9 to C_{21} [47,48]. This triplet is very specific to PE. It is due to the presence of dialkenes that confer specificity. Dialkenes can only be formed upon the pyrolysis of very long, polymeric, aliphatic chains, i.e., PE [26]. Consequently, some authors recommend selecting dialkenes as indicator compounds [13,22,26,29]. However, dialkenes are the least intense peaks upon PE pyrolysis. Therefore, to reach the lower limit of quantification or limit of detection (LOQ/LOD), some other authors selected alkanes and/or alkenes as indicators (see Fig. 4). This was only made possible by removing the interfering NOM. For example, Fisher et al. estimated that below 50 μg , the detection of dialkenes was not possible with their method [35]. They opted for alkanes and alkenes as indicator compounds. They purified copepods by enzymatic digestion and observed no matrix interference if the indicators alkane and alkene

were chosen in the range of C_{16} to C_{26} . Dierkes et al. developed an extraction method based on pressurized liquid extraction. They estimated that there was less interference with 1,14-penta-deca-diene than with 1-pentadecene when working with fish tissues. The other matrices (wood, leaf ...) presented the same level of interference with both indicator compounds [28]. Gomiero et al. analyzed sediments. They developed an intensive separation protocol (based on enzymatic and oxidizing treatment followed by density separation) and selected 1-tetradecene [27]. In conclusion, the selection of an alkane or alkene implies the development of intensive purification methods to prevent interference. The selection of indicator compounds among n-alkadienes offers the perspective of less matrix interference, but the signal of this compound is significantly lower.

4.1.2. Polystyrene

Polystyrene pyrolysis yields mostly styrene. Other benzene derivatives are formed in lesser proportions (Fig. 5) [14]. Styrene oligomers are also formed. The dimers and trimers can easily be detected with classical gas chromatographic conditions. Styrene is also a very common decomposition product of NOM pyrolysis [47]. Proteins, especially those containing the amino acid phenylalanine, are precursors of styrene upon pyrolysis [35]. The interference of PS pyrolysis with NOM was discussed by many of the authors referenced here (Fig. 5). Fabbri et al. selected styrene as an indicator compound even though they did not proceed with sample treatment to remove the NOM from the sediment analyzed [30]. They estimated that for noncontaminated sediment, the styrene/toluene ratio was 0.1–0.4. In the contaminated sediment, the ratio reached 1.4 to 1.7. They attributed this difference to the presence of PS. Assuming that all the styrene was formed from PS, they estimated that the PS concentration reached 3–4 mg/g, corresponding to 10–20% of the organic carbon content in the contaminated sediments. This is the only study we are reporting that considered ratios to monitor the interference of indicator compounds with the matrix. Otherwise, the authors that selected styrene as an indicator compound developed intensive purification of the sample. For example, Dierkes et al. working with pressurized liquid extraction, could eliminate all protein rich materials that generated styrene. They observed interference with wood and assumed that wood was not present in the samples they were analyzing [28]. Styrene can also be formed from other synthetic polymers such as acrylonitrile butadiene styrene (ABS) or styrene-butadiene rubber (SBR) during pyrolysis. Therefore, styrene is related to the presence of all styrene-based polymers [28]. It appears that selecting styrene as an indicator compound raises too many questions. Styrene oligomers are very specific to PS; they should be preferred. Styrene oligomers

Table 2
References for micro(nano)plastic quantification by thermo-analytical method coupled with mass spectrometry.

