CRITERIA AND METHODS FOR MICROPLASTICS MONITORING IN WATERS TO BE USED FOR HUMAN CONSUMPTION WITHIN THE NEW EU LEGISLATION FRAMEWORK

PhD thesis in Pharmaceutical Sciences - XXXV cycle

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PART I: INTRODUCTION AND RESEARCH OBJECTIVES

Microplastics represent unique emerging contaminants due to their complexity and one of the major analytical challenges of the last decade. Their recurrent presence in more and more environmental and biological matrices, even in those usually less prone to contamination, have led the regulatory authorities, the scientific community and interested stakeholder to question about possible effects on human health. The toxicological branch of microplastics research has been a natural evolution of investigations on marine animals. While the toxicity posed by plastics and microplastics (especially those > 300 µm) to marine organisms seems evident, effects induced by smaller particles on humans are yet to be understood. At the same time, since 2004, researchers' efforts also focused on the development of analytical methods for the identification and quantification of microplastics in different environmental and biological matrices. Over the past few years, the focus on microplastics analytical research moved from marine environments also to inland water, drinking water and food, as a consequence of sea contamination pathways identification and regulatory authorities and consumers' concern. In the transition from sea monitoring campaigns to targeted analyses on food and drinking water, several analytical needs changed. As the research focused i.e., more on smaller microplastics (< 300 µm) for toxicological reasons, the necessity of magnification techniques coupled to identification techniques and more accurate sampling/pre-treatment methods emerged. Again, the inclusion/exclusion of certain categories of microplastics due to ECHA intervention (ECHA, 2019; 2020) strongly mitigated the impact of data provided by some studies in the past years, especially those obtained without polymeric identification. Another prominent example regarding the connection of different research area is the ban in the EU and USA market of certain plastics products resulting from specific legislative measures. Microbeads, small pseudo-spherical microplastic particles employed in the past in several cosmetic products, were prohibited in USA (United States, 2015) after they were found in high quantities in the environment thanks to the primitive analytical approaches. Single use plastics products (SUP), the main generators of secondary microplastics, experienced the same fate in EU (European Parliament and Council, 2019). Microplastics research, from the very beginning, has therefore always relied on the dialogue between its analytical, toxicological and regulatory branches. This holistic approach also represented the rationale behind the European Union's regulatory intervention regarding microplastics in water intended for human consumption. Interest in microplastics in drinking water was firstly stimulated by a series of studies in 2018 which reported the presence of microplastics in tap water and in bottled water (Oßmann et al., 2018; Pivokonsky et al., 2018; Schymanski et al., 2018; Strand et al., 2018). With few data on drinking water and more robust data on water destined to human consumption available, WHO published a report in 2019 (WHO, 2019) including a review on analytical techniques, contamination pathways and a first risk evaluation. The WHO report represented the backbone of microplastics research in drinking water and one of the pillars on which the legislative intervention of

EU was based. Microplastics were indeed mentioned for the first time in the revision of the Drinking Water Directive (European Parliament and Council, 2020). Microplastics, in the Directive 2020/2184, were associated with the concept of "watch list". The Commission establishes and regularly updates a "watch list" of substances and compounds of particular concern to the public or the scientific community. According to the text of the Drinking Water Directive, the inclusion of microplastics in the "watch list" is conditional on the development of an effective analytical method, which will be then useful for risk analysis. The European Commission will undertake, by 2024, to find an analytical methodology for the analysis of microplastics in drinking water in order to carry out a complete risk assessment by 2029. Until then, there will be no monitoring requirements as microplastics will not be included in the "watch list". Regarding the experimental activities, the European Union relies on the Joint Research Center (JRC). JRC launched a dedicated project in order to harmonize experience and knowledge about the topic, requiring support from national technical-scientific representatives, industry experts and stakeholders from the EU Member States. This project includes, at the moment, an online survey and a series of workshops designed to collect contribution from stakeholders and experts in microplastics analysis. Following the European Commission and JRC requirements and its institutional role, The Italian National Institute of Health (ISS) with the coordination of the Italian Ministry of Health, has in turn launched a dedicated project regarding microplastics in drinking water. This PhD project has its roots precisely on this project and focused on the different approaches to microplastics mentioned above (Figure 1):

Regulatory approach. The regulatory approach included the definition by the ISS, with the coordination of the Italian Ministry of Health, of a national working group on microplastics in drinking water which includes experts from the National Research Council (CNR), national and local environmental Authorities (SNPA: ISPRA and ARPA), Universities and Water Suppliers (Utilitalia) and its related activities. This group was designed to work on: (i) JRC and EC support on national expertise about microplastic monitoring in drinking water (ii) development of national analytical method for microplastic in drinking water to be presented to the JRC. The composition of the working group and the activities of the first meetings were presented at *XXIX Congresso della Divisione di Chimica Analitica della Società Chimica Italiana (SCI)* (Castello di Milazzo, ME, 11-15 September 2022) and *μMED: International Conference on Microplastic Pollution in the Mediterranean Sea* (Naples, 25-28 September 2022). The contributions of the working group members, together with a detailed bibliographic search on analytical techniques for the analysis of microplastics in drinking water, will provide the basis for a forthcoming publication on the topic in the Rapporti ISTISAN series.

Analytical and Toxicological approaches. The analytical and toxicological approaches included, first of all, a series of publications technical and divulgative - oriented, related to the subject area (Martellone et al., 2020, 2021). In these publications, toxicological and especially analytical approaches, through an intense bibliographical and methodological research, have been deeply investigated. This section of the

PhD and ISS project included an experimental part, carried out with the collaboration of water suppliers, Venice ISP-CNR and Padua University. Experimental was intended for developing a method for microplastic analysis suitable for both drinking water and surface water for drinking water purposes, by comparing sampling and analytical techniques, in order to evaluate their *pros* and *cons* with a view to a routine approach. Another side objective of the experimental part of the PhD project was to understand the possibility to apply the same analytical procedures for both surface water and drinking water. Final data, especially those of drinking water, will be sent to the JRC as part of the European project.

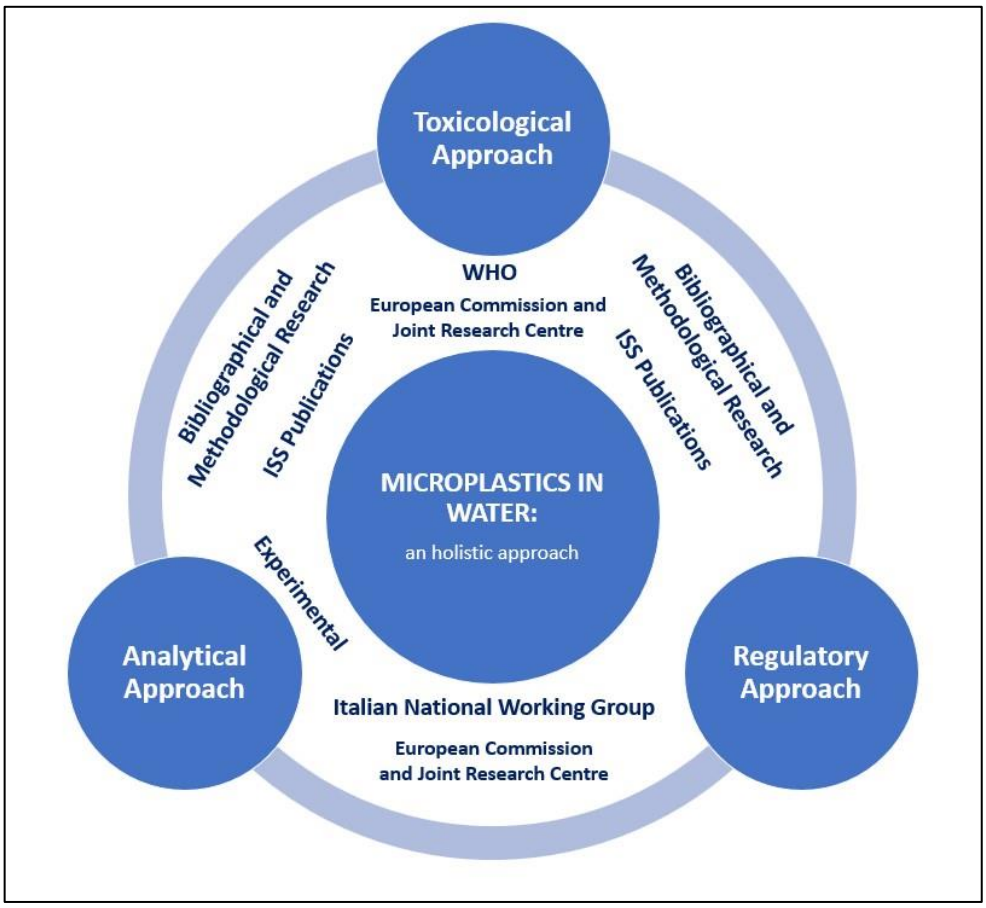


Figure 1. PhD project and its focal points.

PART II: BIBLIOGRAPHICAL AND METHODOLOGICAL RESEARCH

2.1 MICROPLASTICS AND THE ENVIRONMENT

2.1.1 An overview of plastic production and pollution over the last two centuries

Plastic can be considered one of the most versatile materials in different areas as a result of its relatively low cost, its lightweight and its high resistance to deterioration. The production of plastic, conventionally considered since the 1960s, had a massive increase in demand and supply over the last decades, due to its versatile nature and advantages when used in multiple market segments, living spaces and workplaces. The continued, massive production and its properties as material were the main synergistic factors behind the persistent accumulation of plastic debris and waste on our planet, currently one of the most serious concerns on the environmental side and a potential risk to human health. On the other hand, the history of plastic production and plastic pollution is much older and convoluted, probably going back as far as the 19th century, when Alexander Parks registered the "parkesite" (later known as xylonite), a phenol-formaldehyde thermoset derived from cellulose, which was later commonly used in household items, from building materials to radios (Napper et al., 2020). His work opened the floodgates to a torrent of synthetic plastics, as polystyrene (PS), polyester (PES), polyvinylchloride (PVC), polyethylene (PE) and nylon (PA). It was, however, in the 1930s that plastic production drastically increased, with crude oil being used as the raw material of choice for the purpose. Polyethylene terephthalate (PET), invented in 1941 and one of the most widely used plastics nowadays, showed also how versatile these cheap new materials could be. However, it was at the end of World War II that mass production of plastics began in earnest, with annual production of around 2 million tonnes in the 1950s (Geyer et al., 2017; Ritchie and Roser, 2018). Those years were also characterised by the rise of polyethylene (PE), which found full popularity only two decades after its invention, by exploiting its higher melting point enabling previously unimaginable applications and Giulio Natta's discovery in 1954 of isotactic polypropylene (PP). The 1960s marked the definitive establishment of plastic as an unreplaceable part of everyday life and as a "new frontier" in fashion, design and art areas. In the following years, so-called or engineering plastics (or technopolymers) have been developed including polymethylpentene (PTX), polycarbonate (PC), and polyimide (PI); these new materials are characterised by high mechanical and thermal resistance, so high that they have been able to replace several metals in different sectors such as the automotive, electronics and aerospace industries. By the 1970s, world plastic production reached around 35 million tonnes per year, a number that would have doubled in ten years' time (Geyer et al., 2017; Ritchie and Roser, 2018) and a sign of exponential and almost unstoppable growth. The 1970s are also the years that marked the first sights of plastics of different sizes in the marine environment (Carpenter et al., 1972; Colton et al., 1974). The number of

marine campaigns grew significantly in the following years (Shim et al., 2017), partly due to the development of global awareness about environmental issues. Indeed, by the 1970s, the budding environmental movement helped catalyse a growing awareness about the toxicity and wastefulness of synthetics, many of which were designed to be disposable. In 1980, Merrel (1980) reported that the presence of plastic litter on the beaches of an island in Alaska was more than doubled between 1972 and 1974, blaming mainly the waste from Soviet and Japanese fishing activities (Schmidt et al., 2021). Eighties signed the beginning of recycling: prior to 1980, recycling and incineration of plastic was negligible and 100 % of plastic was therefore discarded. From 1980 for incineration, and 1990 for recycling, plastic discarding rates have clearly decreased (Geyer et al., 2017). Some years later, as highlighted by Schmidt et al (Schmidt et al., 2021) there has been an increase in studies regarding plastic litter findings in the stomach of animals. Harvesting campaigns, studies on animals and count of plastic debris during sea campaigns have been the most published works from 1980 to late 2000s, when the topic of microplastics appeared. At the same time, world plastics production amounted to around 120 million tonnes in 1990s and 213 million tonnes in 2000s. These are also the years in which research about biodegradable plastics intensified. In 1989, a series of NOA-A (National Oceanic and Atmospheric Administration) reports regarding marine debris described the presence of areas full of floating plastic waste in the subtropical North Pacific vortex (now known as the Great Pacific Garbage Patch), with density peaks of approx. 1 fragment every 3 m^2 in the Sea of Japan (NOA-A, 1990). Since 2000s, "small plastics" began to receive more attention, also thanks to Thompson's findings (Thompson et al., 2004). In June 2008, the European Parliament issued the Marine Strategy Framework Directive (MSFD) (European Parliament and Council, 2008) to encourage member countries to protect and safeguard the marine environment, with comprehensive description of problems and strategies. In Annex I was reported the *"Descriptor 10"* was *"Properties and quantities of marine litter do not cause harm to the coastal and marine environment"*. In the Directive, for the first time, a distinction was also made between macro- and micro-particles of marine litter, defined during an international workshop by NOA-A as objects with largest measurement over or below a limit of 5 mm; 5 mm was chosen *"to focus the plastic littered discussion on possible ecological effects other than physical blockage of gastrointestinal tracts"*. This point was later developed in *"Marine Litter - Technical Recommendations for the Implementation of MSFD Requirements"* document published by European Commission's Joint Research Centre Institute for Environment and Sustainability (JRC) (JRC, 2011). This technical report described for the first-time appropriate methods for monitoring beaches, surface water, biota and micro-litter, but was not microplastics-specific. Hence, in the first half of 2010s, research on microplastics in marine environments intensifies, both with monitoring campaigns and studies about potential adverse effects on marine biota. Meanwhile, plastic production in 2010 reached 313 million tonnes per even though the cumulative percentage of recycled and incinerated plastics was around 38% compared to that discarded (Geyer et al., 2017; Ritchie and Roser, 2018). The NOA-A Technical Memorandum published in 2015 (NOA-A, 2015) was one the first analytical protocols microplastics-specific as described in

detail methods of analysis (e.g., FTIR microscopy for microplastic identification) for marine water and sand samples. The second half of the 2010s signed the rise of inner water campaigns and the development of acts designed to contain plastic and microplastic pollution with also a look on consequences on human health. The JRC Technical Report published in 2016 (Veiga et al., 2016) described specifications for river system monitoring assuming rivers as a microplastic and microlitter input for marine environments. With Microbead-Free Waters Act of 2015 (United States, 2015), USA in 2017 prohibited the manufacturing, packaging, and distribution of rinse-off cosmetics containing plastic microbeads, followed by several countries as e.g., Italy (Italy, 2017). Regarding other acts designed to limit the spread of plastics in the environment, with Directive EU 2019/904 (European Parliament and Council, 2019) European Member States had to ban from market specific single-use plastics products as dishes, cutlery, straws and polystyrene food containers. In 2019 was also published by WHO a document regarding the state of art of microplastic pollution in drinking water (WHO, 2019). Authors analyzed microplastics inputs in freshwater system and drinking water, analysis strategies, problems related to sample contamination and data collected before 2019 regarding possible effects on human health. 2019 is also the year of ECHA proposal of restriction of microplastic intentionally added in products placed on the EU/EEA market to prevent or reduce their release into the environment (ECHA, 2019). In 2020, world plastic production reached 367 million tonnes (Plastics Europe, 2021), with a slight decrease compared to 2019 (368 million tonnes). A short downturn was also observed between 2009 and 2010 as a result of the 2008 global financial crisis (Geyer et al., 2017; Ritchie & Roser, 2018). In the latest case, Plastic Europe reported that the downturn in the European plastics value-chain both in its production and demand levels was a consequence of the COVID-19 crisis. However, in 2020, the proportion of post-consumer plastics waste sent for recycling in Europe has almost doubled compared to 2006 (Plastics Europe, 2021). Twenty-twenty is also the year of the revision of the Directive on the quality of water intended for human consumption (European Parliament and Council, 2020) in which microplastics are mentioned for the first time in the drinking water context.

2.1.2 Microplastics and their definition

Microplastics are “emerging” heterogeneous contaminants with a complex toxicological profile. The term microplastic appeared for the first time in 2004, when Richard Thompson (Thompson et al., 2004), a professor at Plymouth University, described plastic particles smaller than 5 mm found above the tideline on a beach, in England. Then, research and media interest in the topic rapidly increased from there on. The main challenge in the identification of such contaminants rises from the complexity and heterogeneity of these compounds. Microplastics can indeed be defined as being part of a diversified set of polymers different in sizes, chemical composition, shapes, colours and densities (WHO, 2019) to which various additives are often added for technological reasons. The discussion on the appropriate definition of microplastics, useful both for the development of analytical methods and for toxicological

evaluation, has been going on for many years with the release of sometimes conflicting definitions. Only in January 2019 the European Chemical Agency (ECHA, 2019), in an ECHA proposal of restriction on microplastics in products placed on the EU/EEA market, suggested a detailed definition of microplastics regarding size range as a *"a material consisting of solid polymer-containing particles, to which additives or other substances may have been added, and where ≥ 1% w/w of particles have (i) all dimensions 1 nm ≤ x ≤ 5 mm, or (ii), for fibres, a length of 3 nm ≤ x ≤ 15 mm and length to diameter ratio of >3".* In 2020, ECHA Committees (ECHA, 2020) confirmed the lower limit of 1 nm for particles but also expressed the opinion that a higher limit (100 nm) may be necessary at the time of analysis to facilitate the identification of microplastics in products. Another important document regarding microplastic definition and size range is ISO/TR 21960:2020 standard published in 2020 (ISO, 2020). This document summarizes scientific literature on the occurrence of macroplastics and microplastics, in the environment and biota before 2020. It also gives an overview of testing methods, including sampling from various environmental matrix, sample preparation and analysis. ISO/TR 21960:2020 standard describes microplastics as *"any solid plastic particles insoluble in water with any dimension between 1 µm and 1000 µm"* and large microplastic as *"any solid plastic particle insoluble in water with any dimension between 1 mm and 5 mm"*. ISO/TR 21960:2020 standard describes, as has often been reported in the past (Hartmann et al., 2019) also macroplastics as *"any solid plastic particle or object insoluble in water with any dimension above 5 mm"* and nanoplastics as plastic *"particles smaller than 1 µm".* The proposed microplastics size range, even though it includes (in the definition provided by ECHA) a critical distinction between particles and fibres, represents, in any case, a broad definition as it is extended to compounds in the sub-millimetre range with very different chemical-physical properties and which, therefore, may represent an entirely different environmental, toxicological and analytical problem. For this reason, analyses of microplastics are often conducted on a subset of the definition range. Some authors choose to perform the analysis only on microplastics > 100 µm, which are easier to handle, due to the limitation of some sampling system/analytical techniques while others prefer to focus on the smaller ones, also called Small MicroPlastics (SMPs, usually referring microplastics < 100 µm), with behaviour more similar to nanomaterials. In fact, the definition proposed by ECHA also includes what are traditionally referred to nanoplastics (0.001- 0.1 µm) (Gigault et al., 2020), which are included in the definition of nanomaterials according to JRC (JRC, 2014). On the contrary, ISO/TR 21960:2020 precisely defines nanoplastics marking a clear-cut boundary between microplastics and nanoplastics at 1 µm (Figure 2).

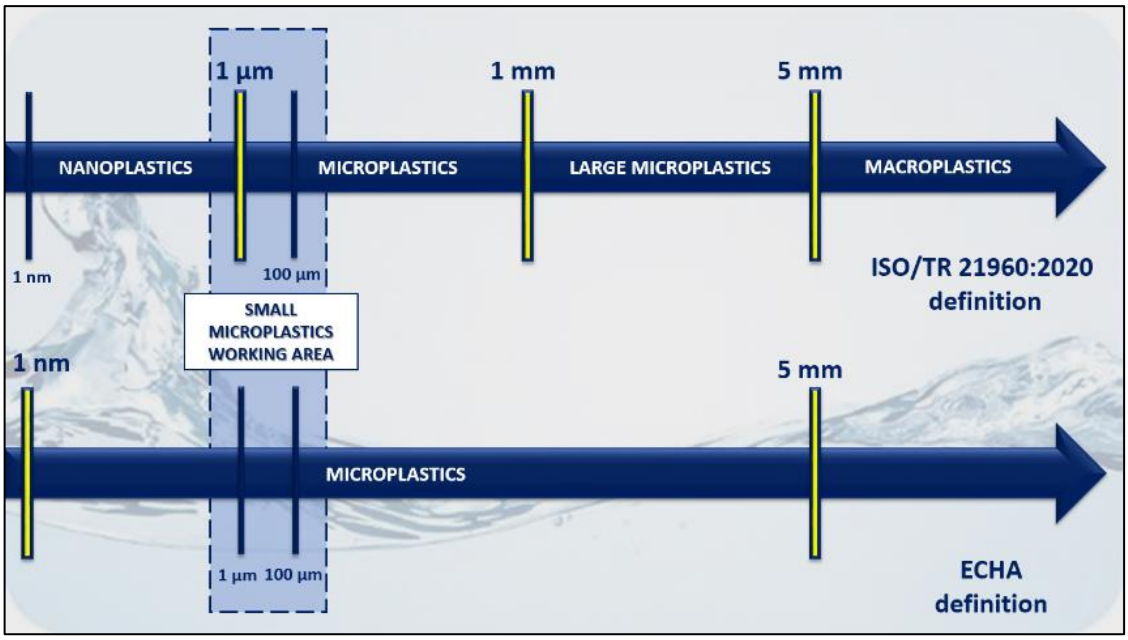


Figure 2. Comparison between ISO/TR 21960:2020 and ECHA size definition of microplastics and small microplastics (SMPs) working area. Yellow bars represent standards set by the ISO/TR 21960:2020 and ECHA definition while thinner bars are operative bounds.

The chemical composition criterion is similarly controversial, but there is general agreement in defining them (Hartmann et al., 2019) as solid polymers of a synthetic (e.g., polyethylene or polypropylene) or semisynthetic nature; tyre wear derivatives are also traditionally included in the group, i.e., particles consisting of a combination of synthetic and natural rubber from the degradation of tyres on road surfaces. ECHA excludes from the definition a) *"Polymers that occur in nature that have not been chemically modified (other than by hydrolysis)"* and *"Polymers that are (bio)degradable, as set out in the criteria in Appendix X".* Regarding biodegradability, the ECHA proposal strictly defines the biodegradability tests that are accepted to determine whether or not a polymeric material is considered a microplastic. Permitted test methods are ISO14851 and ISO14852 Fresh Water Biodegradation, conducted by authorised labs. Modified natural polymers (or semi-synthetic polymers) represent a special case as classification criteria often diverge in the literature and some authors are more prone to classify some polymers in the microplastics group. Hartmann et al. (2019) classified cellulose derivatives e.g., rayon and cellophane as "heavily" modified polymers while "slightly" modified polymers are not considered plastics (Table 1). However, this classification could lead to a significant increase in compounds to be detected and in the resulting number of microplastics found (Corami et al., 2021).

MICROPLASTICS	NOT MICROPLASTICS/MICROLITTER
Synthetic polymers	**Natural polymers (e.g., wool, cellulose)**
Tire wear (and road) particles	**Biodegradable/Compostable polymers**
Copolymers	Semisynthetic polymers/ "slightly" modified polymers (e.g., dyed wool)
Composites with synthetic polymers as essential ingredient	Semisynthetic polymers – "heavily" modified polymers (e.g., Rayon, Cellophane)

Table 1. Chemical composition of microplastics and microlitter. Classification criteria according to ECHA definition is highlighted. Modified from Hartmann et al., 2019.