First author/ Reference	MS method (amu)	Polymer targeted	Indicator compounds	Selected indicator ion (m/z)	Thermo chemolytic trans- methylation ^a	Introduction mode for calibration	Internal standard ^b	LOD/LOQ ^c
Fabbri [30]	Scan mode (45–350)	PS	Styrene		No	Addition of 5 µl PS solution in dichloromethane 1–15 µg	No	Not specified
			Alpha-methyl styrene				No	Not specified
Fabbri [31]	SIM	PS PVC	Styrene Benzene	104 78	No	Weighing polymer to unpolluted sediment sample	No	LOD 0.01 mg/g LOD 1 mg/g
Unice [21]	Scan mode (35–500)	Natural rubber	Dipentene isoprene	68 68	No	Weighing cryomilled standard polymer to artificial soil	D-Polyisoprene D-Polystyrene D-Polybutadiene	LOD 14 µg/g in sediment and 260 µg/g for airborne particulate collected on quartz filter
		Styrene butadiene rubber	Styrene	103				
		Butadiene rubber	Butadiene	54				
		Butadiene rubber	Vinyl-cyclohexene	54				
		Butadiene rubber	Vinyl-cyclohexene	54				
Dumichen [26]	Scan mode (35–350)	PE	1,13-Tetradecadiene		No	Weighing LDPE particles to soils	No	LOD 8 µg/LOQ 40 µg
Fischer [35]	Scan mode 50-650	PE	Eicosane	85	Unaffected	Weighing individually or in mixture of polymer standards to Al2O3	No	LOQ < 4 µg <4 µg
		PP	2,4,6,8-Tetramethyl-1-undecene ^f	210	Unaffected			LOQ <0.6 µg
		PS	Styrene	104	Unaffected			LOQ <1 µg
			Styrene dimer ^g	91	Unaffected			LOQ <6 µg
			Styrene trimer ^h	91	Unaffected			LOQ >1.5 µg
		PVC	Benzene	78	Unaffected			LOQ <2.7 µg
		PET	Bimethyl terephthalate	163	Yes			LOQ <5 µg
		PC	2,2-Bis(4'-methoxy-phenyl)propane	241	Yes			LOQ <0.1
		PMMA	Methyl methacrylate	100	Yes			LOQ <0.4
		PA6	ε-Caprolactam	113	Partial			LOQ >22
			N-Methyl ε-caprolactam	127	Yes			LOQ <10
Eisentraut [13]	Scan mode 35-350	PP	2,4,6-Trimethylnon-1-ene		No	Not specified	D-Polystyrene	LOD 0.4 µg
		PE	1,12-Tridecadiene					LOD 1.6 µg
		PS	Styrene dimer ^g					LOD 0.2 µg
		Styrene butadiene rubber	Cyclohexylbenzene	104				LOD 0.23 µg
			Phenyl[440] bicyclodecene	104				
David [38]	SIM	PET	Benzoic acid	105	No	Weighing cryomilled PET polymer to a standard loamy sand	Cysteine	LOD 700 µg/g LOQ 1700 µg/g
			Vinyl benzene	105				LOD 700 µg/g LOQ 1700 µg/g
			Biphenyl	154				LOD 600 µg/g LOQ 6500 µg/g
Dumichen [20]	Scan mode 35-550	PE	1,12-Tridecadiene	55	No	Weighing the polymers with suspended particulate matter	Use of an internal standard, structure not specified	LOQ 11.4 µg LOQ 9.55 µg
			1,13-Tetradecadiene	55				LOQ 12.2 µg
			1,14-Pentadecadiene	55				LOQ 14.8 µg
			1,15-Hexadecadiene	55				LOQ 14.8 µg
		PS	Styrene	104				LOQ 0.13 µg
			Styrene dimer ^g	91				LOQ 0.19 µg
			Styrene trimer	91				LOQ 0.23 µg
		PP	2,4-Dimethyl-hept-1-ene	70				LOQ 0.4 µg
			2,4,6-Trimethyl-1-nonene ^e	69				LOQ 1.3–1.6 µg
			2,4,6,8-Tetramethyl-10-undecene ^f	69				LOQ 0.88–2.53 µg

Table 2 (continued)