The size and chemical composition criteria of microplastics are relevant to the scientific community as they are strictly necessary in order to assess an appropriate characterization and quantification. However, an extended definition of microplastics could be useful to fully understand their environment distribution patterns and to enable the development of reliable and robust analytical methods. It is therefore useful to appropriately define also secondary characteristics of microplastics such as shape and origin. The shape descriptor, in detail, could be useful for understanding the origin, distribution patterns and human health-related issues of microplastics, especially for SMP (< 100 μm). In the ECHA definition, a fundamental distinction between "particles" and "fibres" relating to their size was made but other shapes were not investigated; in a similar way, ISO/TR 21960:2020 standard referred regarding microplastic shape only as *"microplastics may show various shape" and "plastics are classified by shape (fragments, pellets, cosmetic beads, lines, fibres, films, foams)"*. Compared to what can be found in the literature, several classification methods can be found for microplastics > 300 μm. However, as suggested by Hartmann et al. (Hartmann 2019), this criterion could be useful to adopt a rigorous approach with the aim of reducing the number of shapes detected upon visual inspection, in order to simplify analytical operations. The terms *"sphere"*, *"pellet"* and *"bead"* all represent pseudo-spherical plastics, which differ in origin and use. For all these polymers, the term *"sphere"* or *"spheroid"* could be used to comprehensively describe their shape. The term *"fragment"* is often used to describe irregularly shaped particles, but the term itself suggests that they are plastics derived exclusively from the fragmentation of larger particles. Since some microplastics are already produced in this shape, such as some abrasives used in cosmetics, a more correct term should be *"irregular particles"*. The category of *"films"* is less prone to misunderstanding, as these are microplastics with a planar shape in which one of the three dimensions is considerably smaller than the other two. Plastics with a length-to-diameter ratio >3, on the other hand, are described as *"fibres"* or *"filaments"*, also in the ECHA definition. For smaller

microplastics (especially SMPs) the shape identification is more difficult due to the innate ability of analytical techniques to being able to clearly distinguish the shape of such small particles. For such particles, an expedient related to particles Aspect Ratio (AR) or Elongation Ratio (E) can be employed. Aspect Ratio or Elongation Ratio is a dimensionless morphological parameter calculated as the ratio between the maximum length (L) and the maximum width (W) of the smallest rectangle (bounding box) enclosing the shape of the particle. It allowed to distinguish between elongated and non-elongated particles and by extrapolation it is possible to associate a certain aspect ratio with a certain shape. However, as Rosal pointed out (Rosal, 2021), for the purpose also other descriptors can also be used. As already mentioned, the shape of microplastics often depends by their origin. The concept of the origin of microplastics is related to the way in which these contaminants have entered the environment. The first classification of plastic regarding their origin can be found already in 1987, when Azzarello and Van Vleet (Azzarello and Van Vleet, 1987) distinguished between plastics from ordinary use (i.e., bottles and similar - the most abundant), and those deriving from industry (less numerous). However, the most widely used origin-based classification is the one proposed back in 2011 (Cole et al., 2011) and reported by WHO in 2019 (WHO, 2019), which distinguishes between primary and secondary microplastics. Primary microplastics are defined as plastics directly released into the environment in the form of small particulates. They can be a voluntary addition to products such as scrubbing agents in toiletries and cosmetics (e.g., shower gels). They can also originate from the abrasion of large plastic objects during manufacturing, use or maintenance such as the erosion of tyres when driving or the abrasion of synthetic textiles during washing (Friot and Boucher, 2017). Secondary microplastics, on the other hand, formed as a result of the degradation of larger plastic objects (such as various types of packaging, bottles, and plastic waste) through various physical, chemical and biological weathering processes such as photodegradation or thermal degradation. However, due to aging, it is often very difficult to infer microplastic origin, unless they are clearly identifiable as, for example, microbeads. Regarding other microplastics properties (Table 2), density, which is closely related to the chemical composition of microplastic, could be a useful descriptor as allows a straightforward understanding of the behaviour of microplastics in the environment. This, for instance, affects the area of the water column where the microplastics stratify on and their general inclination to accumulate on the seafloor (WHO, 2019). Another consideration regarding microplastics is certainly that related to plastic additives. Plastic products usually contain additives such as plasticizers, flame retardants, photostabilizers, antioxidants and pigments that could be released in the environment due to degradation; pigments affect the colour of microplastics and can help to recognise them, especially the larger ones. Ngoc Do et al. (2022) reviewed the literature regarding leaching of microplastic-associated additives in water environments. Here are reported some of their considerations. Plastics additives are intentionally added into plastic polymers at different concentrations during production (0.1–70 wt %) in order to keep or enhance their properties (Hahladakis et al., 2018). The weight fraction of plastic additives may vary from up to 70% for plasticizers, up to 25% for flame retardants, and 0.1–3% for

antioxidants and photostabilizers. The type of additive used depends on the plastic material and the performance of the products (Hermabessiere et al., 2017; Hahladakis et al. 2018). For instance, plasticizers are commonly used in plastics to improve flexibility. Plasticizers mainly include bisphenols (Bisphenol A, BPA and analogues, probably among the most abundant plasticizers present in the aquatic environment) and phthalates. Di-2-ethylhexyl phthalate (DEHP) is usually employed in polyvinyl chloride (PVC) while di-n-propyl phthalate, diethyl phthalate (DEP), and di-n-butyl phthalate (DBP) are usually added in polyethylene terephthalate (PET). Flame-retarded polyamides (PA) and polyesters are used in electronic applications and insulation foams, while photostabilizers and antioxidants are employed in many synthetic polymers, including polyolefins as polyethylene (PE) and polypropylene (PP). In addition, pigments are used in a wide range of plastics, while most slip agents are used in polyolefins, and antimicrobial substances are added to PVC, polyurethane (PU), PE, or polyester (PES). The term "microlitter" indicates the fraction of litter of less than 5 mm in size; it includes not only microplastics but also additives and the remaining different type of material released into the environment. Originally, studies on microplastics actually targeted the microlitter as a whole as they were based exclusively on microscopic observation without polymer identification. Lastly, microplastics can appear in different colours. Categorizing plastic debris according to their colour could be useful for identifying sources and potential contamination during sampling preparation. However, as well as for the shape, the colour can't easily be used to infer about the origin and colour information can be biased. Brighter colours are usually spotted more easily during a visual inspection while, dark, transparent, or translucent particles may be underrepresented; additionally, discoloration can take place during weathering as well as sample preparation (Hartmann et al., 2019). Ultimately, for microplastics < 300 µm and nanoplastics classification by colour can be more difficult and often is not carried out. However, for microplastics > 300 µm (large microplastics) and macroplastics in biological contexts, colour could be a useful additional descriptor, where depending on an organism's feeding preferences, some coloured plastic objects may be more or less likely to be mistaken as food (Ory et al., 2017; Bruno et al., 2022).

PRIMARY DESCRIPTORS	OTHER DESCRIPTORS
Size	Shape
	Origin
Polymer type	Density
	Additives
	Colour

Table 2. Large Microplastics (≥300 µm) Descriptors (based on WHO, 2019).

2.1.3 Microplastics contamination of inland water and drinking water

Microplastics are widely spread worldwide (O' Connor et al., 2019; Fu et al., 2020) and have been found in a large number of environmental matrices, including biological ones (Jamieson et al., 2019; Corami et al., 2021; Leslie et al., 2022,) and also unconventional as placenta and faeces (Ragusa et al., 2021; Zhang et al., 2021). Aquatic environments can be considered an important reservoir for these contaminants as microplastics from different sources continuously flow into them. As previously stated, due to the noticeable abundance of floating plastic waste, the marine ecosystem was the first to be investigated for the presence of microplastics. Sea water environment is also the one on which research has focused more in the last decade, because of possible direct effects on aquatic organisms even if other aquatic environments are gradually gaining importance in microplastics analysis, especially in second half of 2010s. Primary microplastics mainly reach sea water through atmospheric deposition and/or via inland waters, while secondary microplastics are the direct consequence of degradation phenomena affection floating plastic waste in the sea (Boucher et al., 2017; Alimi et al., 2018). In the last years, given the concerns about the effects on human health, research interest has partially moved on the ways through which microplastics reach inland waters and effects they may have on drinking water supply (Alimi et al., 2018). These contamination patterns, as reported by WHO (2019), can be summarily divided into three major categories: land-based sources, water sources and atmospheric sources (Figure 3). Contamination of inland water from terrestrial sources mainly occurs due to wear and tear on road surfaces (especially from the degradation of paints used for road marking) and car tyres. City dust, an abrasion product of various plastic items common in populated areas, such as shoe soles and synthetic turf, may play an important role in this issue (National Institute for Public Health and the Environment, 2016; Foundation for Water Research, 2017). The water source most involved in microplastic contamination of inland water are civil or industrial sewage, combined sewer overflows and urban or agricultural run-off. Wastewater accumulates different microplastics used in domestic and civil areas (textile fibres released during washing, considered the most important source of primary plastics (Corami et al., 2020a), plastic wear products, gaskets, paints and microbeads used in cosmetics) but also industrial ones (drilling fluids, cementing pastes containing PET microbeads, rust removal products and paints containing polyester scrubbers). The effectiveness of wastewater treatment plants in removing microplastics is controversial (The Water Environment & Reuse Foundation, 2017; Tang et al., 2020; Elkhatib et al., 2020). Indeed, treatments adopted by each wastewater treatment plant can be very different from each other and are generally not specific for microplastics. Moreover, beyond the overall effectiveness of the process, large volume of water treated in these plants could still contribute to the introduction of microplastics into the aquatic environment. Regarding wastewater, also combined sewers (Foundation for Water Research, 2017), the most common type of wastewater collection systems,

may contribute to microplastics contamination of inland water. These systems allow the simultaneous collection of wastewater and stormwater in order to convey them to the treatment plant. When water flow exceeds the load capacity of the treatment plant (e.g., during heavy rainfall), for safety reasons, the water bypasses the plant and is discharged directly into the receiving water, contributing to the environmental spread of microplastics (Eerkes-Medrano et al., 2019; Mintenig et al., 2019). Atmospheric deposition may also play a significant role in the contamination of inland waters. Although few data are available and the microplastics movement patterns in the air are still poorly understood, a limited number of studies in literature have confirmed the tendency of these particles to be carried by the wind (WHO, 2019). Sources of atmospheric deposition could be textile fibers from clothes drying on the line and components of building materials (WHO, 2019). Moreover, similarly to the case of marine waters, the fragmentation of large plastics can result in the release of secondary microplastics (Gasperi et al., 2014; Morrit et al., 2014). Inland water represents the main route by which microplastics can reach drinking water as in drinking water treatment plants (DWTP) treat water from lake reservoirs and groundwater; in the same way, sea water treated by desalination plants, could contribute to contamination if producing water intended for human consumption. However, could contribute to the contamination of drinking water also pipe components and filters used in drinking water plants, desalination plants and water supply systems (Eerkes-Medrano et al., 2019; Mintenig et al., 2019). Similarly, the bottles and caps of some bottled waters are made of plastic, which themselves may be a source of microplastics in drinking water (Oßmann et al., 2018; Schymanski et al., 2018).

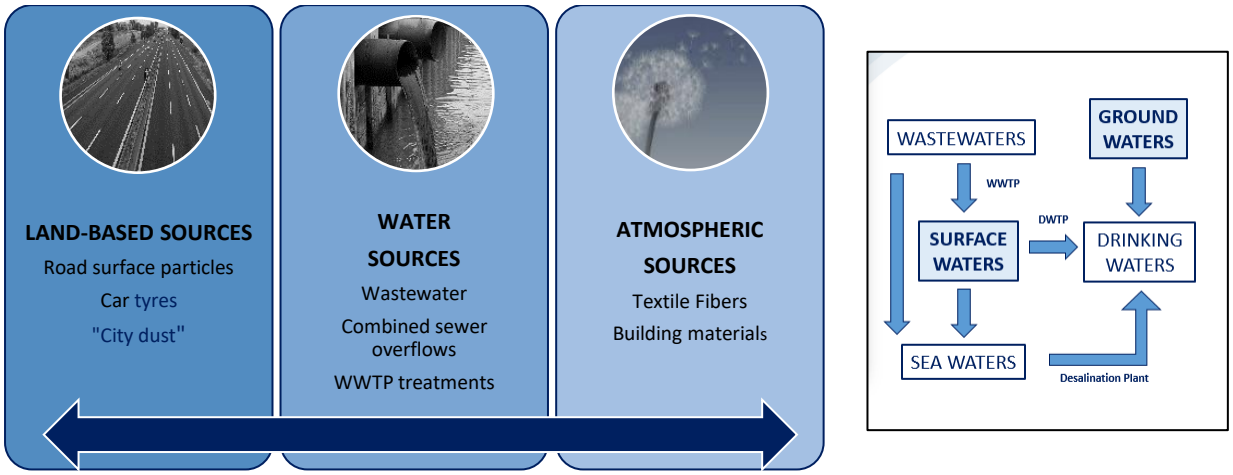


Figure 3. Contamination pathways of inland waters (left, adapted from Martellone et al., 2021) and drinking water (right, adapted from WHO, 2019).

2.1.4 Literature data on microplastics in water and efficiency of removal in DWTPs

Microplastics in drinking water can be considered a fairly new branch of research as interest in it was initially stimulated by some studies in 2018 (Kosuth et al., 2018; Mason et al., 2018) that reported the

presence of microplastics in tap water and in bottled water. Since then, several studies have been published leading to the first data review published by WHO in late 2019 (WHO, 2019). The WHO document, partially based on a systematic review (Koelmans et al., 2019), that WHO commissioned on the occurrence of microplastics in drinking-water, reported not only data retrieved from studies on microplastics in drinking water but also reviewed possible toxicological patterns suggesting recommendations and research needs. Data reviewed by Koelmans et al. (2019) included more than 50 studies on microplastics in drinking water and in or its freshwater sources, i.e., surface water and groundwater, as well as (indirectly) wastewater. However, only 10 studies reviewed by Koelmans et al. focused on drinking water. This data is supported by a review of scientific papers performed by ISS several months later (late 2020) on the MEDLINE database (Martellone et al, 2020) and on an additional literature review (Danopoulos et al., 2020). As can be seen from Figure 4, papers on microplastics in drinking water in the 2010-2020 period were only a small percentage of the total in the MEDLINE database.

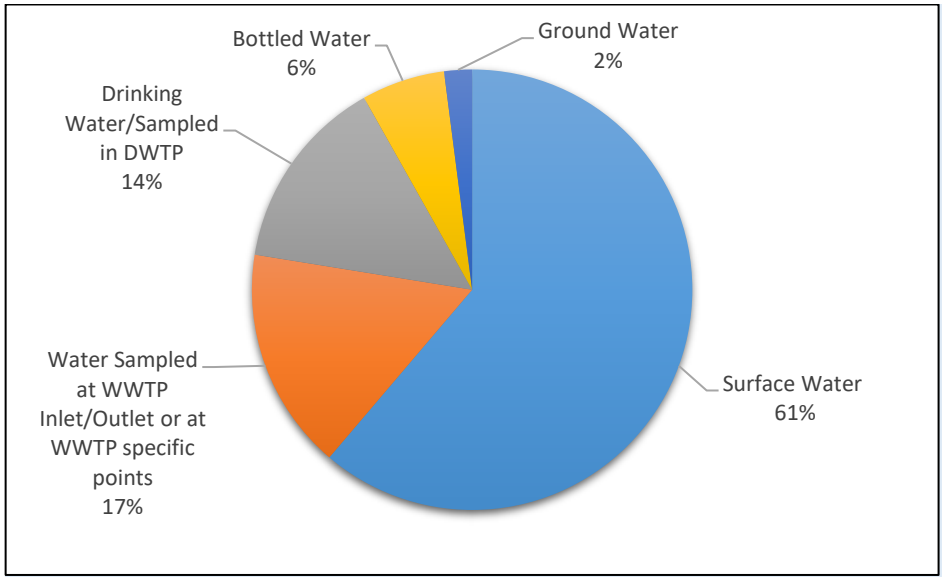


Figure 4. Publications in the MEDLINE database related to the analysis of microplastics in different aquatic matrices (updated to June 2020). Adapted from Martellone et al., 2021.

Following data retrieved by Koelmans et al. (2019), in drinking water concentrations in individual samples ranged from 0 to 10^4 particles/L and mean values ranged from 10^{-3} to 10^3 particles/L. The smallest particle size detected was 1 μm. Particle counts ranged instead from around 0 to 10^3 particles/L in freshwater. However, authors suggested that values could not be compared due to differences in analytical methods. In most cases, freshwater studies targeted indeed larger particles, using mesh sizes that were an order of magnitude larger than those used in drinking-water studies. Regarding plastic types, Koelmans et al (2019) had pointed out that for 32 out of 55 records, polymer types were assessed. Most frequently observed polymer types across studies and records were PE ≈ PP > PS > PVC > PET, with acrylic or acrylic-related compounds, PA and polyester reported in five or more records. Polymer

distribution was justified in term of global plastic demand and polymer density. Regarding shapes, the reviewed studies reported, in the order of decreasing reporting frequency: fragment, fibre, film, foam, pellet, sphere, line, bead, flake, sheet, granule, paint, foil and nurdle. However, the authors suggested that was impossible, at the time of the review, to provide a robust view of shape distribution among microplastics studies for different reasons. These were described in the report and included 1) subset of particles analyzed in certain studies could be not representative for a proper shape assessment 2) studies targeted different size ranges 3) studies are different in their extents 4) some particles' shapes are ambiguous and particles could be described as different even if they were similar in shape. Since 2020, several additional studies have been published and data on microplastics in freshwater water and drinking water increased exponentially after the pandemic period, as can be seen in the number of publications in MEDLINE database (Figure 5).

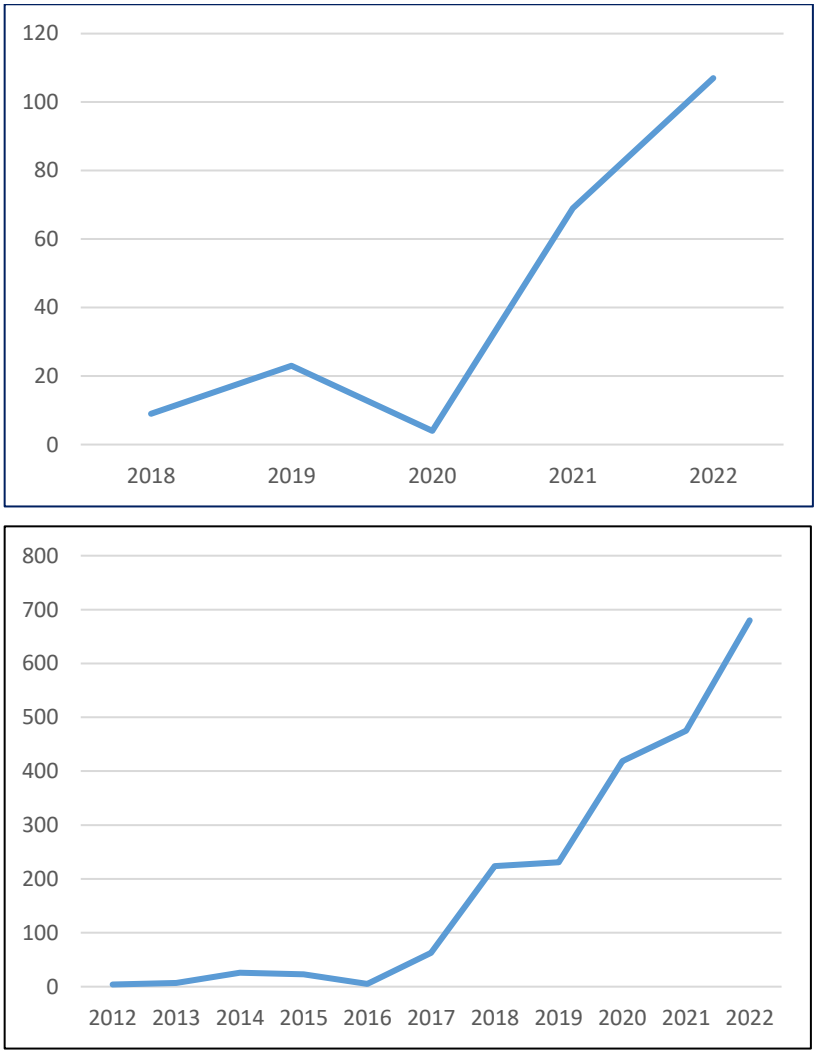


Figure 5. Publications in the MEDLINE database related to the analysis of microplastics in drinking water (up) using "microplastics", "drinking" and "water" keywords (updated to December 2022) and freshwater using "microplastics" "freshwater" keywords (updated to December 2022).

Several papers reviewing data on microplastics in freshwater and drinking water were published since then. Regarding freshwater, a comprehensive review of recent literature on microplastics in freshwater environment was carried out by Wang et al. (2021), Yusuf et al. (2022) and Xue et al. (2022). However, also in this case, heterogeneity in abundances was found and differences in analytical and sampling methodologies did not allow for direct comparisons of abundances. The highest abundance of MPs in rivers, not considering data already reviewed by WHO, was retrieved by Wang et al. (Wang et al., 2020a) in Yangtze River water with 6614 MPs/L. High abundances were also found in Czech rivers (Pivokonsky et al., 2020: ~1300 MPs/L) and in low Yellow River estuary (Han et al. 2020: 930 items/L in dry season and 497 items/L in wet season). All of these studies share the employment of discrete sampling techniques and most of particles retrieved were < 500 μm. Low abundances of MPs were also found in literature. The lowest particle abundance was found by Tan et al. (Tan et al., 2019), with an abundance of 0.56 items/m^3. Sarkar et al. (Sarkar et al., 2020), in Ganga River, found 17.88 MPs/L while Dalmau-Soler in DWTPs of Barcelona Metropolitan area found ~1 MPs/L. However, in many studies with low MPs abundance, especially the older ones, sampling is carried out with nets that can only retain large particles (e.g., > 100 μm). Wang et al. suggested, as also reported by several authors in the past (Liu et al., 2020; Weideman et al., 2019; Zhang et al., 2020a) that abundance of MPs sampled with non-nets methods was significantly higher than using nets, thus explaining the divergence in MPs abundance values found in literature. Regarding polymer type, PP, PE, PS, PVC and PET were the most common polymers identified, matching data reviewed by WHO in 2019. Regarding drinking water, the JRC review (2022) is one of the most recent available. The review collected data from a subset of 207 articles published between 2013 and 2022 reporting studies of drinking water collected from: private and private institutions, households, residential and commercial areas, city plumbing systems, "hydrants'" fountains and from various points of the water distribution network. The amount of microplastic found in drinking water varied between 0.0001 and 440 particles/L. The authors suggested that the huge variation was observed as a consequence of differences in the sampling location (country), the applied detection technique and/or the sampling procedure (collection in containers or *in-situ* filtration). Regarding size and shape, the lower boundaries of the considered size ranges were between 5 and 50 μm (the author reported that it could depend on the pore size filter used for filtration and/or the size limit of detection of the instrumental technique applied) and fibres (found in 50% of studies) together with fragments (found in 44% of studies) were shapes most commonly found. Polyethylene (PE), polyethylene terephthalate (PET), polyester (PEST) and polypropylene (PP) were the polymers most frequently found in drinking water. Other polymers detected were polystyrene (PS), polyvinylchloride (PVC) and polyamide (PA). This data matches those retrieved by Koelmans et al. (2019), even if some of them could be overlapped. A particularly important topic related to microplastics in drinking water is the removal efficiency of drinking water treatment plants (DWTPs) since, as previously reported, inland water can be an important microplastics source.