First author/ Reference	MS method (amu)	Polymer targeted	Indicator compounds	Selected indicator ion (m/z)	Thermo chemolytic trans- methylation ^a	Introduction mode for calibration	Internal standard ^b	LOD/LOQ ^c
Gomiero [27]	Scan mode 35-550	PP	2,4,6,8-Tetramethyl-1-undecene	69	Unaffected	Individually weighing and analyzing between 0.1 and 360 mg of polymer standards	No	LOD 0.5 µg
		PS	Styrene dimer ^e	208	Unaffected			LOD 0.1 µg
		PVC	1-Methylnaphthalene	142	Unaffected			LOD 0.5 µg
		PET	Dimethyl terephthalate	163	Yes			LOD 0.5 µg
		PC	p-Methoxy-tert-butylbenzene	149	Yes			LOD 0.5 µg
		PMMA	Methyl methacrylate	100	Yes			LOD 0.5 µg
		PA-66	Hexene	84	Unaffected			LOD 0.5 µg
Dierkes [28]	Scan mode (50–650)	PE	1-Tetradecene	85	Unaffected	Weighing 20 mg of polymer and diluting it with calcined sand, homogenizing in mortar, and diluting the mixture with calcined sand	D-Polystyrene	LOD 0.7 µg
		PP	2,4-Dimethyl-hept-1-ene	126	No			LOQ 7 µg/g
		PE	1,14-Pentadecadiene	81				LOQ 16 µg/g
		PS	1-Pentadecane	97				LOQ 7 µg/g
			Styrene	104			LOQ 8 µg/g	
Fischer [42]	Scan mode 35-650	PE	1,19-Eicosadiene	82	Unaffected	Weighing between 0.5 and 40 µg of polymer solution in DCMP from 0.01 µg to 1 µg	9- Dodecyl-1,2,3,4,5,6,7,8-octahydroanthracene, anthracene-d10, androstane and cholic acid (each 0.02 µg/µL in n-hexane)	LOQ<0.5 µg
		PP	2,4-Dimethylhept-1-ene	70	Unaffected			LOQ< 0.5 µg
		PS	Styrene trimer ^h	91	Unaffected			LOQ 0.01 µg
		PVC	Benzene	78	Unaffected			LOQ<0.03
		PET	Dimethyl terephthalate	163	Yes			LOQ< 0.5 µg
		PC	2,2-Bis(4'-methoxy-phenyl) propane	241	Yes			LOQ< 0.5 µg
		PMMA	Methyl methacrylate	100	Yes			LOQ< 0.5 µg
		PA-66	e-Caprolactam	113	Yes			LOQ< 0.5 µg
			1257 113 113 N-methyl caprolactam	127	Partially			LOQ< 0.5 µg
			4,4'-Methylenebis(N,N-dimethylaniline)	254	Yes			LOQ< 0.5 µg
Zhou [43]	Full scan (10–550)	PS	Styrene trimer ^h	91	No	Serial dilution of nanosphere dispersion		LOD 1.1 µg/L
	PMMA	Methyl methacrylate			0.6 µg/L			
Funk [29]	Full scan (40–450)	PS	Styrene trimer ^h	91	No	d ₈ -Styrene	Dispersion of nanosphere in ethanol	LOQ 0.03 µg
		PE	1,14-Pentadecadiene	55		Dispersion of microspheres in ethanol		LOQ 0.3 µg

^a Use of tetramethyl ammonium hydroxide.

^b d-deuterated.

^c If LOQ is inferior to a value, the limitation of the calibration is the performance of the balance.

^d Specifically (methyl dimethyl diisocyanate) polyurethane.

^e Two diastereoisomers.

^f Three diastereoisomers.

^g Styrene dimer for 3-butene-1,3-diyldibenzene and.

^h Styrene trimer for 5-hexene-1,3,5-triyltribenzene.

are formed to a much lesser extent than styrene [35]. An optimization of the temperature of pyrolysis towards lower temperature favors their formation (600°C for example) [49].

4.1.3. Polypropylene

PP branched structure leads to a complex thermal decomposition pattern. PP decomposes into six compounds in relatively high abundance: 2,4-dimethylhept-1-ene, followed by two

diastereoisomers of 2,4,6-trimethylnon-1-ene and 4 diastereoisomers of 2,4,6,8-tetramethylundec-1-ene (which can be separated into 3 peaks, Fig. 6). There is a consensus among the already published studies that there is no interference with the matrix for these indicator compounds in tests with spiked samples [13,22,27,35]. However, these aspects are not detailed in any study, and the specificity of these indicator compounds should be examined in the future.