Xue et al. (Xue et al., 2022) reviewed available data up to August 2021 on microplastics in drinking water treatment (DWT), including laboratory- and full-scale studies. The review summarized data from studies comparing microplastics content (abundance, size, shape) in drinking water and in its sources within the DWTP. The removal efficiency and impacts of microplastics in various drinking water treatment processes were also critically reported. Xue et al. (2022) reported that MPs concentration covered a range of 0–6614 particles/L in different drinking water sources (e.g., surface water and ground water), following the same order of magnitude as those of the data reported by WHO (2019). Polyethylene, PP, PET, PVC and PS were the most widely detected microplastics in DWTP source waters, matching types found in drinking water. Among all the shapes, fragments have been more frequently documented as the predominant MP shape in DWTP raw waters. However, in some cases, fibers were more abundant than fragments in DWTP raw waters. Regarding DWTP efficiency in microplastics removal, the author admitted that current data is limited. However, some conclusion can in any case be drawn from the results of the studies conducted so far. According to the available studies, existing DWTPs are generally able to remove over 80% of MPs with a size of 1–100 μm. Most of the DWTPs investigated employed coagulation-flocculation-sedimentation (CFS) and sand filtration steps. In general, more treatment steps were beneficial to the removal of microplastics. DWTPs consisting of coagulation-flocculation (CF) and sand filtration exhibited an overall MP removal of ~40% – ~70% while DWTPs with more steps, such as CFS-filtration-ozonation-GAC filtration achieved > 80% removal. From this data, it emerged that CFS is often the major contributor to MP removal in DWTPs. Data also showed that fibrous MPs were generally easier to remove by CFS than spherical and fragmental MPs, according to their higher density and stronger tendency of forming flocs. Influencing factors in microplastics removal by CFS steps could be water characteristics (e.g., divalent cations) coagulant type and dose, coagulant aid type and dose and treatment conditions. It was also reported that DWTP could contribute to microplastic contamination through reagents and materials employed. Wang et al. (2020a) found the CFS process resulted in a 114%-increase in polyacrylamide (PAM, commonly used coagulant aid) concentrations in the water. Dalmau-Soler et al. (2021) detected microplastics materials in the treatment plant, which had not been detected in the raw water, such as PTFE and epoxy resins.

2.1.5 Exposure pathways and potential health effects

2.1.5.1 Microplastics Exposure Pathways

Poorly is still known about the effects of microplastics on human health. The reasons behind this lack of knowledge are associated to the non-standardised variable related to their characterization and classification, as well as the very limited toxicological investigations. Thus, is currently impossible to quantify human exposure to microplastics, although it is presumed to be smaller than that of marine animals (WHO, 2019). However, the two main exposure pathways appear to be gastrointestinal (GI) pathway (ingestion) and inhalation routes. The human ingestion of microplastics may be due to several dietary products and/or drinking water; microplastics were found also in honey, sugar, sea salt, beer and milk (Hirt and Malapel, 2020). The highest intake of microplastics seems to result from sea food (Hirt and Malapel, 2020), as a direct consequence of marine biota intake. Indeed, some of the species found to ingest microplastic *debris* in nature (such as mussels, oysters, common shrimps and fish) are of commercial importance for fisheries and aquaculture (Lusher et al., 2017; Hirt and Malapel, 2020). Fish are usually prone to accumulate microplastics in their gills, liver and gut, which are not usually consumed by humans (Paul et al., 2020). The same cannot be stated for filter-feeder bivalves such as mussels and clams that are eaten in their entirety. However, recent findings indicate the presence of microplastics also in edible tissues (dorsal muscle) of marine fish, e.g., in *D. labrax*, *T. trachurus* and *S. colias* (Barboza et al., 2020), and freshwater fish as e.g., in *Alburnus chalcoides*, *Barbus capito*, *Leuciscus cephalus* (Makhdoumi et al., 2021). If microplastics are actually ingested, they are exposed to a series of digestive processes as interaction with digestive fluids and intestinal cells, uptake in the intestine and liver and excretion. A major degradation of plastics due to low pH and digestive enzymes is assumed to be negligible due to their high stability. However, a minor degradation/denaturation could occur, especially for smaller plastics as observed in analytic methods development (Martellone et al., 2021), also resulting in the release of additives from microplastics. However, the key step in crossing the gastrointestinal system is most likely the interaction with intestinal tissues. Different scenarios have been proposed (Wright and Kelly, 2017; Paul et al., 2020). The possibility of realisation of these scenarios can be affected by polymer characteristics as size, type and surface properties. In the first instance, particles could not be absorbed and remain in the lumen, until the excretion. This is consistent with the recent finding of plastics in human faeces (Zhang et al., 2021, Ho et al., 2022; Yan et al., 2022). In the second scenario, particles could pass the barrier represented by the intestinal epithelium. Particles could be taken up (uptake) by enterocytes of the intestinal epithelium or other specialized cells (e.g., M cells) and reach the basolateral side (hence the circulatory system) and/or mucosal lymphoid tissues. The uptake and transport of particles < 10 µm into intestinal cells appears possible, especially for M cells. An intracellular uptake of larger particles would be incompatible with the medium size of intestinal epithelial cells of about 10 µm. M cells sample and transport particles from the intestinal lumen to the

mucosal lymphoid tissues. Here, particles could reach basal phagocytes of the Peyer's patch, accumulating in the sub-epithelial dome and being transported to the lymph nodes. In a similar way, through epithelium cells, microplastic could reach the portal circulation. Particles could also cross the epithelium through the paracellular way, through a phenomenon known as persorption. Persorption can be described as the passage of particles into the portal blood through gaps in the single-layer epithelium mainly located at villus tips. Persorption, allowing the passage of particles much bigger than the uptake limits of the cells (until 150 μm), could explain the occurrence of certain plastic particles in the liver and further remote organs like lymph nodes and spleen (Paul et al., 2020). EFSA, in 2016, stated in this regard that only plastic particles smaller than 150 μm in size might cross the intestinal epithelium. Only a few studies using *in-vitro* systems investigated the uptake of plastic particles (Kulkarni & Feng, 2013; Walczak et al., 2015; Abdelkhaliq et al., 2018; Magrì et al., 2018). Most *in-vitro* studies have been carried out with PS and on the cell line Caco-2 and derived co-cultures, a well-established model for human enterocytes. Results indicate the possibility of particle translocation, especially for sub-micron ones, although it is not possible to estimate a linear correlation with size. Fewer data are available regarding bioaccumulation studies following ingestion on animal models. These *in-vivo* studies suggest a low oral bioavailability of microplastic particles, thus implying substantial faecal excretion; particle distribution results (PS particles < 20 μm) provided different results as in one study microplastics were retrieved in liver, gut and kidney and in one only in jejunum and duodenum (Deng et al., 2017; Stock et al., 2019). Stock et al results (2019) confirmed EFSA assumptions in 2016, stating absorption of these microplastics is expected to be limited (≤ 0.3%) and deeply penetrating of microplastics in organs is possible only for the smallest fraction (size < 1.5 μm). It is possible that absorption and distribution may be more significant for nanoplastics than microplastics (up to 7% for nanoplastics <0.1 μm) (FAO, 2017). However, microplastics were also retrieved in blood (Leslie et al., 2022). The analytical technique employed in this study (Py-GC-MS) does not, by contrast, make it possible to infer about the original size of the microplastics investigated. Similar considerations can be raised for the inhalation pathway, despite the structure of the respiratory epithelium is different. In the upper airway, due to the thickness of the lung lining fluid, mucociliary clearance for particles >1 μm is likely. For particles <1 μm, uptake across the epithelium is possible (Wright and Kelly, 2017). If microplastics particle can reach deeper airway, where lung lining fluid is thinner, an increased possibility of translocation can be assumed also for bigger particles (< 10 μm). Here, particles are particularly susceptible to macrophage clearance. Regarding the inhalation pathway, a major role seems to be played by fibers-shaped microplastics and occupational exposure. It was reported that some fibers have the ability to avoid clearance mechanisms and persist in the lung (Wright and Kelly, 2017); fibers 15−20 μm long cannot be efficiently cleared from the lung by alveolar macrophages and the mucociliary. Occupational exposure to plastics fibers has been well documented in the past (Warheit et al., 2001; Muittari et al., 1978; Kramer et al., 1994; Eschenbacher et al., 1999; Boag et al., 1999; Warheit et al., 2001) but updated data is lacking. In textile industry workplaces, workers can be exposed to many orders of magnitude

higher concentrations of suspended plastics in the air than those in the environment. However, the health outcomes evidence may help to understand the possible biological effects of fibers and may suggest exposure consequences if the amount of microplastics in the air will be higher in the future.

2.1.5.2 Hypothesized Toxicological Mechanisms

In recent years, the potentially toxic effects on humans were investigated. Several toxicological patterns have been hypothesized for microplastics from environmental sources, following their manufacturing and chemical/physical properties. First of all, toxicological patterns can be divided into direct damage mechanisms and indirect damage mechanisms (Figure 6). Originally, only direct damage was assumed, as the consequence of the interaction between plastic particles and human cells, tissues and organs (particle damage). As previously stated, the destiny of microplastics in the human body after ingestion/inhalation mainly depends on the possibility of translocation and intake which is in turn influenced by particle size. For particles > 150 µm translocation and intake is negligible and only local Gastro-intestinal (GI) effect can be assumed. For particles < 150 µm, especially for those < 10 µm, translocation appears possible, and effects could be extended to other systems. Only few *in vitro* and *in vivo* toxicity studies, summarized by Paul et al. (Pau et al., 2020), have been conducted so far for understanding particle damage of micro and nanoplastics. However, both *in vivo* and *in vitro* studies, have scientifically disputed results (Paul et al., 2020). Cellular toxicity-related effects (e.g., cytotoxicity) have rarely been detected, mainly in overload situations and with rather unspecific endpoints. The *in vivo* studies reported that smaller size microplastics (< 20 µm) ingested by mice can accumulate in the digestive tract causing damage to the intestine barriers and disrupting the balance of intestinal flora. This could also be the results of plastic accumulation in phagocytes of gut tissue (Wright and Kelly, 2017). However, also extra GI effects were observed as inflammation, oxidative stress and changes in metabolism in liver. Some studies, not mentioned by Paul et al. 2020, focused also on the reproductive toxicity of microplastics < 5 µm (Xie et al., 2019; Hou et al., 2021; Liu et al., 2022). Findings underlined, even if no dose-response relationship was assessed, a correlation between microplastics consumption in mice and changes in gamete (oocytes and sperm cells) quality and quantity. Oxidative stress due to ROS formation was the most frequently hypothesized cause. These studies investigated microplastics toxicity mainly as particles, without considering the possible role played by additives, monomers/oligomers and sorbed chemicals. Microplastics can be indeed a source of a toxicity that can be defined as secondary, in which plastics act as carriers of other pollutants. Monomers/oligomers could be free to leach from plastics when industrial polymerization reaction does not proceed to full completion; plastic weathering may also degrade plastic polymers into monomers and oligomers resulting in diffusion in the environment and the possibility of reaching drinking water. The contribution of this phenomenon on human health, however, is still not understood (WHO, 2019). A similar situation can be observed for additives and sorbed chemicals, with the remarkable difference that these compounds are mainly non-covalently bonded to plastics and are therefore more easily released in the environment due to weathering or directly inside in the human body acting as a *"Trojan horse"* (Hu et al., 2022a; Zuri et

al., 2022). These compounds could be indeed ingested as such as product of water/food contamination or released during digestion processes from microplastics. Plastics additives of concern to human health are, for example, phthalates, bisphenols, brominated flame retardants, triclosan, and organotins. Few studies have described the leaching of additives (bisphenol A and nonylphenol) from plastics in the intestinal tracts of worms and fish (Koelmans et al., 2014). While additives are part of the structural composition of microplastics, sorbed chemicals are the consequence of the interaction of these compounds with the environment. The high surface area-to-volume ratio of these compounds facilitates indeed, the accumulation of organic contaminants (as hydrophobic Persistent Organic Pollutants, POPs, and pharmaceuticals products) and metals (as chromium, zinc or lead) on microplastics surface. Sorption of chemical pollutants on microplastics surface has been widely investigated and demonstrated, as recently reviewed (Yu et al., 2019; Atugoda et al., 2021; Cao et al., 2021). POPs commonly linked to microplastics are organochloride pesticides and polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and other benzene-ring derivatives. Regarding drugs, antibiotics were the most studied compounds, followed by non-steroidal anti-inflammatory drugs (NSAIDs) and β-blockers. In some cases, very high sorption capacities were observed (Atugoda et al., 2021). Since most drugs are usually more hydrophilic than hydrophobic POPs, their mechanisms of interaction with microplastics are probably different (Atugoda et al., 2021). More hydrophilic compounds exhibit greater adsorption affinity toward polar and amorphous polymers while hydrophobic pollutants prefer hydrophobic surfaces. Hence, pharmaceutical compounds tend to adsorb on weathered and aged microplastics more than on pristine microplastics because of induced polarity on the plastic surface. There are no data to quantify the relative importance of microplastics in contributing to toxicity chemical additives in the environment, including drinking-water. On the contrary, the adsorption of contaminants on microplastics surface it is a well-understood phenomenon although the role it might play in toxicity from microplastics it is still unknown. The release of chemicals from microplastics in the GI tract depends indeed on the interaction between the chemical and microplastics particle, as well as the properties of the surrounding environment. For instance, if the gut fluid already contains the pollutants, the reverse phenomenon could be observed, with microplastics removing contaminants from the medium (WHO, 2019). Microplastics' relatively hydrophobic nonpolar surfaces can be also prone to biofilm growth. In this sense, microplastics could act as vectors for the long-distance transport of pathogens and increased transfer of antimicrobial resistance. Biofilm also changes microplastics characteristics such as surface properties, density and size (Wang et al., 2021). In the literature, there are limited data on the distribution of microplastic-associated biofilms, especially in drinking-water. Current knowledge on the adverse effects of microplastic-associated biofilms in fresh water and drinking water is also limited (US EPA, 2016; Li et al., 2018).

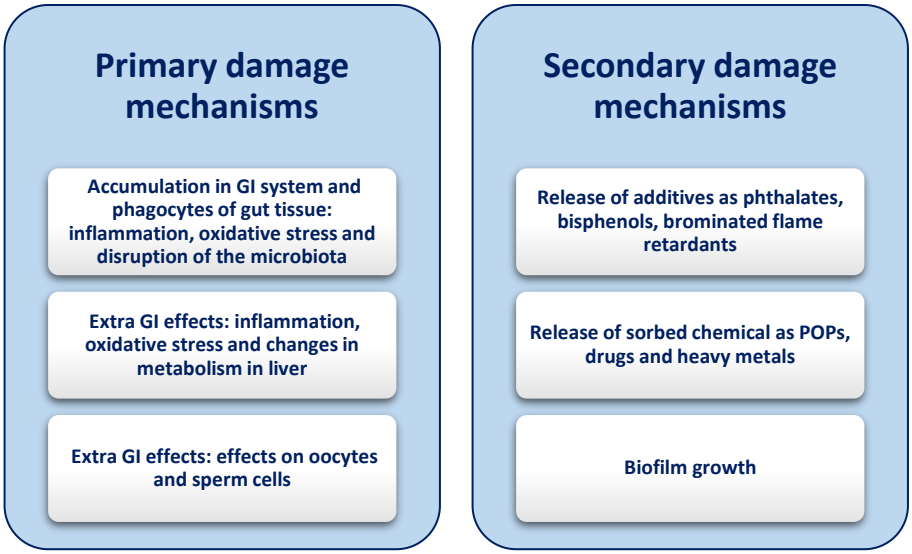


Figure 6. Assumed damage mechanisms for microplastics following *in vitro* and *in vivo* studies on animal models.

2.2 ANALYTICAL APPROACHES FOR ANALYSIS OF MICROPLASTICS IN INLAND WATERS

2.2.1 General Remarks

Several analytical protocols are available for the identification and quantification of microplastics, most of which are similar to those used for marine waters, although there may be considerable differences due to the intrinsic characteristics of the individual matrix. The two main parameters influencing the development of the analytical protocol are indeed the typology of the matrix under investigation and the size of microplastics. These affect the choice of sampling method, pre-treatment reagents and ultimately, the analytical technique involved. The most complex environmental matrices (e.g., wastewater) and biological ones both share the necessity to remove high quantities of interferers that may hinder the analysis; on the other hand, the cleaner ones usually require less elaborate analytical protocols. Certain matrices, especially environmental ones, also require a distinctive sampling method in order to achieve an estimation of the amount of microplastics on extended volume units. Regarding size, it is essential to select an analytical size range from the beginning, as this affects the number and typologies of sampling and analytical techniques applicable. Adopting a sampling method that allows working on a more restricted dimensional range simplifies the following steps even if the proposed size range for their definition is not fulfilled. The typical workflow for microplastics analysis includes a sampling phase, a pre-treatment step and a purely analytical phase (Figure 7). These steps can be very different when considering the analysis of microplastics > 300 μm or microplastics in the "small microplastics" size range. The first ones represent the evolution of the environmental monitoring campaigns in the 1980s and as such follow the methods and requirements of research vessel campaigns while the second ones represent the result of targeted research. Regarding inland waters, differences in microplastics analysis appeared more significant between surface water, usually following procedures similar to seawater,

especially for microplastics > 300 µm and groundwater/drinking water, which involves dedicated developed methods.

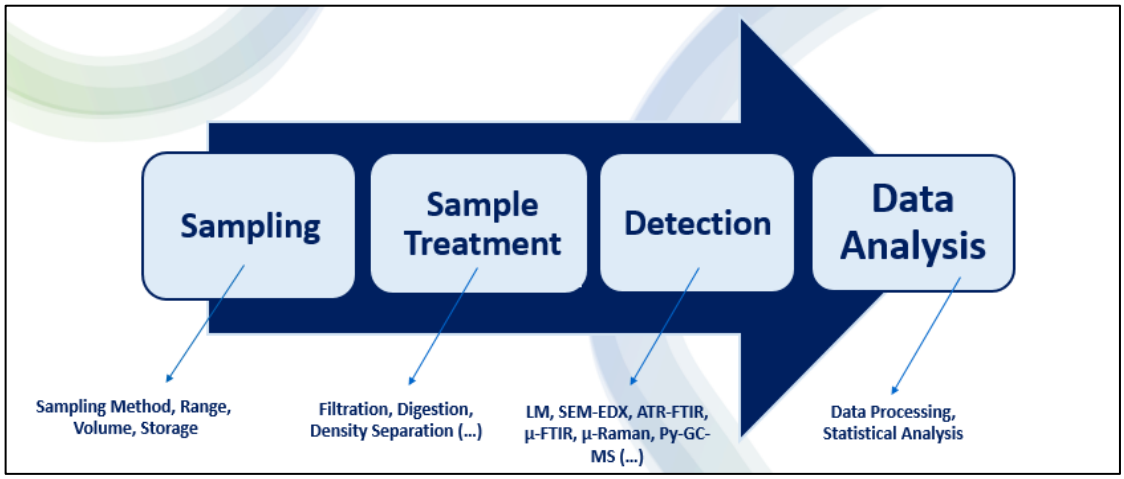


Figure 7. Workflow used in analytical protocols for microplastics analysis.

2.2.2 Sampling

Sampling of microplastics in inland water, as well as for sea water, is one of the most sensitive steps in the whole analytical procedure as it strongly influences the study representativeness. An effective sampling method should ensure the analysis of appropriate water volumes in a short period of time and be effective both for larger and smaller particles. The sample size is indeed one of the most important parameters when analysing microplastics in inland waters. The sample is considered representative when it provides a snapshot of the sampling location with results which reflect the true concentration of microplastics in that specific area. The choice of the appropriate volume for analysis, i.e., the volume of water that should be sampled, is affected by several variables. Once the instrumental availability has been checked, the first criterion to be considered is the statistical one. A sample volume that is too low reduces the chance of finding particles, reduces the power of a study and increases the margin of error (Koelmans et al., 2019). Secondly, specifications of the size cluster and of the water type must be considered in order to perform an appropriate analysis. Some studies showed that smallest particles are actually the most abundant (Cabernard et al., 2018; Weis et al., 2020), hence smaller volumes will be required if the study aims to obtain information on particles smaller than 100 µm. If, on the other hand, only larger microplastics (>100 µm) are investigated, a larger volume will be required in order to avoid underestimation. These considerations should also be related to the type of water on which the analysis is conducted. Koelmans et al., in 2019 (Koelmans et al., 2019), evaluating over 50 different studies, suggested optimal sampling volumes for each type of water studied. These volumes represented guidelines for sampling larger microplastics (> 100 µm) (Table 3). For surface waters, given the expected low concentrations of microplastics > 100 µm, the opportunity of sampling high volumes and the higher susceptibility to atmospheric temporal variables (Ryan et al., 2009), the reference volume was set to 500 L. Larger volumes should also be required if sampling takes place in remote and

hypothetically less contaminated locations, as for groundwater. Regarding WWTP, the authors distinguished between influents and effluents. Influents, due to their origin, are considerably more contaminated than other water matrices and therefore a volume of 1 L is considered adequate. Conversely, on WWTP outlet, similarly to surface water, the recommended volume is 500 L. For drinking water, the recommended sampling value was set to 1000 L, as this water type undergoes specific potability treatments and the expected microplastics concentration is much lower (Mintenig et al., 2019). These guidelines, as the authors stated, do not represent absolute limits. Indeed, there are often issues with some sampling methods (the most common is filter clogging, which usually occurs with high organic content waters) that do not allow this ideal condition to be achieved. In these cases, it is recommended to sample water as far as possible, reporting the volume of water achieved in the data sheet (Koelmans et al., 2019). There are currently no specific guidelines for sampling microplastics < 100 μm (SMPs). If the research objective is only the analysis of SMPs, smaller volumes of water can be sampled (Koelmans et al., 2019). However, as can be seen in the literature (Martellone et al., 2021), in some studies the range of microplastics investigated is also extended to larger microplastics or is not well clarified, compromising the reliability of the research. This represents the main source of data heterogeneity in literature (Wang et al., 2021; JRC, 2022) and one of the most important issues in the analytical field. In any case, the collection of these volumes should be achieved in a sustainable timeframe as the analysis is very time consuming by itself. The most common sampling methods, such as those based on nets or filters, do not allow indeed the sampling of microplastics over the entire range proposed for their definition (e.g., 1 μm - 1 mm). The process on which they rely, a single mechanical filtration, limits sampling to all above the mesh size, resulting in a loss of a considerable fraction of particles (especially SMPs). More sophisticated methods such as sieves placed in series (Ziajahromi et al., 2017; Tamminga et al., 2019; ASTM International, 2020;) and continuous-flow centrifugation (Hildebrandt et al., 2019; Leslie et al., 2017; Dümichen et al., 2017), allowed to increase the size range of sampled microplastics, but they still require further studies to verify their effectiveness, especially for SMPs. An alternative to avoid, at least for the sampling step, particle losses, may be to collect water in containers as bottles. However, a certain filtering step is always required in microplastics analysis so, sooner or later a certain filter medium should be chosen.

WATER TYPE	RECCOMENDED VOLUMES FOR MPs > 100 µm (Koelmans et al., 2019)	SAMPLING TECHNIQUES	MESH SIZES	MAIN ISSUES
SURFACE WATER	500-1000 L	Nets Filtration systems Bottles	Nets: 80-500 µm Filtration systems: < 100 µm	Atmospheric agents Research Vessel (if used) Seasonality
GROUND WATER	> 1000 L	Filtration systems Bottles	Various (3 µm - 450 µm)	Filter clogging
WWTP INFLUENT	1 L	Filtration systems Bottles	Filtration systems: < 100 µm	Filter clogging
WWTP EFFLUENT	500 L	Nets Filtration systems Bottles	Nets: 80-500 µm Filtration systems: < 100 µm	Filter clogging
DRINKING WATER	1000 L	Filtration systems Bottles	Filtration systems: 2,5 - 10 µm	Filter clogging

Table 3. Sampling methods and recommended volumes for each type of water (adapted from Martellone et al., 2021).

Several sampling methods for microplastics in inland waters are available. These approaches tend to resemble each other, especially those for surface water that are very similar to the ones for sea water. Sampling method should be selected according to investigated size class and to matrix nature. Most of the currently available sampling methods are designed for sampling microplastics > 100 µm and are not specific for SMPs. However, in certain cases, they are intrinsic suitable to SMPs (e.g., discrete sampling) or can be adapted for SMPs sampling by merely changing the mesh size of the filter medium (e.g., in filtration systems). Nevertheless, in most cases, the intrinsic characteristics of SMPs are not considered when developing a sampling system and these may not be effectively recovered even if they are included in the analysis. If only SMPs are investigated, an effective and targeted sampling method should therefore be developed. Since there are not currently standardized protocols for sampling SMPs, the development of a specific one should be compulsory as much as a comparison, in terms of recovery rates, with sampling protocols employed for microplastics > 100 µm. Regarding matrix type, in large water bodies (lake, navigable rivers) for logistical reasons, there are more options to choose from. In the case of groundwater, small unnavigable rivers and studies involving WWTPs and DWTPs instead, the availability of sampling techniques is restricted due to the intrinsic characteristics of the medium. For instance, in these areas, the employment of sampling nets could be difficult (often impossible) and

alternative sampling methods such as those based on *in situ*-filtration or bottles are usually preferred. Another parameter to consider, is the instrumental availability. Equipment for microplastics sampling, such as nets, pumps or filtration systems, is often expensive, resulting in a restriction for the entire study. On the other hand, the availability of a pressurized system or the involvement of smaller-capacity bottles allows a great reduction in costs. Sampling techniques for microplastics analysis can be divided into two major categories: *in situ filtration* sampling and *sampling without filtration* (discrete sampling) (Table 4). The first one involves preliminary filtration of considerable volumes of water and is therefore the only ones suitable for microplastics > 100 μm; *in situ filtration* includes net sampling and sampling with filtration systems. The second one does not involve preliminary filtration and requires limited volumes of water. In net sampling, microplastics are collected by filtering large volumes of water by specific sampling nets, dragged by research vessels or attached to hard support in the interested area (e.g., lake, river). During sampling, microplastics accumulate at the end of the net in different types and quantities depending on the mesh opening. Usually, mesh opening for microplastics sampling is in the order of 300 μm, but it is not uncommon to find studies in the literature that make use of larger (less frequently, e.g., 500 μm) or smaller (more frequently, 80-153 μm) openings (Martellone et al., 2021). Nets with 500 μm mesh size allow to easy collect larger microplastics by filtering high volumes without clogging. On the other hand, nets with 80-153 μm mesh size allow to collect of even finer fibres but are more prone to clog and require, if sampling is carried out with a research vessel, a lower trawling speed. 300-333 μm mesh size is indeed considered a fair compromise between sampled size classes and water volumes adequate for the purpose (Martellone et al., 2021), although it still entails severe limitations in order to have a complete view of the distribution of microplastics in water. This compromise indeed entails the exclusion of SMPs from the count as they are usually not retained by nets and still produce very different results, even in apparently similar studies, as observed by Li et al. (Li et al., 2018). Such sampling is, in fact, strongly influenced by a series of parameters as chemical/physical characteristics of the water studied, weather conditions (e.g., wind speed) and those related to navigation as trawling speed, net distance from the vessel and transect length. However, the stronger limitation of the net sampling method is technical. It is not feasible to carry out such studies in non-navigable areas. Studies involving WWTPs or DWTPs influents or effluents necessarily require *in situ* filtration approaches or sampling with containers. The prototype of the net used for microplastics sampling is the Manta Net. The Manta Net, described for the first time in 1981 (Brown and Cheng, 1981), is a modified Neuston Net that was designed to provide a constant depth of immersion and thus sampling. The net is equipped with two plywood, stainless-steel or aluminium wings conferring the characteristic shape of an aquatic manta ray and providing an effective stabilisation on the water surface when trawled. These structures are connected to an aluminium or stainless-steel frame in which a net with variable mesh opening (usually 100-500 μm) is anchored; water passes in the centre of the frame. The deployment of Manta Net, unless automated systems are available, usually required the cooperation of two people at least. Manta nets are suitable for sampling quiet sea water, river water and lake water with a maximum

trawling speed of 5 knots and recommended 2-3 knots (Brown and Cheng, 1981). At higher trawling speeds or in the presence of high waves, the Manta Net could be destabilised, invalidating the sampling. Several other types of sampling nets similar to Manta Net are available, each with their own their own advantages and drawbacks (Martellone et al., 2021). In sampling with filtration systems, the water is conveyed from the sampling area to a cartridge/disc filter, to a series of cartridge/disc filter, to a sieve or a series of sieves with variable mesh size inserted in a closed system and then filtered. Water is usually conveyed to the filtering medium thanks to a pumping system; in case of pressurized systems the filtration medium can be directly connected to the tap and the pump system is not necessary. Although it is feasible for drinking water, which is already in a pressurized system, it is applicable to surface water, groundwater and wastewater only if sampling is carried out inside a treatment plant where water is in a pressurized system too. Smaller mesh sizes (< 100 µm) are usually employed for filtration system samplings compared to those used in nets. It allows to recover also smaller particles but in many cases clogging was observed, resulting in a significant reduction in the overall volume of water filtered. The choice of using multiple filtration media inside the filtration system can provide some benefits as it allows to separate microplastics in different clusters size already at sampling time and may decrease the clogging rate. However, even with this configuration, clogging may occur and represents the major limitation of this kind of sampling. Clogging usually occurs with smaller mesh sizes and water with the high organic continent as wastewater but it was also observed for cleaner media such as drinking water (Ziajahromi et al., 2017; Mintenig et al., 2017; 2019). Clogging does not allow sampling to be completed with the desired volumes of water. This issue, especially for microplastics > 100 µm may not allow an accurate estimation on microplastics contamination and obtained data may not be comparable with those obtained in other studies. A potential solution could be changing filters when they are close to clogging. However, sometimes filters tend to clog rather quickly, especially with particularly turbid water (e.g., those with a high content of organic contaminants or subject to algal blooms) and therefore this operation is not always possible. In addition, changing filters during sampling could lead to particles losses and contaminations. In these cases, the only solution is to install preliminarily larger mesh sizes and sample of SMPs with another sampling procedure. Sampling systems found in the literature appear very different from each other, both in terms of construction and in terms of their characteristics and dimensions. In any case, they are generally voluminous devices, especially those sampling wastewater, requiring more than a single person for operations. However, if a tap direct connection is possible and a pumping system is not required, operations are much simpler. Sampling systems are customized devices and, unlike sampling net that is usually commercialised with the same dimensions, cannot provide reproducible results. Sampling with filtration systems is strictly necessary if those based on nets for microplastics > 100 µm are not suitable. For the option of installing filters below 100 µm and the ability to filter high volumes of water, filtration systems can be very useful for understanding the distribution of smaller particles (SMPs) and fibres in water. Microplastics can also be retrieved by first collecting water in containers and filtering it later (discrete sampling). These approaches do not allow to collect

high volumes of water, but they can be adequate if the target of the analysis is SMPs (Pivokonsky et al., 2018; Koelmans et al., 2019). Discrete sampling is indeed the most frequently used sampling methods for the study of SMPs in wastewater or drinking water. Water can be collected in this way through stainless steel bucket, glass bottles or specific samplers such as Niskin bottles and Van Dorne bottles (Corami et al, 2021; Covernton et al., 2019; Khalik et al., 2018; Zhu et al., 2019).

SAMPLING TECHNIQUES	ADVANTAGES	DRAWBACKS
	***IN SITU* FILTRATION**	
NETS	- Suitable for microplastics > 100 µm sampling in navigable basins - Different nets and mesh sizes available, including standardised ones - Clogging rarely occurs for > 100 µm mesh size	- Require navigable water bodies - Does not allow SMPs sampling - Sampling influenced by weather conditions and those related to navigation as trawling speed, net distance from the vessel and transect length
FILTRATION SYSTEMS	- Suitable also for SMPs thanks to filters with smaller mesh sizes - Suitable for each water type with appropriate adjustments (e.g., pump systems) - Possibility of multiple filtrations	- Clogging is common also for cleaner media - Require design studies as filtration systems are built in-house - Results are not easily comparable
	SAMPLING WITHOUT FILTRATION	
SAMPLING IN CONTAINERS (DISCRETE SAMPLING)	- Quick, easy and inexpensive - Suitable for SMPs - Filtration can be carried out later	- Only small volumes of water retrieved if it is not possible to cumulate samples - Not useful for microplastics > 100 µm assessment

Table 4. Sampling methods for microplastics analysis in inland water. Adapted from Rapporti ISTISAN 21/2.

Samples can be stored using different strategies before the analysis, depending on the sampling method. The material sampled by nets or sieves is usually collected inside glass containers suitably covered for preventing contamination. Disc or cartridge filters from filtration systems, on the other hand, are usually placed in previously decontaminated beakers or Petri dishes covered with aluminium foil after being removed from their housing. Samples from surface water or WWTPs are sometimes treated with

fixatives agents during storage (Moore et al., 2011; Koelmans et al., 2019). Fixatives decrease the evaporation rate of volatile components of the sample and improve its stability. In microplastics analysis, fixatives are mainly employed for preventing the decomposition of organic matter potentially releasing unpleasant odours when the sample is opened. Fixative should be inert towards microplastics in the sample. The most commonly used fixatives for microplastics samples are ethanol and isopropyl alcohol. In literature are also reported acetaldehyde and formalin. Finally, samples of all types should be stored away from sources of light and heat (possibly cooled) and opened only inside the laboratory under a previously decontaminated laminar flow hood.

2.2.3 Sample Pre-Treatment

2.2.3.1 Introduction to Sample Pre-Treatment

Sample from inland water, similarly to those obtained from sediments or sea water, are often rich in interferes from the matrix. Interferes are mainly represented by substances adsorbed on plastics polymers (natural, synthetic origin or biofilms) and particulate matter. Such interferers can change the bulk density of polymers (Morét-Ferguson et al., 2010; Eerkes-Medrano et al., 2015), promoting the formation of aggregates causing problems in microplastics separation from the matrix and prevent their correct identification by spectroscopic techniques. Before proceeding with the analysis, it is thus necessary to develop specific pre-treatment methods in order to remove possible interferers and to extract microplastics from the matrix. The sample treatment method is developed according to the matrix properties, to the sampling method chosen and the subsequent analytical technique (Figure 8). In general, samples from more complex matrices require more sophisticated pre-treatment procedures (Koelmans et al., 2019) however, at the same time, too vigorous pre-treatment should be avoided as it may degrade the microplastics in the sample and prevent their correct identification/quantification. Various pre-treatment protocols are available in literature, such as those from the National Oceanic and Atmospheric Administration (NOA-A, 2015) and the Drinking Water Inspectorate (DWI) (Drinking Water Inspectorate, 2019). Although standard procedures differ in many details, common strategies can be observed. Filtration, digestion and extraction are the major recurring procedures followed by a series of less conventional routes as e.g., staining. These procedures are carried out in different chronological order prior to instrumental analysis. However, a common pattern can also be identified. “Solids” samples from nets and filtration systems, if not involving particles > 100 µm analysed ATR-FTIR spectroscopy, are usually subjected to an additional resuspension step in order to carry out other treatments. Resuspension is usually carried out with solvent extraction (Mintenig et al., 2019) or reverse flow flush step (Strand et al., 2018); a sonication step can also be employed (Prata et al., 2019; Lusher et al., 2020) but its employment should be carefully evaluated given the possibility of breaking up plastic (Löder et al., 2015). Only when samples are in a suspension form (“liquid sample”) can be indeed subjected to extraction, digestion, filtration or staining. Filtration is usually the final step before the instrumental analysis as it is necessary to concentrate the particles on a suitable medium for the analysis.

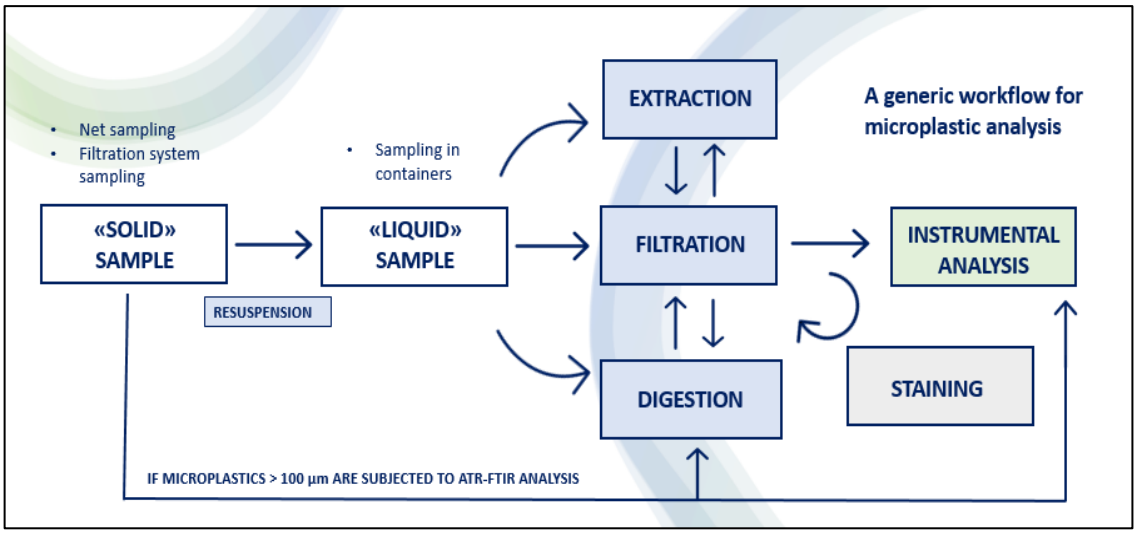


Figure 8. Generic workflow for sample treatment in microplastics analysis.

When developing a digestion, extraction or a filtration procedure for microplastics analysis, as for sampling, is necessary to consider two major parameters as differences in microplastics size and temperature. Particles of smaller sizes are for instance more susceptible to degradation by digestion reagents and high temperatures, could not be successfully extracted with a hypersaline solution and staining may not be effective. In literature, these differences are frequently not considered, and pre-treatment protocols were developed using only bigger particles as a reference. Temperature can also be considered an important key parameter. Some pre-treatment techniques such as Wet Peroxide Oxidation (WPO), enzymatic digestion and drying generally employ high temperatures. In a study about the role of temperature on polymers 125 µm - 1 mm size (Munno et al., 2018), it is suggested to remain below 60 °C to avoid possible polymers degradation, especially of microbeads. Koelmans et al. (2019) suggested a more restricted safety limit, i.e., to stay below 50°C. Several different authors (e.g., Claessens et al. 2013; Drinking Water Inspectorate, 2019; Al-Azzawi et al., 2020) agree on the need to employ "mild" pre-treatment methods for microplastics analysis in order to not denature/degrade plastics. If degradation is observed also for bigger particles, is likely to occur more frequently for smaller microplastics if similar temperatures are used. Regarding temperature-related issues in microplastics analysis, is also important to consider the glass transition temperature (T_g) of the polymers under investigation, especially those that are ubiquitous in the environment such as nylon (transition temperature between 37 °C and 55 °C). For instance, even a temperature of 50 °C, usually considered safe, can contribute to degrade/denature nylon, preventing its recognition and leading to underestimations during particles/fibres count (Collard et al., 2015; Corami et al., 2021; 2022; Rosso et al., 2022).

2.2.3.2 Digestion

Digestion is carried out in order to decompose organic interferers adsorbed on microplastics or contained as such within the sample, as it hinders filtration procedures and the recognition of microplastics.

Digestion should be at the same time effective in the degradation of interferes and not aggressive to polymers. Reagents for digestion should be chosen according to sample properties and size range investigated. Digestion, albeit soft, is strictly necessary for samples from surface water and WWTPs (Koelmans et al., 2019; Drinking Water Inspectorate, 2019), given the high organic content assumed. For other inland water types, digestion is sometimes not carried out, although is often recommended (Drinking Water Inspectorate, 2019). The most commonly used reagents for digestion are acid or alkaline agents, hydrogen peroxide solutions and enzymes, also used in sequence and in combination with each other. Strong acids (e.g. nitric acid, HNO_3 and hydrochloric acid, HCl) proved to be effective in the digestion of organic matter in microplastics sample but can degrade/denature certain polymers (nylon, polyester and polyethylene terephthalate, PET) or change their colour (Polyvinyl chloride, PVC and Polyvinylpyrrolidone, PVP), especially when used in high concentrations and with high temperatures (Catarino et al., 2016; Dehaut et al., 2016; Karami et al., 2017). Acid combinations have shown good digestive activity, but with clear polymer degradation (Enders et al., 2015). Alkaline agents (NaOH, KOH) showed similar effectiveness but also similar effects on polymer integrity and colour (Munno et al., 2018). Polyamides (e.g., nylon), Polyethylene (PE) (also low-density, LDPE), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polycarbonate (PC) and cellulose acetate (CA) are negatively affected by alkaline agents (Cole et al., 2015; Dehaut et al., 2016; Löder et al., 2017; Munno et al., 2018). Hydrogen peroxide (H_2O_2), as an oxidant reagent, is one of the most effective reagents for the digestion of microplastics samples. Using H_2O_2 results in only minimal polymer degradation if the treatment time does not exceed 48 hours and if the temperature is not exceeded (Qiu et al., 2016; Löder et al., 2017). Oxidising properties of hydrogen peroxide are also amplified in the presence of Fe^{2+} ions. In the presence of Fe^{2+} catalysts, hydrogen peroxide acts as a precursor to oxidising radical initiators according to the Fenton reaction. Fenton reaction proceeds spontaneously at room temperature but can be accelerated by increasing it in a process called Wet Peroxide Oxidation (WPO). However, Fenton reaction is negatively impacted by excessively high temperatures as an exothermic reaction; above 57°C is also promoted the volatilisation of the hydrogen, reducing the overall effectiveness of the process (Venny et al., 2012). Several studies using WPO can be found in the literature. The NOA-A (NOA-A, 2015) and the DWI (Drinking Water Inspectorate, 2019) suggest adopting WPO for samples from surface water and WWTPs, but it is not uncommon to find it also among the digestion procedures in studies involving water with lower organic content. However, WPO is not suitable for SMPs analysis as the temperatures involved have a real chance of degrading particles. Instead, enzymatic digestion can be considered an alternative or complementary digestion technique to those already mentioned. It is a less aggressive technique for polymers and safer for the operator, since reactions with interferes do not show high reactivity, unlike, for example, WPO. However, it is more expensive than traditional methods and is limited to a few specific compounds given the selectivity of these catalysts. Enzymes have also a well-defined working temperature (often around 50 °C) and a pH range that must be complied in order to achieve optical organic content removal. Another issue of using

enzymes is the long incubation time, especially when multiple steps are carried out leading to additional sample manipulations necessary with an increased probability of contamination (Di Mauro et al., 2017; Lee et al., 2021). In addition, if multiple enzymatic digestion steps are performed, the risk of contamination significantly increases as supplementary sample manipulation and washing steps are carried out. Enzymatic digestion is often used in combination with H_2O_2 digestion for samples from sea water, surface water, WWTPs and as a DWI-recommended purification technique (Drinking Water Inspectorate, 2019) for samples from other types of inland water as groundwater and drinking water, which may still have traces of biofilm that can hinder visual inspection. Proteinase K showed a digestion efficiency of up to 97% at 50°C in seawater samples (Cole et al., 2015); a sequential use of protease, cellulase and chitinase interposed with WPO showed of 98.3% for the same matrix type (Löder et al., 2017). Very high digestion (72-88%) was also obtained by using only enzymes (trypsin, collagenase, and papain) although the matrix taken as a reference was of biological one (Courtene-Jones et al., 2017b). Sodium dodecyl sulfate (SDS), an ionic surfactant capable of denaturing proteins, disrupting cell membranes, and promoting the detachment of organic material from microplastics and microplastics themselves from filters, is often used in conjunction with enzymes or other reagents (Enders et al., 2015). Extraction techniques are employed to isolate microplastics from the medium where they were dispersed. In the past, a widely used extraction technique for microplastics > 100 µm was density separation with hypersaline solution. Density separation is a gravimetric separation technique which allows the separation of microplastics from heavy interferers (especially inorganic ones). This procedure is mainly used for sewage sludge and sediments, due to their high inorganic content and viscous consistence, but it was also used in several studies on samples from dirtier waters, such as wastewater and surface water (NOA-A, 2015; Fischer et al., 2016; Ziajahromi et al., 2017; Mintenig et al., 2017). It is usually performed after the digestion step and consists of mixing the solution obtained during the digestion process with a hypersaline solution. The resulting solution is then placed in a separating funnel, allowing the heavier components to settle. This technique exploits the tendency of microplastics with densities close to water (0.8 - 1.4 g/cm^3) to float in the surface (flotation) when exposed to a hypersaline solution; sediments and other interferers tend, instead, to precipitate. The main problem with density separation is that not all microplastics can be effectively retrieved from the matrix and is as effective to water matrices as densities are similar to those of hypersaline solution. Fluorinated polymers, elastomers and even some rubbers, as they are much denser than other microplastics (density usually > 1.4 g/cm^3), do not float with hypersaline solution treatment and are lost during the extraction (Lusher et al., 2020). Furthermore, SMPs may not have sufficient hydrostatic pressure to be separated by hypersaline solution and bubbles formed during the flotation process may further interfere with their separation (Lusher et al., 2020). Another issue related to density separation is observed after the filtration of the solution used for separation. Some residues may adhere to the filter surface and even form crystals after drying, hindering the subsequent quantification and identification of polymers (Corami et al., 2021). Hypersaline solutions are usually prepared with sodium/zinc halides (ZnX_2) or potassium formate

(HCOOK) on the basis of availability and separating ability. According to an intercomparison study on microplastics in sediments (Quinn et al., 2016) separating ability appears to be higher as higher is the final density of solution (saturated solutions). For instance, $ZnBr_2$ and $ZnCl_2$ (1,7 g/cm^3) presented higher particle recoveries than NaCl (1,2 g/cm^3). Specifications of salts used for density separation were accurately described in Rapporti ISTISAN 21/2 (Martellone et al., 2021). An alternative to density separation for microplastics extraction (Table 5) is oleo-extraction. Oil extraction is a promising extraction method exploiting the lipophilicity of polymers over water and interferers contained in the sample. Oleo- extraction was firstly employed in 2017 by Crichton et al. (Crichton et al., 2017) in a pilot study regarding sediments demonstrating higher recoveries of canola oil over density separation with NaI and $CaCl_2$. Different oils provided different results as observed with canola oil, castor oil, olive oil and sunflower seed oil (Mani et al., 2019; Lechthaler et al., 2020; Scopetani et al., 2020; Corami et al., 2021; 2022). In Lechthaler et al. tests (2020), castor oil achieved the highest recovery rate (99.9%), followed by canola oil (96.2%) and olive oil (93.5%). Similar results were obtained in Mani et al. test (2019), in which castor oil produced recoveries of approx. 99%. Olive oil showed good recovery rates in Scopetani et al. (2020) experience: low, medium and high-density polymers reached a mean recovery rate of 90%, 97% and 95% respectively. Sunflower seeds oil in Corami et al. experiments (2021, 2022) showed ≫95% recovery rates. Oil extraction is also reported to be more effective than density separation for smaller particles (Lechthaler et al., 2020). The popularity of oil extraction from sediments and water matrices grows exponentially in recent years. Oil extraction was preferred over density separation, especially for sediments and biological matrices, for its shortest processing time, lower cost per sample and because its efficiency is relatively independent from density of plastic particles (Crichton et al., 2017). The latest mentioned advantage allows microplastics to be recovered from the medium regardless of their density (e.g., fluorinated polymers).

DENSITY SEPARATION		OIL EXTRACTION	
ADVANTAGES	**DRAWBACKS**	**ADVANTAGES**	**DRAWBACKS**
- Tried and tested method, especially for sediments and biota. - Several salts with different density a saturated solution.	- Negligible effectiveness for water and SMPs. - Fluorinated polymers, elastomers and even some rubbers, are lost during the extraction.	-Promising technique also for water matrices. -Shortest processing time, lower cost per sample and independence from plastic density.	-More studies are needed to confirm its efficiency. - Oils composition is variable.

Table 5. Differences between Density Separation and Oil Extraction. Modified from WHO, 2019.

2.2.3.3 Staining

A secondary pre-treatment technique common for water samples is tagging of plastics polymers with an appropriate dye (Shim et al., 2016; Erni Cassola et al., 2017; Maes et al., 2017). Staining allows microplastics to be distinguished from other particles in the sample, simplifying microscopic counting without the necessity of polymer identification. In order to achieve this purpose, dyes should effectively bind to each type of microplastics in samples, do not interact with the remaining material or the filter, and produces a clear colour when visual inspection is carried out. Different dyes have been tested for this purpose, but only few of them have proven to be effective and selective. Unsatisfying results are reported for lipophilic dyes such as Eosin B, Hostasol® Yellow 3G, Oil Red EGN and Rose Bengal (Maes et al., 2017; Lares et al., 2019). Currently, the most promising dye for microplastics is Nile Red. Nile Red is a lipophilic fluorescent dye useful to stain lipids in biological samples (Greenspan et al., 1985) and suitable for synthetic polymers (Jee et al., 2009). Some authors (Shim et al., 2016; Erni-Cassola et al., 2017; Maes et al., 2017; Tamminga et al., 2019) have developed sample incubation protocols with promising results. The main drawback of Nile Red is due to its interactions with lipid impurities in samples, returning false positives. Furthermore, some polymers such as PC, polyurethane (PU), PET and PVC produced weak fluorescence signals (Erni-Cassola et al., 2017) and fibres proved to be difficult to stain (Tamminga et al., 2019). Therefore, count with Nile Red could provide underestimation in microplastics number. In any case, even assuming high efficiency of digestive methods and selective staining, a confirm of polymers identity based on spectroscopic methods or mass spectrometry is always required to discard false positives, as stated by ECHA (ECHA, 2019). For this reason, staining alone cannot provide a clear picture of microplastics content in certain matrices and should be considered only a support/screening technique.