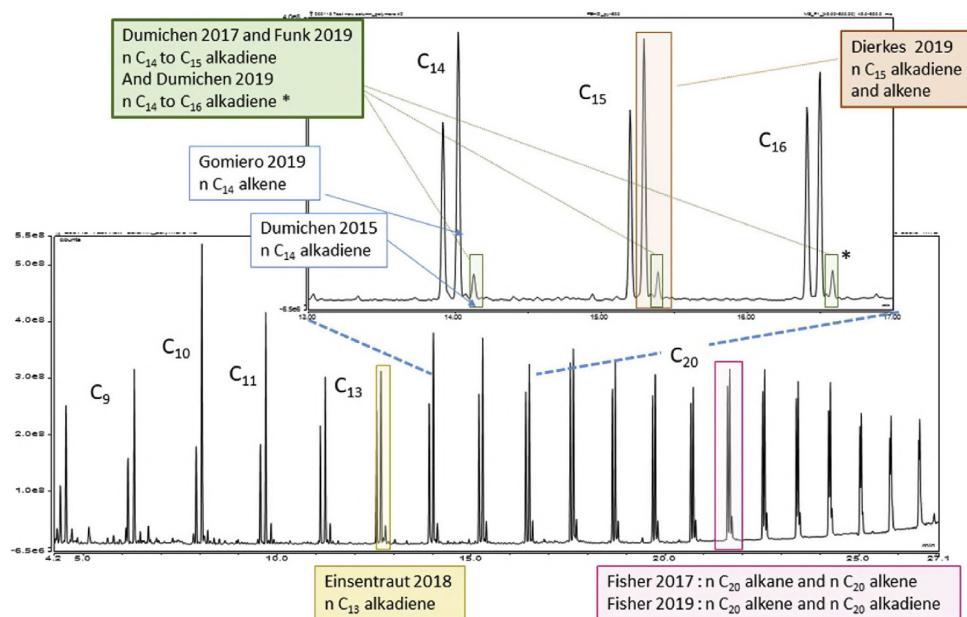


Fig. 4. Chromatogram of polyethylene analyzed by Py-GC-MS in full scan. The indicator compounds selected for polyethylene quantification are presented with the references. Hydrocarbons containing between 13 and 16 carbon atoms were selected, presenting an instauration or not. Among the most recent publications (2018–2019), a consensus was reached for the selection of alkadienes.

4.1.4. Polyethylene terephthalate

All poly(alkylene terephthalate)s produce terephthalate derivatives upon thermal decomposition. Polyethylene terephthalate is by far the most produced terephthalic acid-based polyester. Authors generally use the term PET but even if it is not specified, this term includes all poly(alkylene terephthalate)s. Upon pyrolysis, PET emits multiple signals of benzoate and terephthalate derivatives and oligomers. These decomposition products are polar, and the gas chromatography column used is usually nonpolar. The compounds

produced show broad peaks with tailing [13]. With thermo-analytical method in combination with thermochemolysis using tetramethylammonium hydroxide (TMAH) as a derivatization agent, dimethyl terephthalate is formed in important proportions. The use of TMAH significantly enhances the detection sensitivity for PET and has been chosen by many authors [13,27,35]. The use of TMAH does not interfere with the pyrolysis of other polymers, such as PE, PP and PS [35].

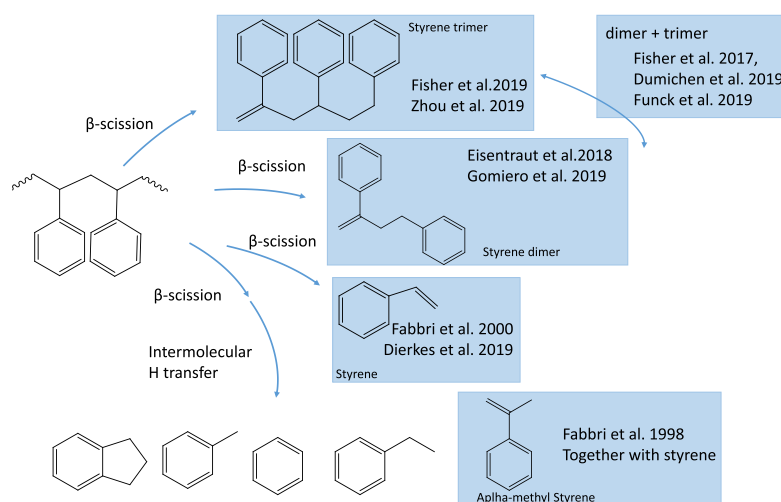


Fig. 5. Major pathways involved in polystyrene pyrolysis. Styrene is produced through the depolymerization of macroradicals formed by random thermal cleavage of the main chain, whereas styrene oligomers are formed by intermolecular reactions [50]. The molecules selected as indicator compounds for microplastic quantification are highlighted.