2.2.3.4 Filtration

Filtration is the leading technique for microplastics sampling employing to remove reagents and other potential interferers. A filter also represents the substrate/support on which analyses by Fourier transform infrared microscopy (MicroFTIR or μ-FTIR) and Raman microscopy (MicroRaman or μ-Raman) is usually performed. The filter, if used for this purpose, should therefore interfere as little as possible with the analytical techniques involved. In the case of spectroscopic techniques, the filter should transmit or reflect the incident radiation (Table 6) without presenting absorption at the interested wavelengths, namely those in the infrared region. Stainless steel filters, as well as glass microfiber and cellulose filters, are used exclusively for sampling or, if necessary, to filter solutions from one step of the pre-treatment procedure to the next one. They are not suitable for spectroscopic analysis because they have absorptions in the infrared region (e.g., glass microfiber filters) or because they hinder the visual inspection. Aluminium oxide filters are some of the most widely used as support for spectroscopic analysis (Velasco et al., 2020; Kelly et al., 2020). Aluminium oxide filters, available in different diameters (13 mm, 25 mm, 47 mm), are rigid and fragile filters that are particularly suitable for analysis by transmission IR spectroscopy, although they are transparent to IR radiation only in the range 1250-

3800 cm^{-1} (partial interference with fingerprint region). Aluminium oxide filters are also suitable for reflection IR spectroscopy but better alternatives in terms of the spectral quality generated are available. These filters are also suitable for Raman microscopy as do not produce characteristic signals in Raman spectra even a weak fluorescence can be observed (Käppler et al., 2015). Silicon filters are rigid, fragile and more expensive than other filters used for spectroscopic analyses. They can be used in analyses with transmission IR spectroscopy (IR-transparent in the window 4000-600 cm^{-1}) and in those with Raman spectroscopy (Käppler et al., 2015). Silicon filters have in the past shown weak vibrational bands due to impurities in the filter matrix (asymmetric Si-O-Si stretching), which can hinder analysis and must be corrected (Käppler et al., 2015). Silicon filters, in addition of being expensive, have also a rectangular shape, which is difficult to adapt to standard filtration systems (need gasket development) and are available only with a small filtration area. Silicon filter are indeed available, for now, only in one size (1 mm x 1 mm). The small size might be an advantage, as it allows the subsequent analysis of the entire filter or a considerable part of it in a short time, but it can be a problem if high volumes of sample must be filtered. High volumes may indeed lead to the overlapping of microplastics and other particles over the filters, hence silicon filters are usually suitable only for drinking water or water with low organic content (e.g., groundwater). Other suitable filters for spectroscopic analysis are those made of gold-coated polycarbonate (Cabernard et al., 2018; Von der Esch et al., 2020) and silver (Horton et al., 2021, Cunsolo et al., 2021). These filters showed good results for analysis by reflection IR spectroscopy or Raman spectroscopy. Both are more expensive than aluminium oxide filters used in transmission but do not reach the rating of silicon filters. For spectroscopic analysis can also be used zinc selenide (ZnSe) optical windows, which are not filtering media (Simon et al., 2018; Vianello et al., 2019). In this case, particles need to be manually moved on the windows for the analysis. This, for obvious reasons, is possible only for manually handable particles (> 100 µm). Filters also serve as a support for the analysis when staining techniques (Nile Red, Rose Bengal) are used for particle counting. Polycarbonate membrane filters of the PolyCarbonate Track-Etched (PCTE) subtype showed good results for the use of Nile Red. Their hydrophilic surface proved to reduce the retention of unbound dye by decreasing background fluorescence during analysis. In addition, they are translucent when exposed to ethanol, making it possible to use bright-field microscopy in addition to fluorescence microscopy (Erni Cassola et al., 2017). Finally, if scanning electron microscopy coupled with energy dispersive spectrometry (SEM-EDS or SEM-EDX) is used as an analytical technique, polytetrafluoroethylene (PTFE) filters have proven to be preferable (Pivokonsky et al., 2018).

SUPPORT TYPE	EMPLOYMENT	ANALYTICAL TECHNIQUE	FEATURES	COST PER UNIT
Stainless steel, Glass microfiber, Cellulose Filters	- Sampling and Intermediate filtration	None	- Useful only for sampling and intermediate filtrations	+
Aluminium Oxide Filter	- Filtration and Spectroscopic analysis	- Transmission FTIR microscopy - Raman microscopy	- Cheaper than other filters for spectroscopy - Available in different sizes	++
Silicon Filter	- Filtration and Spectroscopic analysis	- Transmission and Reflection FTIR microscopy - Raman microscopy	- Wider spectral window - Square shape One size available Expensive	++++
Gold Coated PC and Silver Filters	- Filtration and Spectroscopic analysis	- Reflection FTIR microscopy - Raman microscopy	- Highly reflective surface which can hinder the detection of certain particles	+++
Zinc Selenide Optical Window	- Spectroscopic analysis	- Transmission FTIR microscopy	- Good results for FTIR microscopy - Particles need to be manually moved on the windows	++
Polycarbonate track-etched (PCTE) Filters	- Filtration and Visual inspection	- Visual Inspection with Nile Red staining and Fluorescence Microscopy	- Best choice for Nile Red staining and Fluorescence Microscopy - Also useful for bright-field microscopy	+
Politetrafluoroetilene (PTFE)	- Filtration and Visual inspection	- Visual Inspection with SEM-EDS	- Best choice for Visual Inspection with SEM-EDS - Filter is made of plastics	++

Table 6. Support media for microplastics analysis. Adapted from Martellone et al., 2021.

2.2.4 Instrumental Analysis

2.2.4.1 General Remarks

Analytical techniques for microplastics analysis in aquatic environments, also employable for other sample typologies as sediments and biota, can be classified on the basis of physical variable being measured. A distinction is therefore made between *"particle-based"* techniques, which investigate the properties of microplastics as particles (type, number, size distribution, and shape) and *"mass-based"* techniques, which involve investigation on the mass of plastic present in the sample together with chemical identification of polymers (Brandt et al., 2021, Ivleva, 2021) (Table 7). The first group of techniques, currently the most popular, includes generally non-destructive techniques, frequently based on magnification methods (microscopy) coupled with spectroscopic measurements and usually requiring very elaborate sample preparation. In the group, it is therefore possible to distinguish between microscopy techniques, spectroscopy techniques, and microscopy techniques coupled with spectroscopy. These include optical microscopy, fluorescence microscopy, electron microscopy (coupled or not with EDX spectroscopy, Energy Dispersive X-ray Analysis), Fourier Transform Infrared spectroscopy (coupled or not with microscopy), and Raman spectroscopy (coupled with microscopy). Several laboratories are beginning to approach these techniques, especially infrared and Raman microscopy, in the sequential analysis of samples of the same type. The second group includes instead destructive techniques based on mass spectrometry and pyrolysis that usually require less elaborate sample pre-treatment with shorter analysis time. This group mainly includes Pyrolysis coupled to Gas Chromatography/Mass Spectrometry (Py-GC-MS) and Thermal Extraction/Desorption coupled to Gas Chromatography/Mass Spectrometry (TED-GC-MS). *Mass-based* techniques are newer and constantly evolving techniques, which still need harmonization in order to employ them to environmental samples for the purpose of routine analysis.

PARTICLE-BASED SPECTROSCOPIC TECHNIQUES (ATR-FTIR, FTIR microscopy, Raman microscopy	ADVANTAGES	DRAWBACKS
	- Possibility to retrieve several information on different descriptors as polymer identity, size and shape with a single analysis (FTIR or Raman microscopy); - Greater versatility; easy and quick analysis on single large particles (> 500 µm) with Attenuated Total Reflectance FTIR; - Less expertise required thanks to dedicated software and instrumental automation; - Calibration non required; - Typically non-destructive techniques.	- Time-consuming analysis often performed only on a sample subset (low representativeness) - Analytical limits related to particle size; - Additives not always detectable; - Elaborate sample pre-treatment usually required.
MASS-BASED TECHNIQUES (Py-GC-MS, TED GC-MS)	**ADVANTAGES**	**DRAWBACKS**
	- Reduced analysis times and the possibility to study more easily the whole sample; - Particularly suitable for particles < 100 µm and nanoplastics; - Possibility of targeting additives separating from microplastics; - Elaborate sample pre-treatment often not required; - Extensive adjustment of instrumental parameters.	- Information loss (e.g. size, shape); - Not suitable for larger particles (> 500 µm) unless special arrangements; - Expertise required for method development; - Issues in calibration and sample introduction; - Results expressed in weight of plastics; - Destructive techniques.

Table 7. Differences between *particle-based* spectroscopic techniques and *mass-based techniques*

In the literature, several analytical protocols using one or more of the previously reported analytical techniques, even belonging to different groups, can be found (Table 8). Analytical techniques can indeed be used in series or in parallel for the simultaneous determination of multiple microplastic parameters as number, size, shape and polymer identity (Hendrikson et al., 2018, Corami et al., 2020a, Jiang et al., 2020). Multi-techniques approaches are always preferred as no technique, not even coupled techniques (e.g., Raman microscopy) is currently able to characterize them in their wholeness according to commonly used definitions. On the other hand, this approach is often not sustainable for the development of a routine analysis workflow, as it requires different instrumental equipment, which is often inconsistent with other types of analysis and therefore not so attractive to individual laboratories not belonging to the research area. Criteria for selecting an analytical technique or analytical techniques for microplastics analysis are therefore dictated by the choice of properties to be investigated and the available equipment, implicitly related to the needs of the referring laboratory. A third criterion indirectly affecting the choice of analytical technique is the matrix typology. The matrix intrinsic characteristics determines the selection of sampling and pre-treatment methods prior to instrumental analysis and should therefore be considered at planning stage. Regarding the microplastics descriptors under investigation, first and foremost it is necessary to select the working size range along with the need or not to perform a count/weight determination. Some analytical techniques have indeed a size range cut-off that does not allow discrimination of particles below that value and is not possible, unless certain sampling strategies, to work simultaneously on the entire range proposed for microplastics definition (e.g., 1 µm – 1 mm for the ISO/TR 21960:2020 standard). In order to identify larger particles (> 100 µm), assuming that investigations are carried out on one particle at a time and without microscopic count, Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) can be used as a reference technique. ATR-FTIR is particularly effective for screening investigations on marine and surface water samples with sampling techniques targeting microlitter (e.g., sampling with manta net). For medium sized particles (1 mm – 100 µm) and small microplastics (< 100 µm) magnification technique (microscopy) coupled with a spectroscopic identification technique is strictly required. The magnification-spectroscopy coupling represents an optimal solution for microplastics analysis, as it allows performing a particle count together with polymer identification and observing the morphology of plastics. These coupled techniques have as its main limitation the inability to discriminate particles below a certain threshold (usually 10 µm for infrared microscopy and usually 300 nm for Raman microscopy) (Schymanski et al., 2021). However, it should be considered that < 1 µm particles are nanoscopic; nanoplastics, as nanomaterials, exhibit substantially different chemical and physical behaviour from microscopic particles. In the qualitative analysis field, an alternative to magnification-spectroscopy approaches are mass-based techniques. These techniques allow the identification of particles with the only lower dimensional threshold represented by the identification limit (LOD), but they require considerable expertise and involve the loss of some critical information. In most studies, polymer identification is also associated with a count or a weight determination

(quantitative analysis). For microplastics counting was originally employed light microscopy, but it does not allow, even with dyes, chemical identification of polymers and can therefore be used only as a preliminary screening technique for particles > 100 µm. Suspected particles with a compatible size can be selected and identified later with other analytical techniques. For an efficient microscopic counting and chemical identification for smaller particles, the two most widely employed techniques are infrared microscopy and Raman microscopy; these techniques allow an automatic microscopic counting of particles within the sample along with chemical identification of polymers. If quantitative determination by weight is desired, an alternative to the previously mentioned techniques may be Py GC- MS and TED GC-MS. However, in this case, issues related to the processes of calibration and results expression must be considered. Another microplastics descriptor of common interest is the morphological profile. Most microscopy-based or microscopy-coupled techniques allow assessing the morphology of microplastics on the support more or less efficiently. However, the choice technique in this field is electron microscopy. Electron microscopy also provides reveals of abrasions and aging signs on microplastics surface and can therefore be used to formulate hypotheses about the age of the polymers found and their origin. If plastics-related contaminants are also investigated, Raman microscopy and *mass-based* techniques may be particularly useful for the purpose. Regarding issues related to matrix typologies, it should be considered that the analysis approach, as previously reported, tends to change substantially when moving from one matrix to another, especially in the sampling and pre-treatment phase. Aquatic environments include a countless variety of different matrices, which require the implementation of distinct strategies for interferers' removal. From this point of view, the most difficult matrices for processing are sediments and water with high organic content (wastewater, surface water and seawater). For these matrices, a winning strategy could be to choose, downstream, a technique that is less affected by interferers, such as *mass-based* ones (Enders et al., 2020) or to extensively work on the sampling and pre-treatment approaches in order to reduce the matrix effect as much as possible. The second path appears to be the most widely pursued nowadays given the only recent interest in *mass-based* techniques for microplastics analysis and critical issues associated with them. In contrast, for cleaner matrices such as groundwater and drinking water, the purification process is usually less elaborate, and the matrix effect tends to be less intense. Particular attention should still be paid to the water hardness parameter, since calcium and magnesium salts can result in aggregates formation hindering the analysis, especially for spectroscopy-based techniques. The low number of particles, particularly those > 100 µm, usually found in groundwater and drinking water also increases the relevance of techniques capable of discriminating small particles; in this sense, using optical microscopy without dyes, even if only for a screening operation, should be strongly discouraged.

TECHNIQUE	TYPOLOGY	SUBTYPE	DESCRIPTORS	CUT-OFF
LIGHT MICROSCOPY (LM)/ FLUORESCENCE MICROSCOPY	*Particle-based*	Microscopy technique	- Count (screening) - Polymer identity (screening with dyes)	200 nm
ELECTRON MICROSCOPY WITH OR WITHOUT EDX COUPLING	*Particle-based*	Microscopy technique/ Microscopy coupled with spectroscopy technique	- Information about elementary composition (only with EDX spectroscopy) - Size - Shape	10 nm
ATR-FTIR	*Particle-based*	Spectroscopy technique	- Polymer type	Particles manually handable, 40 µm
FTIR MICROSCOPY	*Particle-based*	Microscopy coupled with spectroscopy technique	-Polymer type - Count - Size - Shape	5-10 µm, 40 µm for ATR-FTIR accessory attached to FTIR Microscopy
RAMAN MICROSCOPY	*Particle-based*	Microscopy coupled with spectroscopy technique	- Polymer type - Count - Size - Shape	300 nm
Py-GC-MS	*Mass-based*	Pyrolysis technique	-Polymer type - Weight	< 500 µm, compatible with maximum crucible load (weight and size)
TED-GC-MS	*Mass-based*	Pyrolysis and Thermal Desorption technique	- Polymer type - Weight	< 500 µm, compatible with maximum crucible load (weight and size)

Table 8. Differences between analytical techniques for microplastics analysis.

2.2.4.2 FTIR Spectroscopy and microscopy in microplastics analysis

Infrared (IR) spectroscopy is a spectroscopic analytical technique employed for qualitative and quantitative analysis as well as for studying chemical bonds. More in detail, it can be employed for identifying functional groups of organic compounds, for identifying an unknown compound and for determining sample purity and the strength of chemical bonds. This technique exploits the interaction of compounds with electromagnetic radiation with wavelengths within the mid-infrared (500 cm^{-1} - 4,000 cm^{-1}). Infrared radiation is absorbed by organic molecules and converted into vibrational and rotational energy. When molecules absorb infrared radiation, there is an increase in bonds vibration, an amplification of the oscillations of interatomic distances and bond angles that naturally occur in the fundamental state. All these movements can be summarised in two main types of molecular vibration: bending and stretching. The spectrum of these frequencies provides useful information on molecules structure and can also be used for quantitative analysis thanks to the Lambert-Beer law, an empirical law that correlates absorbance with optical path and concentration. Modern IR spectrophotometers are Fourier Transform spectrophotometers (FTIR). In FTIR, spectra are recorded simultaneously (at various wavelengths) thanks to a mechanical device, the interferometer, and a mathematical operation, the Fourier transform. The introduction of Fourier Transform instruments resulted in several advantages in spectra acquisition as shortened analysis times and analytical noise. IR Spectra can be acquired in transmission or reflection. Attenuated Total Reflectance FTIR (ATR-FTIR) is a particular type of reflection IR spectroscopy employed for samples that are usually difficult to process (solids, but also liquids and pastes) particularly suitable for polymeric films and fibres such as microplastics. In ATR-FTIR, sample is placed in close contact, by pressure, with a crystal with a high refractive index. ATR crystals consist of different materials such as AgCl, ZnSe, ZnS, Ge, silicon and diamond. ATR-FTIR exploits the reflection phenomenon occurring when an electromagnetic radiation passes from a denser medium to one with a lower density (such as the sample under investigation). Briefly, an infrared radiation passes through the crystal, reaches the adhering sample and is reflected. It has been demonstrated that during this reflection process the radiation acts as if it penetrates for a small distance (2-3 μm) into the less dense medium before reflection occurs. The radiation that penetrates is called an evanescent wave. If the less dense medium absorbs the evanescent radiation, there is attenuation at the wavelengths of the absorption bands. The reflected radiation then returns to the ATR crystal, which, thanks to its high refractive index, redirects the radiation back to the sample. The reflection phenomenon is then repeated a different number of times. After a few reflections, the attenuation of the intensity of the IR beam is adequate for being detected by the spectrophotometer, returning an IR spectrum in attenuated total reflectance. However, the attenuation detected is weak and can only be detected by a Fourier Transform instruments. As previously reported, ATR-FTIR is an extremely useful technique for a quick identification of a single microplastics > 100 μm. Although the technical cut-off of ATR-FTIR is 40 μm (Do et al., 2002), this technique can be employed only for manually handable particles. Particles need indeed to be manually placed on the crystal in order to be analyzed. FTIR Microscopy or

µ-FTIR is a coupled analytical technique exploiting the combination of a light microscope with an infrared Fourier Transform spectrometer. FTIR Microscopies enable simultaneous visual inspection of the sample and qualitative investigations using wavelengths in the mid-infrared region. Infrared microscopy, given its versatility and low analysis time, has several applications in industrial and naturalistic fields, as well as on investigations related to cultural heritage. Infrared microscopy allows investigation on very small particles, approximately up to a limit of 10 µm (Renner et al., 2017; Cabernard et al., 2018; Primpke et al., 2018; Weisser et al., 2021). However, differences in terms of resolution strictly depends on by the instrument's trademark and resolution also of 5-7 µm are reported. Infrared microscopy, as for other microscopy techniques, requires that analytes are transferred onto a support medium, which in some cases is represented by a mechanical filter previously used to separate unknown compounds from the matrix as part of the pre-treatment procedure. The support material must allow visual inspection and must also be compatible with infrared spectroscopy analysis, considering the prerogatives of the different acquisition modes. As normal FTIR spectrophotometers, also FTIR Microscopy spectra can be acquired in transmission or in reflection. Transmission analysis generally provides good quality spectra. However, particles thicker than 100 µm or coloured (Schymanski et al., 2021) can result in total absorption of characteristic bands, providing low-quality spectra. In this case, reflection analysis should be considered. Reflection spectra with FTIR microscopies are usually acquired with an ATR accessory as scattering phenomena frequently occurs with direct specular reflection. However, this technique requires the sample to be placed on a solid surface that does not break when the ATR crystal is pressed onto it (Primpke et al., 2019). Furthermore, particles tend to stick to the crystal, making any new measurement of that area of the sample virtually impossible. Analysis of microplastics is usually carried out in transmission mode for its easier application and lower energies required to acquire high-quality spectra. ATR-FTIR microscopy is also limited to 40 µm particles. However, the choice of acquiring spectra in transmission or reflection should be made considering the matrix characteristics previous experience with the same sample typologies. Infrared microscopes, thanks to dedicated software, allow the operator to manually select particles on the support to visualise and identify them or to work automatically, without operator interventions, to analyse specific areas of the filter or the entire filter. The ability to automatically identify particles and/or work automatically depends on the instrumental specifications and features of the dedicated software. Manual analysis (often known as "*Point and Shoot*" analysis) is useful if there is a low number of particles on the support or if only an exploratory investigation of its contents is desired. However, if the number of particles to be studied is huge, manual analysis is rarely viable as long analysis time and possibility of human errors in counting (if necessary) are often an obstacle. Automatic analysis, on the other hand, can be performed with two different approaches: *Imaging/Mapping* and *Particle Measuring* (Figure 9) (Primpke et al., 2020; Ivleva, 2021; JRC, 2022). In *Imaging/Mapping*, FTIR microscopies scan a previously selected area of the support or, if possible, the entire support, without any *a priori* knowledge of particles positioning on it. This operation is performed by acquiring spectra in series, one per pixel, on a

predefined pattern (mapping). Particle information is obtained by grouping adjacent pixels with identical spectral classification (Primpke et al., 2017; 2019; 2020). Imaging/Mapping analyses can be time-efficient for mapping restricted areas or areas with considerable spacing between points; conversely, for larger areas with minimal spacing between points, as is often for microplastic analysis, analysis can be extremely time-consuming. A significant decrease in analysis time is possible thanks to multiple-scan detectors such as Linear Arrays (LA) and Focal Plane Arrays (FPA). These detectors allow the operator to acquire spectra in parallel (imaging) over specific selected areas. Linear Arrays can scan a single line simultaneously while Focal Plane Arrays can scan several lines at the same time, i.e. allow the simultaneous acquisition of several spectra in a real two-dimensional area. Each two-dimensional area acquired simultaneously by the detector is called "tile", while the entire mapped area is called "mosaic". Focal Plane Arrays consist of a variable number (up to 256x256) of cadmium mercury telluride (MCT) detector units. However, *Imaging/Mapping* data collection mode, even when FPA detectors are employed, has its drawbacks. The most important issue with *Imaging/Mapping* is that a considerable amount of redundant data is usually produced, because most of the spectra acquired are from the support and not from the sample. However, the amount of data generated (and consequently analysis time) depends on the lens chosen for the imaging, which determines the size of the individual pixels composing the mosaic. Redundant data and the lack of a well-defined technique for counting particles can result in very long analysis times. *Particle Measuring* data collection approach, on the other hand, involved two separate and consecutive analytical operations. Firstly, FTIR microscope acquires an optical image of a specific and previously selected area, identifying, via software, particles in relation to the background of the medium where they are located (optical contrast). Subsequently, FTIR microscope performs a spectroscopic analysis on the identified particles, providing one or more (depending on the number of scans per particle previously established) infrared spectra for each particle. *Particle Measuring* data collection approach allows shorter analysis times while also less redundant data. However, this advantage is strongly mitigated in case of filters with a high number of particles and has a higher degree of uncertainty compared to *Imaging/Mapping* approach due to the particle preselection (not based on a chemical identification) made during the acquisition phase. Another issue with *Particle Measuring* is that FTIR microscopes are often equipped with components for acquiring low-resolution optical images, which can result in blurred images that software cannot resolve.

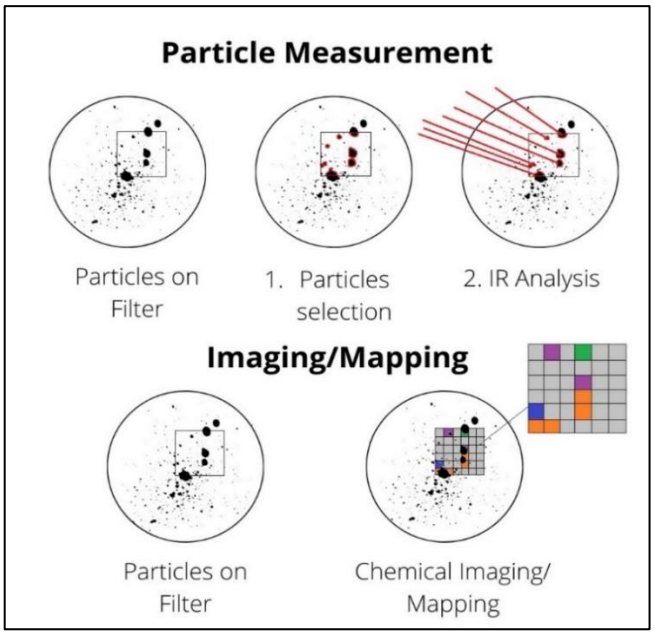


Figure 9. Differences between *Particle Measuring* and *Imaging/Mapping* data collection approach on a restricted filter area. Adapted from JRC, 2022.

In literature, several studies on microplastics with *Imaging/Mapping* and *Particle Measuring* approach in FTIR microscopy can be found. The choice of operating with one or the other data collection mode depends on instrumental capabilities and the need to optimise time with the ultimate aim of developing analytical protocols for routine analysis. However, the recent introduction of software, including free-licensed ones, capable of automatically recognising particles on the area scanned by *Imaging/Mapping* (Vianello et al., 2019) allowed to significant decrease the amount of data and time required by operating in this mode, often leading researchers to prefer it over *Particle Measuring*, if available. In any case, with both data collection modes, the overall analysis time mainly depends on the choice of performing the analysis of all particles on the filter (*total surface/all particles*) or only of some of them. The first approach is always the most desirable option but is only achievable if the number of particles (effective or estimated) is low or the support size is such that a complete analysis can be carried out within sustainable time according to stakeholders needs (Vianello et al., 2019). Hence, a subsampling operation on a representative section of the support from which data is extrapolated is very often necessary, even if this will necessarily be followed by some error. The aim of subsampling is to perform a correct selection so all particles on the filter, regardless their size and shape, have the same chance of being selected (Gy, 1979). Subsampling for microplastics analysis can be performed with two different strategies; analysis software of FTIR and Raman microscopies can operate with one or the other approach for the analysis of microplastics, but both are often not available. The *particle-based subsampling* model involves a selection of particles on the support from the original batch. It requires a priori knowledge of the filter content and is therefore only applicable for the *Particle measuring* data collection approach. In essence, it is a random sampling of particles independent from their distribution on the support for statistical purposes, so that each particle has the same probability of being selected.

Commonly also known as *random subsampling*, this model can be applied in two versions: *full random* (selection of particles regardless of their size) or *stratified random* (selection of particles stratified by size classes). This subsampling model follows the prerogatives of *Particle measuring* approach and shares its advantages and drawbacks; moreover, it is not available with every FTIR microscopy software (Schymanski et al., 2021). Instead, the *windows subsampling* or *box-based subsampling* model involves an investigation on representative areas (count fields) of the filter (Schwaferts et al., 2021). This approach does not require any a priori knowledge of the filter content and is the best solution for *Imaging/Mapping* protocols (Schymanski et al., 2021) but introduces a higher risk on error. The number, shape and size of count fields selected on the support differ from one study to another. The arrangement of these counts fields on supports can follow a pattern or be arbitrary (random) (Figure 10). In the first scenario, the most representative models are the *cake model*, the *cross model* and the *helix model*. The *cake model* pattern considers the inhomogeneous distribution of particles between the centre and the edges by scanning an area of the filter as if it were a slice of a cake. However, in order to obtain a more representative estimation related to heterogeneity between opposing regions of supports, it would be necessary to scan other "slices" of the cake; alternatively, the *cross model* can be adopted, as it more suitable to take account of these differences. Another option is the *helix model* (or *spiral model*), in which windows are selected with a helical pattern, globally representing both the edges and the centre of the support. In all such models, the error can theoretically be minimised by decreasing the counting fields' area and by increasing their number, until the ideal condition is represented by *particle-based subsampling/random subsampling*, which is based on a selection of individual particles and not areas, is achieved. Once the analysis on count fields is performed, data must be corrected for the whole filter according to the count factor, which depends on the overall filter area and the area of the filter that is sub-sampled (total area of all count fields).

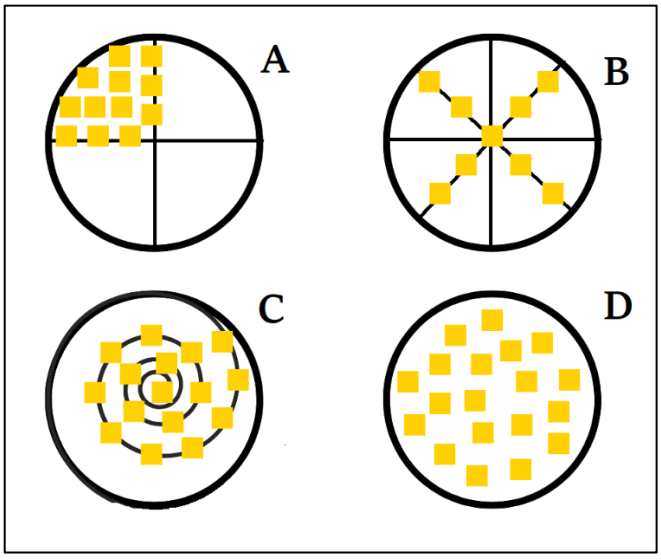


Figure 10. Differences in *"windows subsampling"* patterns: a) cake model, b) cross model, c) helix model and d) random model. Adapted from Corami et al., 2021.

2.2.4.3 Raman spectroscopy and microscopy in microplastics analysis

Raman spectroscopy is a vibrational spectroscopic technique mainly used for qualitative investigations of molecular structure exploiting the Raman Effect, discovered by Indian physicist C.V. Raman in 1928. Raman spectroscopy allows retrieving similar information to those obtainable from IR spectroscopy on inorganic and organic species and even biological systems. This analytical technique, similar to IR, exploits the interaction of compounds with an electromagnetic radiation. However, unlike IR, Raman spectrophotometer do not evaluate absorption, transmission or direct reflection of the radiation by substances, but scattering (Smith et al., 2019a/b). Scattering includes a wide category of phenomena in which waves or particles are deflected (i.e., change in trajectory) due to collision with other particles. Scattering, differently from other reflections and refractions, which instead result in a regular and well-determined change of trajectory, involve messy deflections. If particles are much smaller than wavelength of the incident radiation (< 1/10 of the wavelength), as in the case of molecular systems, scattering will be of the Rayleigh type, i.e., it will occur in all directions (isotropic scattering) with intensity depending on the Rayleigh law applied to molecules. The Rayleigh model assumes that no energy is transferred between the molecule involved and photons of incident radiation; this means that Rayleigh scattering can be considered as the result of an elastic collision in which photons do not gain/loss energy due to the impact with the molecule. If source emits a monochromatic radiation, the scattered radiation will therefore have the same wavelength as the incident radiation. However, Raman observed that a very small part of the incident radiation is scattered as an inelastic collision. This means that some photons loss or gain energy from the interaction with molecules and some scattered radiations have therefore a different wavelength compared to the incident radiation. Thus, the molecular system is, in a certain sense, excited by this interaction as part of the energy was absorbed causing a deformation in the electronic cloud (change in polarizability). The difference in energy between the incident beam and inelastically scattered radiation (Raman shift) corresponds to vibrational motions of molecules (the first vibrational level of the fundamental state) which in turn depends on its chemical structure. Raman spectra will therefore have signals in proximity of molecule's IR absorption bands, although with some differences. Raman spectra are therefore vibrational spectra, distinctive of the molecule observed because of its functional groups and similar in appearance to infrared spectra. Generally, with some exceptions due to different selection rules, Raman spectroscopy allows to see frequencies of vibrational modes that are not observable in IR and vice versa. Similarly, covalent bonds usually provide strong Raman signals, while partially ionic bonds produce often weak signals; with IR spectroscopy, the opposite happens. This is why infrared spectroscopy and Raman spectroscopy are usually considered two complementary analytical techniques. To observe Raman scattering, electromagnetic radiation of any wavelength can theoretically be used, since the recorded wavelength difference is independent of the original frequency of the incident light. However, a radiation as monochromatic as possible should be employed (as Raman shifts are very small) and intense, as the intensity of the scattered light is very weak. The most common sources for Raman spectroscopy, as monochromatic, coherent and highly

collimated, are lasers operating in the UV, in the visible and in the near infrared (NIR). One of the main issues in Raman spectroscopy is background fluorescence. Since Raman signals are relatively weak, using certain wavelengths (particularly the more energetic ones) emphasise fluorescence phenomena of molecules, which can cover them. This can be a problem if using more energetic wavelengths is needed to increase the weak signal of the scattered light. Lasers can also damage samples (particularly biological ones) through bleaching phenomena. Another limitation of Raman spectroscopy is its not very wide diffusion as IR that results, for example, in spectral libraries with limited contents. On the other hand, Raman spectroscopy has several advantages compared to IR spectroscopy for the identification of unknown species. First, water and carbon dioxide are weakly active in Raman spectroscopy, allowing a mitigation in humidity and air interferences. In addition, at the cost of a (usually) less signal-rich spectrum, Raman bands are usually narrower and easier to identify, also allowing studying vibrational modes that are impossible to observe with an IR spectrophotometer. Raman instruments are usually more expensive than those for infrared spectroscopy are, but in recent years, differences have decreased. In Raman microscopy an optical microscope is coupled with a Raman spectrophotometer. Similarly, to infrared microscopy, Raman microscopy allows a visual inspection of particles on support along with qualitative investigation to assess their chemical composition. For these reasons, this technique is employed for the same purposes, but with differences (Table 9) due to the distinctive characteristics of physical phenomena related to Raman spectroscopy. The Raman Effect exploits wavelengths in the visible or near infrared area; therefore, glass optical components (as in standard light microscopes) can be used in Raman microscopes, unlike infrared instruments, allowing an easier visual inspection. The use of such wavelengths, combined with the unique features of laser sources and of confocal optical microscopy, also results in an increase in resolution enabling the identification of particles up to 1 μm and beyond (Schymanski et al., 2021). In addition, the lower spectral interference induced by water and polar molecules facilitates analysis of microplastics in samples from aqueous matrices or with significant residual humidity. However, this potential benefit is offset by the low intensity of signals returned and by the interference caused by fluorescence phenomena. Fluorescence can be mitigated by adopting an appropriate sample pre-treatment protocol, by changing instrumental parameters and choosing a suitable support for particles analysis. Sample pre-treatment for Raman microscopy analysis generally follows the workflow performed for infrared microscopy analysis and other analytical techniques and includes, among others, filtration, extraction, and digestion techniques. In particular, with regard to fluorescence phenomena, critical interferers necessary to remove may be clay minerals, dust particles, humic substances and impurities with microbiological origin (Schymanski et al., 2021). Regarding instrumental parameters, the most important one is the choice of excitation wavelength. In a recent paper, Nava et al. (Nava et al., 2021) reviewed the literature about microplastics analysis by Raman microscopy and comprehensively described the advantages and drawbacks of using certain wavelengths as well as other instrumental parameters. Their observations are given below. In the analysis of microplastics by Raman microscopy, the two most commonly used wavelengths were the

laser in the near infrared at 785 nm (red) and the laser in the visible at 532 nm (green). The 785 nm laser, currently the most widely used laser for the analysis of microplastics, represents a good compromise between signal intensity, fluorescence suppression and overall performance. Conversely, the 532 nm laser, since Raman scattering is inversely proportional to the fourth power of the wavelength, produces a much more intense Raman signal. However, as the energy of the excitation electromagnetic radiation increases, an increase in fluorescence phenomena commonly corresponds too. Since Raman scattering is generally less intense than fluorescence, using this wavelength sample, filter or impurities fluorescence phenomena, could saturate the detector preventing a correct particle identification. In addition, it should also be considered that the spectral resolution decreases as the wavelength used decreases. On the other hand, high-energy wavelengths, operating in the UV (< 300 nm), are rarely used because there is a concrete risk of sample degradation together with those of observing interference due to UV absorption of different organic molecules, including microplastics. In microplastics analysis by Raman microscopy, the same membrane filters adopted for infrared microscopy analysis are usually employed. However, given the distinctive characteristics of Raman scattering phenomenon and the higher instrumental resolution, it is necessary that filter surface being as uniform as possible to facilitate automatic recognition of particles, especially those smaller than 10 µm. As previously reported, the most employed supports for Raman microscopy are silicon filters, alumina oxide filters and gold coated PC filters. When particles are not being analyzed directly on the filter, one expedient to avoid substrate-related interference may be to manually remove them and place them on a microscope slide (Bottari et al., 2019); glass does not present interfering bands in Raman spectra (Gilbert et al., 2019). However, this method is only feasible for larger microplastics (> 100 µm). Raman microscopes can employ data collection modes of IR microscopes allowing, thanks to dedicated software, a *Point and Shoot* analysis or semi-automated analysis as *Imaging/Mapping* and *Particle Measuring.* However, in a recent comparative study (Von der Esche et al., 2020), Raman *Imaging* appears to be less effective than *Particle measuring*, performed with IR microscopy equipped with an FPA detector. Similar to IR microscopy, it is always desirable to perform analysis of all particles on the filter (*total surface/all particles*) than performing a subsampling. For Raman microscopy, this is even more complex given the larger number of particles generally observable with this technique due to the higher instrumental resolution. For Raman microscopy, this is even more challenging given the higher number of particles generally observed with this technique because of the higher instrumental resolution. The *total surface/all particles* approach is indeed compatible with routine Raman microscopy analysis only if a small filter, such as a silicon filter, is employed. In any case, even by minimizing the actual analysis area in this way, if the number of particles in the sample is excessively high, it is strictly necessary to apply a subsampling model for sustainable analysis times. Subsampling models *(particle-based models* and *box-based models*) employed in infrared microscopy can also be applied with Raman microscopy. In a comparative study of microplastic analysis by Raman microscopy (Brandt et al., 2021), none of these models (*particle-based models* and *windows models*) proved to be better than the others.

Differences were observed only in edge scenarios, such as with filters with few particles and inhomogeneous distribution. In those cases, *particle based* subsampling proved to be more accurate than *windows* subsampling. In most samples, the subsampling error observed was due to counting error (extrapolation from a small number of measured particles) and the inhomogeneity of the particle distribution was negligible.

	ATR-FTIR	IR MICROSCOPY	RAMAN MICROSCOPY
RESOLUTION	Limited to contact point	10 µm	300 nm
POLYMER IDENTIFICATION	Possible semi-automatically	Possible also semi-automatically	Possible also semi-automatically
PARTICLE COUNT	No	Possible also semi-automatically	Possible also semi-automatically
MORPHOLOGICAL ANALYSIS	No	Possible also semi-automatically	Possible also semi-automatically
SURFACE PROPERTIES ANALYSIS	No	Some surface additives can be identified	Some surface additives can be identified
SAMPLE PRE-TREATMENT	Limited to surface cleaning of particles to be analysed	Normally extended for matrices with high organic content	Normally extended for matrices with high organic content
SUPPORTS FOR ANALYSIS	No support required	*Transmission:* Aluminium Oxide filters, Silicon filters, ZnSe windows *Reflection:* Gold-coated filters, Ag filters	Aluminium Oxide filters, Silicon filters, Gold-coated filters, PTFE filters
ACQUISITION MODE	Attenuated total reflection (ATR)	Transmission and attenuated total reflection (with ATR accessory)	Raman scattering
SEMI-AUTOMATED ANALYSIS MODE	No	Imaging and Particle measurement	Imaging and Particle measurement
CRITICAL ISSUES	Analysis limited to one particle at a time and only applicable to particles > 100 µm	Humidity, carbon dioxide, glass	Fluorescent specimens, laser choice, limited spectral database

Table 9. Differences between ATR FTIR, FTIR Microscopy and Raman Microscopy.

2.2.5 Quality Assurance/Quality Control (QA/QC) in microplastics analysis

The quality of the analysis can be influenced by several factors partly independent from microplastics-related analytical issues. Contamination is considered one of the most important (Prate et al., 2021; JRC, 2022) because it can lead to an overestimation of the microplastics in the sample and an erroneous risk assessment. Contamination during microplastics analysis can occur in different analysis phases (sampling, sample treatment and analysis) and from different sources. Airborne contamination (i.e., contamination of samples due to airborne polymer particles and fibres) has been described as a major issue in microplastic analysis (Prata et al., 2021; JRC, 2022). Plastic particles may already be floating in the air during the operations or results from wear and tear of operators' clothes. Nylon or acrylic clothes can indeed release particles or fibres, especially during outdoor sampling. The potential effect of this type of contamination on microplastics analysis was studied by Scopetani et al. (2020). In this study, an experimental model was developed to understand the magnitude of cotton fibres released from clothes during microplastic sampling procedures. The obtained value was then normalised to the results of a so-called *rubbing experiment*, which aimed to compare the release of textile fibres from cotton clothes and synthetic clothes. It was reported that the release of fibres from synthetic clothes could contribute to an overestimation of microplastics in the samples up to 15%. Contamination may also occur due to the release of plastics from equipment and reagents employed during the entire analytical procedure (cross-contamination). Nets, assembled filtration systems and containers in which sample are stored usually have several plastic structural components which can release plastics (Martellone et al., 2021). Also paints of research vessels employed in open-areas studies are indicated as a possible source of contamination (Setälä et al., 2016). During sample treatment, particular attention should be paid to reagents employed for digestion, extraction, filtration and staining, which should be tested as plastic-free. Appropriate plastic-free reagents should also be used in cleaning protocols for glass and other equipment employed. The employment of non-plastic free reagents and inappropriately cleaned equipment can indeed be another important source of contamination. Other possible contamination patterns during microplastics analysis are illustrated in Figure 11.

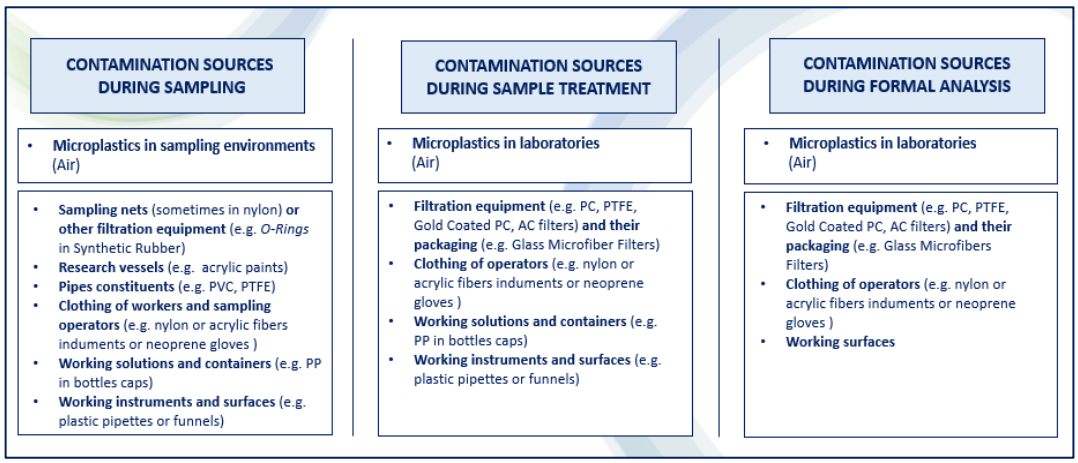


Figure 11. Contamination patterns during each phase of microplastics analysis.

Contamination can be mitigated by using general and specific (i.e., step-dedicated) precautions and quantified by blanks (Corami et al., 2021; Prata et al., 2021). General and specific precautions include e.g., operating in a clean working environment, employing only glass and plastic free equipment, wearing only cotton robes, sample handling in a laminar flow cabinet or in a clean air laboratory and developing a cleaning protocol for equipment and surfaces. However, the complete removal of plastic from analytical environment is almost never achievable and the analytical result obtained should always take the presence of contamination into account. Blanks (negative controls) are employed for quantify the occurring contamination. Final data of a microplastics analysis campaign are indeed usually corrected for blanks by subtracting particles from samples with values retrieved in blanks. Blanks in microplastics analysis can be distinguished into three types: field blanks, reagent blanks and procedural blanks. Field blanks are usually carried out in order to understand the effect of atmospheric deposition on empty filters. Field blanks are particularly useful in the case of long outdoor sampling campaigns (e.g., manta net sampling in research vessels) because of the prolonged effect the wind might have. Reagents blanks allow to understand and exclude reagent contamination from consideration. Reagent blanks are usually carried out by filtering a limited amount of reagent (e.g., ultrapure water, reagents for extraction and/or staining) on the analytical support. Procedural blanks allow to understand and potentially exclude cross-contamination of equipment from consideration. Procedural blanks are usually runned with the same procedure of normal samples but using ultrapure water instead of the matrix under investigation. According to Koelmans et al. (2019) the number of procedural blanks required to verify/correct for contamination or to demonstrate the absence of contamination is n = 3 replicated procedural blanks. In a more recent review, Schymanski et al. (2021) suggested that for a small series with less than ten samples that are all processed within one day, a single (process) blank sample could be sufficient while for large series, multiple (process) blank samples should be analyzed alongside the sample series (e.g., at least one process blank sample per five or ten samples). In most studies on MPs, QA/QC data include solely blanks analysis for control of sample contamination (Schymanski et al., 2021). The scarcity of other QA/QC parameters in microplastic analysis data was already highlighted by Koelmans et al. in 2019 (2019) in an intercomparison study between over 50 papers about microplastics contamination in drinking water; only three studies provided e.g., full data on positive controls indicating that it was not a very common practice. Most recent data (Muller et al., 2020; Schymanski et al., 2021) showed that nowadays the situation is not much different and positive controls are rarely carried out. Positive controls are useful to quantify particle losses. Losses of particles may occur during various steps of the analysis and could lead to an underestimation of results if not correctly evaluated; positive controls are also useful to study the effectiveness and the reliability of the analytical procedure in terms of sampling, sample treatment and analytical techniques. Positive controls are usually prepared by spiking ultrapure plastic-free water with a predetermined number of microplastics particle types from reference materials. However, preparing positive controls for microplastics analysis is an analytical challenge. Unlike other conventional contaminants, microplastics are not dissolved in aqueous

samples, but are present in particulate form. Therefore, they are not homogenously distributed, and the reproducibility tests could be compromised. Researchers also have to face, for positive controls, issues related to microplastics definition. Since microplastics are defined as particles with different type, size and shape it's virtually impossible to adopt a standard mix for each polymer type, size and shape, so a sort of compromise is inevitable. Particles similar in type and size could indeed exhibit different behaviour in relation to their shape (e.g., fibres and non-elongated particles) and wear and deterioration degree (differences in surface properties) (WHO, 2019). In 2021, Schymanski et al. (2021) reported that suitable *certified* reference materials were not available. However, since 2019, different uncertified reference materials were employed in inter-laboratory tests. Reference materials are usually purchased from specific distributors or prepared directly by researchers from plastics. Uncertified material is reported to be suitable if the size is checked by optical microscopy. The particles with a certain size should be determined correctly within a tolerance of ± 10% (Schymanski et al., 2020; 2021). During the 2nd JRC workshop on *"a methodology to measure microplastics in drinking water"* (21st September 2022) the topic related to the lack of reference or representative test material and how to overcome the issue emerged. The suggestion was to consider a mixture of different shapes. It was also noted that information describing such test materials, how to fabricate and finally how to use them practically is missing from the scientific literature. In addition, the simpler option of using commercial spherical particles was mentioned but it was commented that these may be poor candidates because of shape-specific behaviour which influences their recovery and detectability. In 2020 Muller (2020) et al reported the results of the *"first international comparative study of commonly applied analytical methods for microplastic analysis"*. Results in terms of particle number, polymer type, abundance of particles and/or particle mass for each polymer type from 17 laboratories of eight different countries were compared by exploiting FTIR microscopy, Raman microscopy, light microscopy, SEM and *mass-based* techniques. Two different stock suspensions were employed: one with five types of reference microplastic particles (polyethylene, polyvinyl chloride, polyethylene terephthalate, polymethylmethacrylate and polystyrene) with a mean diameter > 50 µm (large size fraction and moderate number of particles ≈100-900) and one with three types of reference microplastic particles (polymethylmethacrylate, polyethylene and polystyrene) with a mean diameter < 50 µm (small size fraction and large number of particles 2000-11000). Particles for mix suspensions were retrieved from a German distributor. The size classes were selected in order to cover a wide range from 8 µm to 140 µm and polymer types were chosen based on their broad industrial use and relevance in former microplastic studies. The authors reported that only FTIR microscopy exhibited an acceptable performance for quantifying/identifying large particles while FTIR microscopy and Raman microscopy for smaller particles are reported as questionable. Py GC-MS was judged "questionable" for particles > 50 µm while for particles < 50 µm appeared inadequate. Moreover, FTIR microscopy and optical microscopy was considered "sufficient" for the quantification of generic total polymer number. In the latest JRC Technical Report on *Analytical Methods to measure Microplastics in Drinking Water* (JRC,