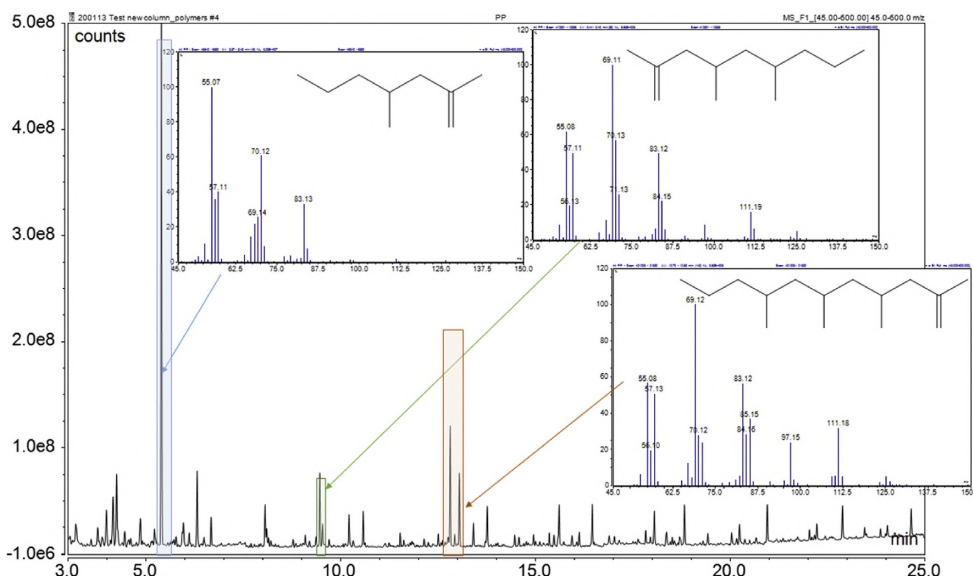


Fig. 6. Chromatogram of polypropylene pyrolysis analyzed by gas chromatography-mass spectrometry in full scan. The indicator compounds selected for polypropylene quantification were chosen among the three molecules represented here. There was no noticeable matrix interference reported in the literature data.

4.1.5. Polyvinylchloride

PVC pyrolysis does not provide any specific decomposition compounds. The small aromatic molecules produced upon PVC pyrolysis present matrix interference even after sample purification [35]. PVC analysis in environmental samples by thermo-analytical method coupled with mass spectrometry is only possible with intensive sample purification. Gomiero et al. selected 1-methylnaphthalene and did not observe matrix interference after using a multistep procedure based on combined enzymatic and oxidizing treatments followed by a density separation phase [27].

4.1.6. Polycarbonate and poly(methacrylate) polymers

The use of TMAH also facilitates the analysis of PC by selecting methylated bisphenol A. PMMA can also be easily detected by thermo-analytical method coupled with mass spectrometry. This applies for all poly(methacrylate)s polymers. After thermochemolysis with TMAH, they all form methyl methacrylate and are usually all categorized under the term PMMA disregarding their former molecular weight or substituent [27,35,42,43]. PMMA and PC pyrolysis with thermochemolysis produces very specific molecules with no interference from NOM.

4.2. Matrix interference

We discussed the case where the indicator compounds are also formed upon NOM pyrolysis in the previous paragraph. Another interference from the matrix is the catalytic effect of some ions during pyrolysis. Metal ions impact the thermal decomposition mechanism of polymers, although their effects are not yet completely understood [51]. PS pyrolysis was reported to interfere with clay minerals [30]. The authors observed important changes in the product distribution. Styrene was the predominant pyrolysis product in the presence of calcite and quartz (90% of the total area), but its proportion dropped in the presence of clay to 70% with kaolinite and 12% with montmorillonite. If possible, it is recommended to evaluate the possible catalytic effect on the product distribution of the matrix, especially when working with sediment

or suspended particulate matter that could contain important amounts of inorganic matter.