2022) another intercomparison study was reported. This study was undertaken by the Southern California Coastal Water Research Project (SCCWRP) in order to develop standardized methods for quantifying and characterizing microplastics in drinking water (De Frond et al., 2022). The study included 22 laboratories from six different countries that evaluated performance of optical microscopy, FTIR microscopy and Raman microscopy; *mass-based* techniques were not included. Three spiked samples of simulated clean water and a laboratory blank were sent to each laboratory with a prescribed standard operating procedure for particle extraction, quantification, and characterization. The samples contained known amounts of microplastics over four size fractions (1-20 µm, 20-212 µm, 212-500 µm, >500 µm), four polymer types (PE, PS, PVC, and PET), and six colours (clear, white, green, blue, red, and orange). The total number of particles were around 609 (±132) of which 249 (±60) were in the range above 20 µm. The samples also included around 80 false positives (natural hair, fibres, and shells) as example of particles that can be mistaken for microplastics. Particles were extracted by cascade filtering/sieving and analyzed through optical microscopy to count all particles above 20 µm. Chemical characterization by FTIR microscopy or Raman microscopy was done on a filter sub-sample. The final result was then extrapolated to the full filter area. Particle recovery as determined using stereomicroscopy was 76% ±10% (SE). For particles in the three largest size fractions (> 20µm), mean recovery was 92% ±12% SD. The blank samples count was 91 particles (±141 SD). Regarding chemical identification, the test showed that both Raman and FTIR microscopy were accurate (> 90%) in determining the nature and number of particles in the size fraction above 20 µm. When applied to particles in the range 1-20 µm the FTIR method exhibits an important decrease in accuracy/recovery to around 30%. In contrast, the Raman method maintained a good level of accuracy down to 1µm. Another newly published intercalibration study is the one co-organised by QUASIMEME, EUROqCHARM and the NORMAN network (Van der Veen et al., 2022). In the third round of the study 98 laboratories were included from different European countries evaluating both *particle-based techniques* and *mass-based* techniques. The samples included 'soda' tablets to be dispersed in water (simulating water samples) with different polymers in the seize region of 50-300 µm, and three sediment (or sand) samples. More in detail, tablets consisted of a mixture of sodium hydrogen carbonate ($NaHCO_3$) citric acid ($C_6H_8O_7$), a binder (lactose) and polymers which dissolve completely in water. Microplastics were prepared by cry milling of pre-production pellets and separated by dimension by sieving with a vibratory sieve shaker. A selected number of 50-300 µm PE, PET and PS irregular particles were added in one tablet while a selected number of PP, PC and PVC irregular particles were added in a second test tablet. The choice of the appropriate pre-treatment and instrumental analysis technique was left to each individual laboratory. Only a few laboratories used density separation (n= 5) and/or digestion/oxidation (n= 8). Test samples were filtered using a variety of different filters including alumina filters, quarts, glass fiber, PFTE, stainless steel and gold filters. For the final analysis most laboratories employed IR based methods, including FTIR microscopy, ATR-FTIR, Raman microscopy and Py-GC-MS.

2.3 MICROPLASTICS AND REGULATORY AFFAIRS

2.3.1 EU measures for preventing the diffusion of plastics in the environment

To deal with the increasing levels of plastics and microplastic pollution, the European Commission is adopting a number of actions under the European Green Deal (European Commission, 2019) and the Circular Economy Action Plan (European Commission, 2020). Some examples include regulatory measures to: a) Limit the intentional use of microplastics in consumer or professional products, b) Develop labelling of consumer goods, through which consumers would be informed about the appropriate waste disposal options for their products and which disposal methods should be avoided, c) Monitoring the presence of microplastics and other measures to prevent the unintentional release of microplastics in the environment. In addition, EU research programmes (European Parliament and Council, 2013, 2021) support the development and harmonisation of methods for measuring microplastics and, more generally, filling gaps in scientific knowledge about the risk and presence of microplastics in the environment. Regarding the first two topics, a remarkable legislative act is Directive (EU) 2019/904 (European Parliament and Council, 2019), transposed in Italy with D. Lgs. 196/2021 (Italy, 2021). Formally referred as *"on reducing the impact of certain plastic products on the environment"* the Directive aims to prevent and reduce the impact of certain plastic products on the environment, and to promote a transition to a circular economy throughout the European Union (EU) by introducing a combination of measures tailored to the products covered by the directive, in particular, by ensuring that single-use plastic (SUP) products, for which more sustainable alternatives are available and affordable, cannot be placed on the market. The directive applies to some SUP products fully or partly made of plastic and fishing gear containing plastic, that are typically intended to be used just once or for a short period of time before they are thrown away. The list of SUP products banned from the market is shown in Figure 12.

DIRECTIVE (EU) 2019/904 LIST OF SUP PRODUCTS BANNED FROM THE EU MARKET
Cutlery (forks, knives, spoons and chopsticks)
Plates but not glasses and bottles
Traws and cotton bud sticks (except those used with active implantable or other medical devices)
Sticks to be attached to and to support balloons and their mechanisms, except balloons for industrial or other professional uses and applications that are not distributed to consumers
Food containers made of expanded polystyrene (i.e., boxes, with or without a cover) for immediate consumption without any further preparation, typically consumed from the container or ready to be consumed without further preparation
Products made from oxo-degradable plastic, as plastic materials that include additives which, through oxidation, lead to the fragmentation of the plastic material into micro-fragments or to chemical decomposition
Beverage containers made of expanded polystyrene, including their caps and lids
Fishing gear containing plastics

Figure 12. List of SUP products banned from the EU.

In addition to market restriction, the Directive also set requirements for SUP bottles, which are not banned from market. The directive sets a collection target of 90% recycling for SUP plastic bottles by 2029 (with an interim target of 77% by 2025). These bottles should contain at least 25% recycled plastic in their manufacture by 2025 (for PET bottles), and 30% by 2030 (for all bottles). Those with caps and lids made of plastic may be placed on the market only if the caps and lids remain attached to the containers during the products' intended use stage. The Directive also set standards for labelling; some disposable plastic products placed on the market must carry a visible, clearly legible and indelible marking affixed to its packaging or to the product itself. These products include sanitary items, wet wipes, tobacco products with filters and filters marketed for use in combination with tobacco products and cups for beverages. These labels should inform consumers about the appropriate waste disposal options for the product/ what type of waste disposal should be avoided for the product and the presence of plastics in the product, along with the negative environmental impact of littering. Finally, the Directive introduces extended waste-producers' responsibilities, incorporating the "polluter pays" principle. Producers will have to cover the costs of a) waste collection and of cleaning up litter, b) data gathering in relation to wet wipes, balloons and tobacco products with filters and filters marketed for use in combination with tobacco products and c) awareness-raising for some SUP products as food and beverage containers, beverage containers, cups, packets and wrappers, lightweight carrier bags, and tobacco products with filters and filters marketed for use in combination with tobacco product.

2.3.2 Microplastics and Directive 2020/2184

Directive (EU) 2020/2184 (European Parliament and Council, 2021) is a revision of the Directive 98/83/CE (European Parliament and Council, 1998) on the quality of water intended for human consumption. Water intended for human consumption means *"(a) all water either in its original state or after treatment, intended for drinking, cooking, food preparation or other domestic purposes, regardless of its origin and whether it is supplied from a distribution network, from a tanker, or in bottles or containers; (b) all water used in any food-production undertaking for the manufacture, processing, preservation or marketing of products or substances intended for human consumption unless the competent national authorities are satisfied that the quality of the water cannot affect the wholesomeness of the foodstuff in its finished form".* The Directive shall not apply to: *"(a) natural mineral waters recognised as such by the competent national authorities, in accordance with Council Directive 80/777/EEC of 15 July 1980 on the approximation of the laws of the Member States relating to the exploitation and marketing of natural mineral waters (1); (b) waters which are medicinal products within the meaning of Council Directive 65/65/EEC of 26 January 1965 on the approximation of provisions laid down by law, regulation or administrative action relating to medicinal products"*. The Directive is being transposed by the various European Member States and is designed to set new standards for drinking water quality in order to protect human health from the negative effects of

contamination of water intended for human consumption and ensuring its healthiness and cleanliness. The major innovations of the Directive include:

a) A new risk-based approach to water security covering the entire supply chain modelled on the Water Safety Plans (WSPs) developed by the WHO;

b) Water parameters list updated with some changes;

c) Homogenisation of different national approval systems for materials in contact with water intended for human consumption;

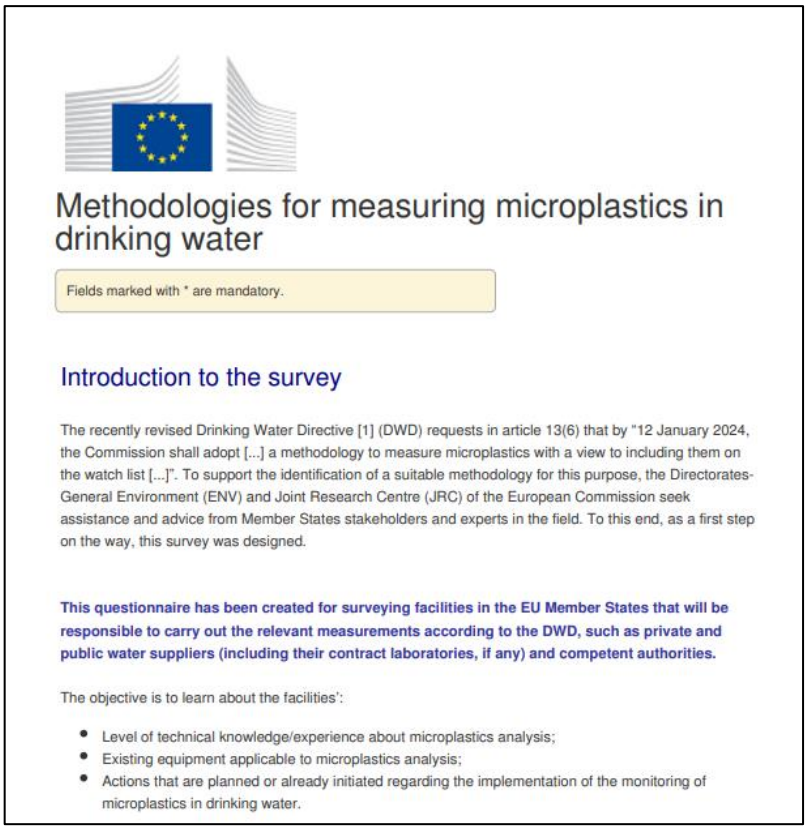
d) Regulation of information provided to consumers.

Regarding water parameters list, more restrictive values were set for Lead (Pb) and Chromium (Cr) while less restrictive values were set for Antimony (Sb), Boron (B) and Selenium (Se). New chemical parameters are also introduced as Bisphenol A, Chlorite and Chlorate (when used as disinfection methods), Aloacetic Acid, LR-Microcystin, PerFluoroAlkyl Substances (PFASs) and Uranium. However, the most important innovation involves the introduction of the "watch list" mechanism for emerging compounds as s, such as endocrine-disrupting compounds, pharmaceuticals and microplastics. The Commission establishes and regularly updates a "watch list" of substances and compounds of particular concern to the public or the scientific community. The watch list mechanism, as reported by the Directive, will make it possible to respond to growing concerns in a dynamic and flexible way. It will also enable follow-up on new knowledge about the relevance for human health of those emerging compounds and on new knowledge about the most appropriate monitoring approaches and methodologies. This watch list mechanism for water intended for human consumption is part of the response to various relevant Union policies, as set out in the communication of the Commission of 11 March 2019 'European Union Strategic Approach to Pharmaceuticals in the Environment', the communication of the Commission of 7 November 2018 'Towards a comprehensive European Union framework on endocrine disruptors' and the Council Conclusions of 26 June 2019 'Towards a Sustainable Chemicals Policy Strategy of the Union'. Substances and compounds are included in the watch list when they are likely to be present in drinking water and may present a potential risk to human health. For this purpose, the Commission refers, in particular, to scientific research of the WHO. The "watch list" proposes an indicative value for each substance or compound and, if necessary, a feasible and not expensive analytical method. Member States shall introduce monitoring obligations with respect to the eventual presence of substances or compounds included in the "watch list" along the drinking water supply chain. As required by the Directive, the Commission established a first "watch list" of emerging compounds on 19 January 2022, which includes beta-estradiol and nonylphenol; Member States have until 12 January 2023 to implement the monitoring requirements throughout the drinking water supply chain and to take measures if the parameter is exceeded. For the first time, microplastics appeared in drinking water Directive, have been defined as emerging compounds and were related to the "watch list" mechanism: *"Where surface waters are used for water intended for human consumption, Member States should pay particular attention in their risk assessment to microplastics*

(…) and should, where necessary, require water suppliers to also monitor and, where necessary, carry out treatment for those and other parameters included in the watch list if considered a potential danger to human health". The Directive specifies also for method development and risk analysis for microplastics in drinking water: *"By 12 January 2024, the Commission shall adopt delegated acts in accordance with Article 21 in order to supplement this Directive by adopting a methodology to measure microplastics with a view to including them on the watch list (…) once the conditions set out under that paragraph are fulfilled"* and *"The Commission shall, no later than 12 January 2029, and thereafter where appropriate, submit a report to the European Parliament and to the Council on the potential threat to sources of water intended for human consumption from microplastics (…), and on the relevant associated potential health risks"*. Basically, the inclusion of microplastics in the watch list is conditional on the development of an effective analytical method, which will be then useful for risk analysis.

2.3.3 JRC activities under Directive 2020/2184

In order to identify a suitable analytical methodology, the European Commission's Joint Research Centre (JRC) launched a dedicated project in order to harmonize experience and knowledge about microplastic analysis in drinking water, requiring support from national technical-scientific representatives, industry experts and stakeholders from the EU Member States. This project includes an online survey (Figure 13) and a (series of) workshops designed to collect contribution from stakeholders and experts in microplastics analysis. The survey, including questions related to preparedness, investigated parameters, size range, sampling techniques, pre-treatment reagents and instrumental analysis has been closed on March 6th, 2022. Results were presented at the first two workshops on September 6th and 8th, 2022.



Methodologies for measuring microplastics in drinking water

Fields marked with * are mandatory.

Introduction to the survey

The recently revised Drinking Water Directive [1] (DWD) requests in article 13(6) that by "12 January 2024, the Commission shall adopt [...] a methodology to measure microplastics with a view to including them on the watch list [...]". To support the identification of a suitable methodology for this purpose, the Directorates-General Environment (ENV) and Joint Research Centre (JRC) of the European Commission seek assistance and advice from Member States stakeholders and experts in the field. To this end, as a first step on the way, this survey was designed.

This questionnaire has been created for surveying facilities in the EU Member States that will be responsible to carry out the relevant measurements according to the DWD, such as private and public water suppliers (including their contract laboratories, if any) and competent authorities.

The objective is to learn about the facilities':

- Level of technical knowledge/experience about microplastics analysis;
- Existing equipment applicable to microplastics analysis;
- Actions that are planned or already initiated regarding the implementation of the monitoring of microplastics in drinking water.

Figure 13. JRC survey on Methodologies for measuring microplastics in drinking water.

Survey results, involving water suppliers, control authorities, regulatory authorities, contract labs and universities showed a complex and variegated situation. Forty-one entities from 20 countries responded to the survey, 44% of which were water suppliers or control authorities. About half of the respondents rate their preparedness for a future monitoring of microplastics to be low or very low and only a quarter deem it high or very high. So far, particle number and size were the properties most frequently determined, especially using FTIR microscopy, Raman microscopy and Py-GC-MS. For a future monitoring, the majority opted for a size range of 1-1000 µm and deemed the determination of mass, number and size being equally important. Data from the survey also showed that different approaches to sample collection are not yet well understood by stakeholders and the majority of them reported sample volumes < 100 L. Filtration/sieving was the most employed sample treatment technique, followed by digestion and density separation while FTIR microscopy was the most employed analytical techniques followed by Raman microscopy. Size range of particles retrieved appeared variable but about a half of involved laboratories focused also on microplastics < 100 µm. Polyethylene, polypropylene, PET and nylon were the microplastics most commonly reported to be investigated/found. During the 1st and 2nd workshops were also illustrated different possible approaches in microplastics analysis. Analytical methods should be distinguished into in "screening methods" and "specific methods". A "screening method" can be defined as (relatively) quick and cheap method which aims to detect plastic particles without identifying the precise composition (type of polymer) of the particles and with a concrete possibility of false positive or/and false negative results. It can be used only for an overview of

the sample before being subjected to a “specific method” (e.g., fluorescence microscopy). A “specific method” allows instead the detection of the particles’ individual polymeric composition. Specific methods should be classified as techniques with high to moderate time consumption and moderate to high investment costs (e.g., FTIR microscopy, Raman microscopy and Py-GC-MS). Another option described was “indicator analysis”. In “indicator analysis” only a small number of selected polymers with a limited size range (‘indicators’) that are indicative of the overall presence of microplastics in the sample are analyzed. “Indicator analysis” employs the same analytical techniques used for “specific methods” but are faster compared to them. However, only a part of potentially relevant microplastic is captured with a concrete risk of underestimation of the true quantity in the investigated medium. A 3rd workshop on September 21st, 2022, was instead conceived as a forum to discuss the information on microplastics analysis presented at the previous workshop. Prior to workshop, another poll was launched to capture written information from the participants and questions to discuss. From the discussion emerged that the level of microplastics in drinking water samples is low with values being in line with published data. Moreover, measurement of blanks and background levels were considered very important. It was suggested to report blanks data separately from microplastics data. Shape was considered an important descriptor and a precise description of all the relevant different shapes (e.g., spheres, fibres, fragments, films) should be required in microplastics analysis. In contrast, reporting of particle colours is deemed to be too strongly affected by human bias. Regarding volumes, difficulties emerged for obtaining lower particle cut-off size; a general agreement on the necessity to filter several hundreds of litres was observed even if a distinction between smaller particles (SMPs) and bigger particles behaviour was not made. A focus was also made on the lack of reference or representative test material and how to overcome the issue.

PART III: INSTITUTIONAL AND EXPERIMENTAL ACTIVITIES

3.1 ACTIVITIES RELATED TO THE ITALIAN NATIONAL WORKING GROUP

On the basis of JRC's activities, the Italian National Institute of Health (ISS), with the coordination of the Italian Ministry of Health, has defined a national working group which includes experts from the National Research Council (CNR), national and local environmental Authorities (SNPA: ISPRA and ARPA), Universities and Federation of Water Suppliers (Utilitalia). This group was designed to work on: (i) JRC and EC support on national expertise about microplastic monitoring in drinking water (ii) development of national analytical method for microplastic in drinking water to be presented to the JRC. The recruitment phase took place on March 2022, with a "call to action" by the Italian National Institute of Health and following discussions with interested stakeholders (ISS, 2022). The inclusion criteria were: (i) experience in microplastic analysis regardless the matrix type (SNPA, Universities, CNR), (ii) proven expertise on emerging contaminants and involvement in the issue (Water Suppliers). Therefore, after collecting applications and checking inclusion criteria, with the agreement of the Ministry of Health, the group was formed. The group, apart from representatives of ISS and Ministry of Health, consist of 41 members: 18 from SNPA (ISPRA and ARPA), 12 from Water Suppliers and 11 from Universities and CNR. The first meeting of Italian National Working Group for microplastics in Drinking Water took place on June 16th, 2022. During the first meeting, participants were invited to introduce themselves giving an overview on the overall experience in microplastic analysis. The information requested from group members were based on the JRC survey. As some members of the working group collaborate, they present themselves as a working team (23 in total). Results of the national survey (Martellone et al., 2022a; 2022b) were exposed at *XXIX Congresso della Divisione di Chimica Analitica della Società Chimica Italiana (SCI)* (Castello di Milazzo, ME, 11-15 September 2022) and *μMED: International Conference on Microplastic Pollution in the Mediterranean Sea* (Naples, 25-28 September 2022). Obtained data showed how, despite there is a diversified knowledge among the different institutions, the magnitude of the problem has been well understood by all the members, even if some of them, especially water suppliers, have approached it only recently. From the contributions presented by Italian National Working Group members, it can be observed that there are heterogeneous analytical strategies but with common elements; experts, despite belonging to different organizations with different roles and objectives, are extensively working on the water matrix (Figure 14), particularly on surface water, drinking water and sea water. Issues of common interest are also wastewater, for the possibility of massive contamination from civic and industrial sources and groundwater, as hypothetically less contaminated from microplastics. Moreover experts, especially groups belonging to public institutions, are expanding their research lines also to other types of matrices such as biota or sediments.

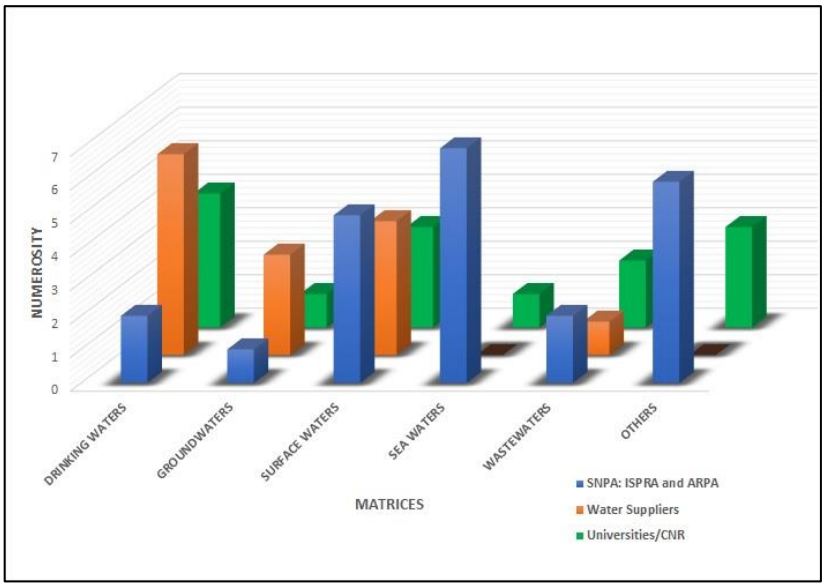


Figure 14. Distribution of studies related to the presence of microplastics in the different matrices analyzed among the various authorities and institutions belonging to the working group.