4.3. Introduction of the sample

Thermo-analytical method coupled with mass spectrometry was not frequently used for quantification. The first legitimate issue was the repeatability of the process. Studies realizing multiple injections of standards have proven that the repeatability of the method, is good either with Py-GS-MS with relative standard deviation (RSD) between 10 and 15% [21,28], or with TED-GC-MS, with RSD between 6 and 12% [20], depending on the indicator compounds selected. The main issue is not the repeatability of the analytical instrument. Controlling the amount of solid introduced into the system is the most challenging issue. This aspect is more developed in the calibration section.

There was also a question of the impact of the particle size analyzed on the repeatability of the method. It is said that the particle size has no effect because most common thermoplastics melt before they decompose independent of the particle size, morphology and structure [26]. In the case of thermochemolysis (use of TMAH), some authors recommended that the particles should be as small as possible to enable the optimal reaction surface [42].

4.4. Representativeness of the subsample analyzed

Analyzing a few milligrams of a sample raises questions about the representativeness of the subsample analyzed. Inhomogeneity in samples is a major challenge in microplastic analysis regardless of the analytical method chosen. We discuss this aspect depending on the method of sample preparation:

- i) If sample purification is performed, the MPs are generally deposited on inorganic filters (glass or alumina based) for analysis [27,35]. The use of TED-GC-MS allows the introduction of the whole filter and avoids subsampling and questions

regarding the homogeneity of the filter. In the case of Py-GC-MS, grinding the filter is recommended before analysis of a sub-sample [22,28].

- ii) If no sample purification is performed, for sediment or suspended particulate matter, cryomilling the sample is recommended to ensure a homogeneous distribution of the polymers in the matrix [13,21,26,28,30]. When using this method, the reproducibility and reliability of the methods were demonstrated. With regard to laboratory duplicates, the relative standard deviations were generally below 15% [21,28,30,42]. Field duplicate analysis demonstrated that collocated samples yielded repeatable results [21].

4.5. Chromatography and MS development

The optimization of pyrolysis and transfer line temperature together with the GC oven program was detailed [24]. The conditions are very similar in the reported articles. Mass spectrometry development has not been discussed much. Most investigations were undertaken with full scan mode. A few authors used the single ion monitoring mode (Table 2). Very interesting improvements are expected in the future with the use of high-resolution mass spectrometry.

We are reporting two studies that do not correspond to the scope of the review but that already investigated the potential of mass spectrometry development for microplastic analysis. The first investigation used alkali-assisted thermal hydrolysis to depolymerize two plastics containing ester groups (PC and PET). The building block compounds were monitored by LC-MS/MS [52]. The second article reports the coupling of liquid chromatography to high-resolution mass spectrometry (LC-HRMS) for PS analysis. This allows LODs of 32.5 and 110 pg/L⁻¹ for freshwater and sea water, respectively [16]. These performances have not yet been reached with thermo-analytical method coupled with mass spectrometry and present very promising perspectives in terms of mass spectrometry development. The coupling of thermo-analytical method to gas chromatography and high-resolution mass spectrometry or tandem-mass spectrometry is expected.

4.6. Calibration

The use of serial dilution in a solvent is excluded for most polymers because their solubility is very poor to nonexistent at room temperature. PS is soluble in toluene and chloroform, and these solutions were used for external calibration [42]. Styrene butadiene rubber and isoprene rubber solution in chloroform also allows the establishment of calibration between 30 and 180 µg [21] for tire dust detection. An option for insoluble polymers is to introduce a controlled mass of pure polymer. This option was often adopted [20,22,26,27,30,35,38,42]. All these authors agreed that there are methodological limitations in the calibration curve range caused by the lowest trustable mass measurable. There are also transfer losses because of electrostatic effects. An interesting option is the use of serial solid dilution of the polymers in an inert matrix (calcined sea sand, for example [28]). Finally, the use of colloidal dispersion was proposed for liquid serial dilution. Funk et al. used micro- and nano-particle dispersions in ethanol. Using 25 µm microspheres for PE and 504 nm nanospheres for PS [29]. Zhou et al. used commercially available nanospheres as standards (25 and 65 nm and 1 µm PS nanospheres and 25 and 75 nm PMMA nanospheres) [43]. The use of micro- and/or nano-particles in dispersions is limited to commercially available ones. We recommend working with nanospheres rather than microspheres because microspheres are much more prone to sedimentation. This

recommendation limits the use of colloidal dispersion to a short list of polymers because very few are commercially available. For example, PE nanospheres are not commercially available.

4.7. Use of an internal standard

Because of the difficulties in introducing a controlled mass of an internal standard (IS), most authors have opted for external calibration [35]. The authors who could use an IS reported a considerable improvement in the signal sensitivity and linearity [21,38]. The use of cysteine was proposed [38]. Cysteine pyrolysis produces a strong signal at $m/z = 33$ corresponding to the SH-ion. They demonstrated that this IS was absent in the pyrolysis of the polymers monitored (PET) and the oil organic matter. For tire particles quantifications, Unice et al. used deuterated polymers (polyisoprene, polybutadiene and polystyrene) introduced in chloroform solution; this is possible because these polymers are soluble [21]. Deuterated PS was also used by some authors [13,20,28]. As PS is easily soluble in chloroform and toluene, we highly recommend spiking a solution rather than weighting very small masses of polymer for better accuracy and gain in time. Some important matrix effects discussed earlier with PS pyrolysis might complicate the use of deuterated PS [30] because of proton exchange with the catalytic effect of some inorganic matter. Recently, Fisher et al. proposed using 9-dodecyl-1,2,3,4,5,6,7,8-octahydroanthracene, anthracene-d10, androstane and cholanic acid [42]. The use of cholanic acid is interesting since its acid moiety is methylated during the online derivatization performed and is a way to monitor the thermochemolysis reaction. We do not recommend the use of anthracene or other aromatic compounds as IS if working with an autosampler. If the samples are prepared a long time in advance, there are important losses due to sublimation. The use of 9-dodecyl-1,2,3,4,5,6,7,8-octahydroanthracene seemed promising [42].

4.8. LOQ/LOD

Almost all publications cited here have expressed an LOD of 3 as the threshold signal-to-noise ratio. For the LOQ, the threshold was set at 10. Dierkes et al. experienced false positives and defined LOQs of the average of eleven procedural blank samples. The procedural blank consisted of proceeding to the sample preparation protocol with sand [28].

As discussed in the Calibration section, the poor to in-existent solubility of polymers is a limitation on the establishment of proper calibration. For most studies, the limit of quantification was directly related to the limit of the balance. As the thresholds for LOD and LOQ were not reached, the limit of quantification was geared to the lowest calibration standards [35,42]. The range of LOQ was typically approximately 0.2 µg for PS considering PS trimers as the indicator compound and 0.4 µg for PP, and range of LOQ for PE was approximately 10 times superior [20,22,29,42]. Depending on the indicator compound selected for PE, the LOQ was 1.6 µg (for tridecadiene) [22], 9.5–14.8 µg (for C₁₃ to C₁₆ dialkene) [20] and 0.3–0.4 µg (for C₁₃ to C₁₅ dialkene) [29]. When the polymer is easily soluble in a solvent, such as PS, a dilution series of PS in dichloromethane was performed, and an LOD of 59 ng was obtained for the styrene-trimer [42]. Using PS and PMMA nanosphere dispersions allowed LODs of 1.1 µg/L and 0.6 µg/L to be reached for PS and PMMA, respectively [43].

4.9. Contamination

Sample contamination due to airborne polymer particles and fibers is described as a major problem in microplastic analysis [2].

Therefore, to avoid contamination, certain measures need to be taken. This includes avoiding synthetic components in clothing, wearing cotton lab coats, and prerinsing and cleaning all materials used as well as laboratory (bench and laminar flow cabinet) surfaces. Most of the cited articles addressed these measures.

Field blanks were not systematically recorded. Funk et al. processed field blanks [29]. Procedural blanks were more systematically described [21,27,28,35,42,43]. For sediment analysis, the procedural blanks consisted of processing washed [21] or calcined silica sand [28]. For some authors, no polymers were detected in the procedural blank samples [27]. Other authors signaled the presence of a styrene signal that was attributed to the packaging of the glass filters [22,35]. Tempering the filters at 590°C (muffle furnace) before use solved the problem [35].

Cleaning the glass equipment, the pyrolysis crucibles and the filters at 590°C was a systematic procedure in the articles cited. Cleaning at high temperature is very appropriate with the low sensitivity of mass spectrometry detection and is recommended.

4.10. Validation method

The level of quality assurance deployed is not described with the same level of precision in the cited articles. The quality of microplastic research has been debated recently and has been quantitatively assessed for studies on microplastic ingestion by biota [2] and from freshwater and drinking water [53]. An evaluation of various aspects of the analytical procedure was proposed, and it provides a total reliability score for data [53]. Very few of the references cited here were evaluated in these review articles. In future developments, it would be interesting for the authors to go through autoevaluation during procedure development.

The use of an IS greatly improves the performance of the quantification method, but there are still important obstacles. Very few polymers are soluble in a solvent and have deuterated standards available. Most polymers are not soluble, the use of IS solution is not possible, and the introduction of a controlled mass of IS is difficult. The use of molecules instead of macromolecules as IS was proposed. Cysteine [38] and some polycyclic aromatic hydrocarbon derivatives [42] were proposed. None of the molecules proposed have unanimous support yet, and further developments are expected.

4.11. Uncertainties in the estimation of plastic concentration

Some factors affect the pyrolytic behavior of plastic. These aspects were minimally addressed in the quantification method developments published. These main factors are listed:

- 1 The chemical structure of the polymer.** In the plastic industry, fabrication processes lead to a great diversity in the polymer structures adapted for the properties required for their application. The average molecular weight and chain branching of the polymers could be very different from one object to another, for example. However, the pyrolysis product distribution is correlated with the chemical structure of the polymer. Molecular weight [54] or branching [55] affect the pyrolytic fingerprint of the polymer. How to integrate this factor in the calibration curve has not been discussed much.
- 2 Effect of formulation.** Plastics are made of polymers mixed with additives. These additives could interfere with polymer pyrolysis. It is known that some metals have a catalytic effect and modify the yields of the pyrolysis products [51]. The extent of the impact of plastic formulation on polymer pyrolysis is not much discussed. For tire particle detection, it was addressed,

and the conclusion was that the additives did not affect the pyrolysis behavior of the polymers [21].

- 3 Amount of polymer in the plastic.** The mass of a polymer does not necessarily represent the total mass of a plastic. For example, polyethylene additives and fillers could represent up to 50% of the weight of a plastic [56]. This mass deficit is not considered in the calculation of the microplastic mass concentrations.
- 4 Polymer weathering.** Upon photodegradation, the polymer undergoes oxidation of the polymer backbone (formation of ketones ...), formation of insaturation or branching. All these chemical modifications favor β -scission during pyrolysis and consequently modify the pyrolysis product distribution. It was discussed that for PE, micro(nano)plastic could present a pyrolytic fingerprint distinct from that of pristine polymers [11].

All these aspects imply detailed structural investigation by thermo-analytical method coupled with mass spectrometry. At the least, the uncertainties generated by these factors in the development of a quantification method must be evaluated and, if possible, corrected.

5. Conclusion

The analysis of micro(nano)plastic poses new challenges for environmental analytical chemistry. This critical review emphasizes the ability of thermo-analytical method coupled with mass spectrometry for the simultaneous, selective, and sensitive analysis of micro(nano)plastic in environmental samples. Recent developments have allowed quantification in addition to detection. The latest advances in mass spectrometry development have not been incorporated yet to the determination of microplastics which will certainly improve the present performance.

With the selection of characteristic indicator compounds and related ions, it was demonstrated that minimum sample purification is possible for some polymers. This option offers the perspective of handling a larger number of samples. Plastic pollution monitoring lacks cost-efficient and fast methods to evaluate contamination and make risk assessments.

To summarize it was reported in this analysis of the latest publications that thermo-analytical method coupled with mass spectrometry allows the simultaneous detection of several polymers (typically 7 to 8) in one run after minimum sample purification to reach levels of detection in the nanogram range and is compatible with nanoplastics analysis.

Mass-related analysis of micro(nano)plastic is complementary to the spectroscopic characterization of particles (μ -FTIR or μ -Raman). All the methods contribute to a differentiated understanding of micro(nano)plastic behavior in the environment. In addition, multiscale characterization will help us to better understand the complex interactions of micro(nano)plastic with natural constituents and their reactivity, and mass spectrometry is compatible with many of these innovative development [10].

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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