Drinking water is obviously the most frequently studied matrix by water suppliers, but many of them are extending their research to surface water and groundwater, as waters intended for drinking water production and possible source of microplastics. On the contrary, national, and local environmental Authorities focused more on sea water as part of monitoring related to marine strategy (European Commission, 2011); ARPA and ISPRA were also involved in sediment and biota analysis, surface water and groundwater. Only a small percentage of ARPA and ISPRA researchers were studying drinking water when they were recruited, even though interest in the development of an analytical method for drinking water was rapidly growing among them. Universities and CNR are mostly involved than ARPA and ISPRA in drinking water analysis, with also a focus on surface water, other matrices (sediments, biota) and wastewater. Only a small percentage focused on sea water and groundwater. These choices, as a direct consequence, affects the development of sampling techniques and analytical methods; in fact, clusters of sampling methods can be identified from the survey according to the type of water sampled. Manta net appears to be the most suitable sampling method for seawater samples; all the group members who approached the issue of marine pollution originating from microplastics refers to Manta net as their standard sampling method. Indeed, for other sample types, the situation appears different and more varied: in these cases, other sampling methods, such as glass bottles or "*in situ*-filtration methods" i.e., systems connected to sieves/filters, have been developed. These systems allow filtration of large volumes of water without sample loss but significant increases in costs and sampling time (Martellone et al., 2021). For drinking water, the most common solutions appear to be sampling with bottles for water suppliers while Universities and CNR are also interested in trying alternative solutions as "*in situ-filtration* methods". This *status* describes the different objectives and roles of the organization involved. Water suppliers need a simple, quick sampling method and bottle sampling from taps, despite low volumes of water collectable, are obviously preferred. On the contrary, researchers from universities

and CNR are interested in more complex methods for representativeness (high volumes of water filtered) and lowering-contamination (closed systems) reasons. The survey trend also shows that working group members are developing analytical protocols for microplastics with or without a pre-treatment phase. An untreated sample, especially full of organic matter samples (surface water, wastewater) could be problematic in terms of signal-to-noise *ratio* and goodness of results but not requires effectiveness and plastic-degradation studies and could globally speed up the entire analytical procedure. On the other hand, with dedicate pre-treatment protocols, results could be significantly better, but operations and the development of the procedure could be also time-consuming. In this respect, it is noted that groups that have chosen to apply pre-treating protocols, opted for (i) digestion with, for example, acids or hydrogen peroxide or (ii) digestion followed by an extraction step. As seen in sampling phase, the water type influences the choice of analytical criteria, so pre-treatment methods depend on what water type working group members analysed. Sea water samples, mainly retrieved by manta net sampling and conducted by ARPA members, are usually dried, sieved and submitted to digestion protocols (hydrogen peroxide, with or without heating). For groups who experienced drinking water analysis, a pre-treatment step with light digestion (acids or peroxides) without temperature programs and followed by a filtration on a substrate is very common. The most popular substrates for drinking water filtration are silicon square filters, which showed good results for Raman and Fourier Transform Infrared (FTIR) microscopy analysis; alumina round filters have also been used with good results in Raman and FTIR microscopy analysis. On the contrary, surface water samples followed the direction of the distinctive research group: analysis of surface water carried out by ARPA members followed the marine strategy protocols (manta trawl – drying – sieving – digestion) while analysis of surface water carried out by water suppliers are usually carried out in a similar way of drinking water (bottle sampling – light digestion – filtration on substrate); only few authors carried out density separation (with or without digestion) using an hyper-saline solution. Ultimately, even from an instrumental point of view, there is heterogeneity. As reported by the survey, the most frequently used analytical techniques for the characterization of microplastics were Microscopy (optical microscopy and electron microscopy), FTIR spectroscopy (normal and microscopy-coupled), Raman microscopy and Gas Chromatography-Mass Spectrometry coupled to Pyrolysis (Py-GC-MS), as shown in Figure 15.

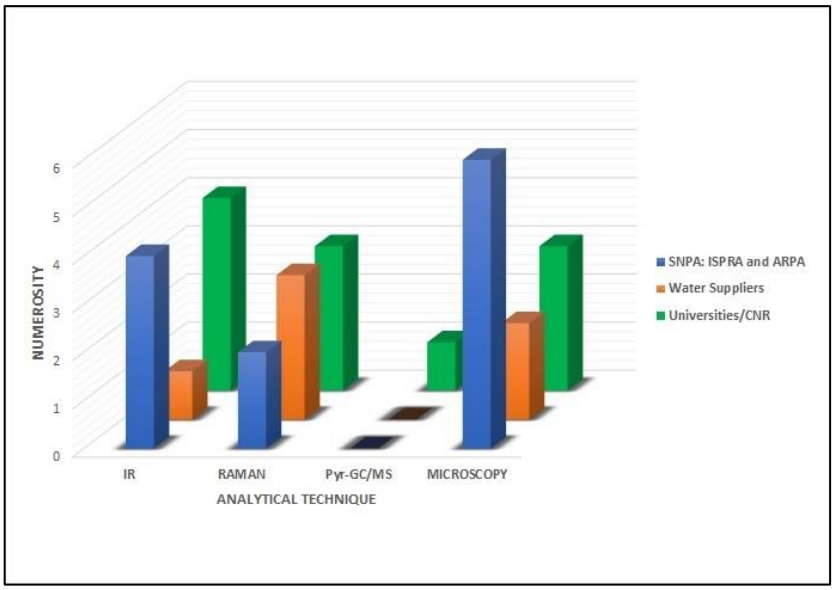


Figure 15. Distribution of selected analytical techniques in microplastics analysis among the various authorities and institutions belonging to the working group.

The employment of spectroscopy (FTIR and Raman) was very common among working groups regardless of affiliation. Microscopy-only techniques are also frequently used, especially from ISPRA and ARPA members. On the contrary, Gas Chromatography-Mass Spectrometry coupled to Pyrolysis, as an emerging technique in microplastic analysis, is currently used only by a single group affiliated to university. It is also necessary to consider that about 17% of working teams still do not have the instrumentation for microplastic analysis in drinking water (analyses were performed externally) although most of them are moving to purchase and/or are waiting for an analytical method approved by EU for the purchase. This is explained by the very recent interest of some ISPRA and ARPA members to drinking water analysis and of some water suppliers in the topic. The survey also confirmed that the choice of analytical techniques follows the type of water investigated. In this context, 3 major clusters can be observed: sea water samples, surface water samples and drinking water samples. Sea water samples are usually observed with microscopy (optical or electron microscopy), as they include large microplastics (> 300 µm) retrieved from Manta nets. Some research groups also confirmed the identity of some polymers with FTIR spectroscopy. Drinking water samples, as they often originate from water filtered on a substrate, are usually analysed with microscopy coupled to spectroscopy techniques as FTIR microscopy and Raman microscopy. Water suppliers usually preferred Raman over FTIR given its ability to be insensitive to water vibrational signals during the analysis. Surface water samples, as seen in pre-treatment phase, followed the direction of the distinctive research group: ARPA members usually performed microscope analysis while water suppliers were prone to use FTIR microscopy and Raman microscopy. From the panel discussion and survey suggestions, scientific priorities, and common objectives to be pursued emerged. The two major issues appeared to be variability in results and the relative lack of microplastics certified standard; also blanks preparation and contamination prevention

were discussed. Variability, as suggested by various group members, could be minimized by using similar analytical techniques with a standard procedure; also, microplastic standards could be useful. Some working group researchers in the past experienced proficiency tests with standard and agreed to share information about where to find/how to fabricate them. In term of contamination prevention, it was stated that is virtually impossible to prevent every source of contamination and is important to provide environmental blanks, procedural blanks and reagent blanks in order to avoid results overestimation. In conclusion, the *status* of microplastic analysis in Italy appears to be heterogeneous and constantly evolving. Researchers from organizations with different roles and objectives were already working on an analytical method for microplastics identification and quantification in drinking water, but without agreement on sampling method, pre-treatment, or analytical technique. The main difficulty that has emerged is to align the different targets of public research authorities and agencies and water suppliers. To overcome this issue, it will be necessary to develop a method that considers the needs of both, and, in particular, that is routinely applicable. The working group, as well as for method development, will be also useful to share know-how and data, waiting for more information about health implication of microplastics. In line with the working group aims, during the next months the participants will contribute to the drafting of a technical paper focusing on analytical techniques for the analysis of microplastics, while starting to work on the common analytical method.

3.2 EXPERIMENTAL ACTIVITIES

3.2.1 Objectives, Research planning and Experimental setup

Specific aims of the experimental part of the PhD thesis were to develop a method for microplastic analysis suitable for surface water and drinking water, by comparing sampling and analytical techniques, in order to evaluate their *pros* and *cons* with a view to a routine approach. Another side objective of the experimental part was to understand the possibility to apply the same analytical procedures for both surface water and drinking water. As microplastics analysis, especially in drinking water, is a fairly new branch of research (WHO, 2019; JRC, 2022) a careful planning step was required. A lot of time, also due to the pandemic, was spent on bibliographic research, understanding microplastics spread patterns in inland waters and analytical methods to properly characterise them. Bibliographic research, concluded with the publication of a technical report (Martellone et al.,2022) underlined the need to employ advanced and not straightforward analytical techniques, unlike other emerging contaminants. It also emphasised problems in microplastics sampling and sample treatment due to differences between bigger and smaller particles (small microplastics, SMPs) and between particles and fibers. In addition, high-quality data, especially for microplastics below 300 µm and for drinking water was lacking. In order to conduct a study such as the one described before and complicated by the issues mentioned above, national and international collaborations have been established. Collaborations helped the planning phase and gave logistical support for the sampling, sample pre-treatment and analysis phases. For the sampling phase, some video conference with Cranfield University (UK) and available Italian

drinking water suppliers were held. Discussions with Cranfield focused on sampling methods and the design of a new sampling system capable of filtering large volumes of water without clogging. Instead, with drinking water suppliers was discussed the opportunity of sampling water entering and leaving a drinking water treatment plant with adduction from surface water. For the sample pre-treatment phase and analysis phase, we collaborated with Istituto di Scienze Polari – Consiglio Nazionale delle Ricerche (ISP-CNR) in Mestre (Venice, Italy) and the Department of Chemistry of Padua University. Venice ISP-CNR gained remarkable experience over the years in microplastics analysis with FTIR microscopy, especially in biota, sediments and seawater matrices. They, above all, focused on small microplastics following the scientific approach of "low temperature" (mild) pre-treatment methods, consistent with problems related to small microplastics analysis. After discussing the analytical methodology to be employed in the research, both organisations gave us the availability to perform analysis in their laboratories, following the scheme previously reported. Once the corresponding positions have been established, a first draft of the timetable was drawn up. The timetable was later impacted by different elements, first of all the possibility of conducting the analyses due to the pandemic limitations. The actual project schedule is accurately described in Table 10.

DEVELOPMENT PHASE	SUB-PHASES	COLLABORATING ORGANIZATIONS	STUDY TIME PERIOD
RESEARCH PLANNING	Bibliographic Research		Autumn 2019 – Winter 2019
	Selection of study areas and Sampling Planning	Drinking Water Suppliers Cranfield University	Spring 2020
	Analysis Planning	Venice ISP-CNR Padua University	Summer 2020
SAMPLING	Sampling methods development and sampling protocol development	Cranfield University	Autumn 2020 – Spring 2021
	Sampling campaign	Drinking Water Suppliers	Summer 2021
SAMPLE PRE-TREATMENT	Surface and drinking water filtration-system samples: resuspension and homogenisation	Venice ISP-CNR	Summer 2021
	Surface water samples: digestion, extraction, filtration	Venice ISP-CNR	Spring 2022
	Drinking water samples: digestion, extraction, filtration	Venice ISP-CNR	Autumn 2022
FORMAL ANALYSIS	Surface water analysis (FTIR)	Venice ISP-CNR	Summer 2022
	Surface water analysis (Raman)	Padua University	Summer 2022
	Drinking water analysis (FTIR)	Venice ISP-CNR	Autumn 2022
DATA MANAGEMENT AND FINAL DRAFT	Data management #1 – Surface Water	Venice ISP-CNR	Autumn 2022
	Final Draft	Venice ISP-CNR	Winter 2022

Table 10. PhD project plan.

3.2.2 Material and Methods

3.2.2.1 Study Areas

In order to study microplastics contamination in freshwater environments and develop an analytical method useful for both types of waters investigated (surface water and drinking water), study areas were identified according to the design of the project. Choice criteria were the following: (i) proximity to anthropic activities and to the terminal tract of the river, (ii) possibility of sampling inside a drinking water treatment plant (DWTP) with multiple approaches and (iii) proximity to analysis sites. Following these criteria, three different areas were selected. Natural waters from the three longest Italian rivers (Po, Adige and Tiber river) destined to human consumption and the same water after the drinking water treatment was sampled. Po, which originates from Monviso in Piemonte, is the most important fluvial system of Northern Italy and the longest river in the Country with a very large drainage area (about 71.000 km^2) including several large cities (e.g., Turin, Piacenza, Cremona, Ferrara and Milan) as well as area of intensive industrial and agricultural activities. In the final tract, Po splits into a delta flowing into the Adriatic Sea in the proximity of Venice lagoon. Several data on Po ecosystem pollution emerged in the last decades. Data includes not only recent studies about plastics and microplastics contamination (Atwood et al., 2019; Munari et al 2019; Giardino et al., 2023) but also researches about other pollutants as heavy metals (Farkas et al., 2006) and contaminants with endocrine active potentials (Viganò et al., 2015) even though a lot of studies appear now outdated. The sampling site for Po area has been set at DWTP #3. DWTP #3 captures not only surface waters (60-80% of the treated waters) but also groundwaters in order to produce drinking water. Different waters undergo different treatments but both of them eventually converge in the drinking water sent to water supply network. Surface waters, before other treatments, enter ponding basins. After that, surface waters undergo clariflocculation, filtration on sand filters, ozonation, filtration on Granular Activated Charcoal (GAC) filters and finally disinfection by chlorine dioxide (ClO_2). Groundwaters, on the contrary, undergo oxidation in different lines and filtration on sand filters before the mixing with treated surface water prior to GAC filtration step. Adige, which originates from Reschenpass in Alto Adige close to the borders with Austria and Switzerland, is the second longest river in Italy with a drainage area of approximately 12.000 km^2. Adige River flows for 410 kilometres through most of north-eastern Italy, bathing, among others, the cities of Trent, Verona and Rovigo. As well as Po, also the Adige River flows into the Adriatic Sea, but closer to Venice lagoon. As far as it knows, data about microplastic in Adige River is lacking while other organic and inorganic pollutants have been studied in past years (Chiogna et al., 2016). The sampling site for Adige area has been set at DWTP #2. DWTP #2 is smaller than DWTP #3 and capture only surface water. However, drinking water sent to the water supply network is mixed in a minimum part with drinking water produced by another DWTP, which also treated water from Adige River. Surface waters undergo clariflocculation, filtration on sand filters, filtration on Biological Activated Charcoal (BAC) filters and finally disinfection by chlorine dioxide (ClO_2). Tiber river, which originates from Apennine Mountains, more precisely Fumaiolo Mountain in Romagna, is the most important river in Central Italy and the third

longest river behind Po and Adige. With a drainage area of approximately 17.000 km^2, Tiber flows through Tuscany, Umbria and Lazio until it meets Tyrrhenian Sea, between Ostia and Fiumicino cities. Tiber river runs through, above all, the city of Rome, where it receives water from the Aniene River resulting in a greatly increase of its flow capacity. Several data on Tiber pollution emerged in the last decades. Data includes not only plastics but also, among others, polycyclic aromatic hydrocarbons (PAHs) (Montuori et al., 2016a), heavy metals (Montuori et al., 2016b) and pharmaceuticals (Pérez-Fernández et al., 2014; Patrolecco et al., 2015). The sampling site for Tiber area has been set at DWTP #1. DWTP #1 is a unique treatment plant used in case of hydric emergencies. Surface waters in DWTP #1 undergo clariflocculation, filtration on sand filters, filtration on GAC filters and finally disinfection by ClO_2.

3.2.2.2 Sampling

Sampling Methods Development

One of the purposes of the research was to study differences between sampling techniques for microplastics analysis, especially sampling techniques suitable for small microplastics analysis (< 300 µm) and which can be used for both types of water (drinking water and surface water). Therefore, research focused on bottle sampling and *in-situ* filtration sampling, leaving aside sampling techniques more suitable for the larger fraction of microplastics (> 300 µm) and for navigable water bodies as sampling with nets. As described in the previous section, bottle sampling is the simplest, quickest and cheapest sampling technique for microplastics analysis, allowing an easier storage of samples in view of a routine analysis. However, bottle sampling, especially for drinking water matrix and for the collection of small microplastics, could provide an underestimation of results due to the lower volume of water that can be collected. *In-situ* filtration approaches, on the other hand, provide higher filtration volumes but require expertise, a design study and longer sampling times. At each sampling point, both sampling techniques were used in order to compare them and understand differences in results. The aim of this part of the study was also to understand the opportunity to apply the same sampling approach to two extremely different matrices such as surface water and drinking water. For bottle sampling, 2L cleaned and decontaminated Duran glass bottles were used. In order to prevent contamination by plastics caps an aluminium foil was applied between the tap and the neck of the bottles and around the whole cap. Aluminium foil was inserted both before and after water sampling. In addition, stock PP blue caps have been replaced with Polybutylene Terephthalate (PBT) red caps (Sial, Italy). PBT is a thermoplastic material from polyester family with similar properties to polyethylene terephthalate (PET), difficult to find in surface waters. For *in-situ* filtration approach, a filtration system was built, inspired by Mintenig et al. (2019), Drinking Water Inspectorate (2018) and other authors' works. The filtration system has been designed for filtering high volume of waters without clogging (preferably > 500 L) while simultaneously collecting microplastics > 5 µm. In order to fulfil this purpose, two filtration units were

assembled in sequence, one with a 300 µm filter, useful to retain impurities and bigger particles and one with a 5 µm (analytical filter). Three simple criteria were observed when designing the filtration system: (i) using plastic-free materials, (ii) using materials that can withstand possible high-pressures (iii) building up a closed system in order to reduce airborne contamination. When purchasing, brass parts were preferred over stainless-steel part only when stainless steel counterparts were too expensive or were not available. The assembled filtration system consists of (Figure 16, Figure 17):

1) A flexible silicon hose with stainless-steel adapters or jubilee clips. The silicon hose (12.5 mm Ø, 70° Shore), thanks to adapters or jubilee clips, can be connected to different tap sizes or to a pumping system for sampling without tap systems. In some cases, to better adapt to certain sampling taps with higher pressures, a flexible stainless-steel tube with a brass core has replaced the flexible silicon hose or was inserted along with it.

2) *A brass non return-valve*. The installation of a non-return valve (ARCO, Italy) is necessary in case of low pressures as it prevents water from flowing back. The non-return valve allows also the placement of the filtration system on an angle that increases the possibility of water backflow.

3) A brass "T piece" connector followed by two brass switches. A "T piece" just after the non-return valve allows the channelling of water in due directions: (i) to priming hose, (ii) to sampling systems. The brass switch (A1) located just before the first filtration unit allows the isolation of the entire filtration system when water does not flow through it and is opened when sampling begins. The brass switch (B) followed by a priming silicon hose allows to ensure the system to the tap avoiding airborne contamination.

4) The first filtration unit. The first filtration unit consists of a 10" length cylindrical stainless-steel filter housing (Spectrum, UK) in which a 300-µm stainless-steel cartridge filter (Wolftechnik, Germany) was inserted. The design of these filter housings allows water to flow in one-direction and capture particles on the large surface area of the filter. This unit has been designed to hold bigger particles, which surface water is usually full, that would clog the following 5 µm analytical filter.

5) A system of two "key" stainless-steel connectors. These particular connectors placed just before and after the isolation valves (A2, A3) of the second unit, allow an easy release of the second filtration unit for replacement during sampling.

6) The second filtration unit with two brass switches that act as isolation valves. The second filtration unit is similar to the first one with the difference being the mesh size of the filter: 5 µm (Wolftechnik, Germany) instead of 300 µm. The second filtration unit is the analytical unit that isolate microplastics from the water medium for the subsequent identification. Brass switches allow the isolation of the unit when the filter housing is replaced, decreasing the risk of an airborne contamination.

7) A flow meter connectable to the secondo filtration unit. The water meter (SISMA, Italy) is required to record the volume of water passed through the filtration system in order to normalise the results obtained.

8) A flexible stainless-steel tube with a brass core serving as discharging hose. A discharging hose is necessary to discharge water that has been filtered through the system.

When designing the filtration system, deployment of flat discoidal stainless-steel housings with 5 µm disc stainless steel inserts were considered (Strand et al., 2018; Weber et al., 2021). These filtration units, employed only for drinking waters, allow the distribution of particles on a smaller area (ca 10 cm^2) reducing possible losses and facilitating their subsequent identification. However, for surface waters, a smaller area could have resulted in an easier clogging and overlapping of particles so cylindrical units with cylindrical filter (ca 500 cm^2 filter area) were employed. In order to avoid cross-contamination, two identical and separate filtration systems were built, one for surface water and one for drinking water.

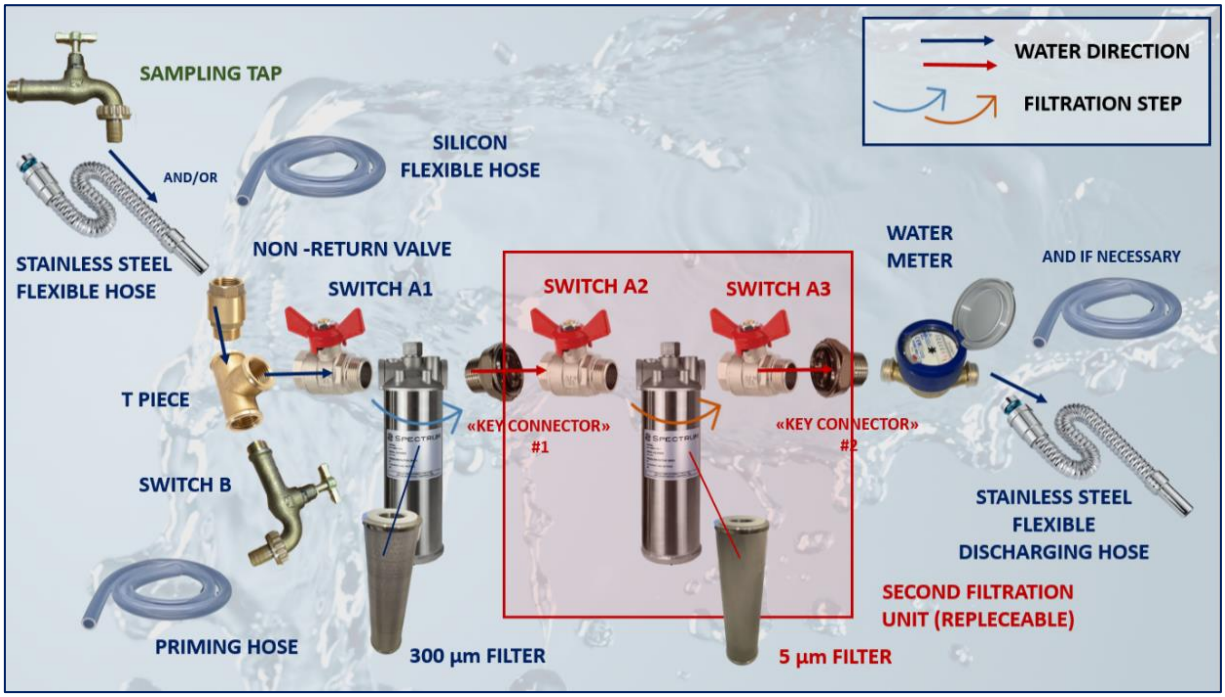


Figure 16. Filtration System Design.

Figure 17. Filtration System components.

Sampling protocol development

Before sampling operations in DWTPs, a sampling protocol was drawn up (Figure 18), both for bottles and for the filtration system sampling. The protocol includes a cleaning step performed in the laboratory before and after field sampling and was drawn up on the basis of the time availability of the drinking water treatment plant operators combined with the experimental requirements. Thus, sampling time in drinking water treatment plant was set has been set for up to six hours. To achieve desirable volumes for each type of water (> 500 L) in this period of time, sampling with filtration systems must be performed at the same time with two different filtration systems, assuming pressures at taps were adequate. For filtration system sampling, both for surface drinking water, a replacement of the second filtration unit with a new empty one after a set period of time was also employed. This gimmick minimizes the possibility of clogging, which was likely expected to occur in waters with high turbidity. Once the targets and restrictions were set, the protocol was finalised.

SAMPLING PROTOCOL	
1)	Once all parts have been decontaminated following cleaning protocols (*see QA/QC section), the filtration system is assembled, switching valves (A1, A2, A3 and B) are closed and the open ends are sealed with aluminium foil as for bottles. Bottles and filtration systems are then moved to the sampling spots;
2)	Before sampling, water quality data should be retrieved (turbidity, hardness, pH and conductivity) in order to better understand water behaviour and any potential effects on the filtration system;
3)	At sampling spots, tap is left running for 5 minutes before the sampling begins. Once this time passes, bottles are filled with water. Briefly, 12L of surface water and 12L of treated drinking water were collected from sampling tap into 2L glass bottles for a total of 24 L of water for each sampling site. Aluminium foil is then applied between the tap and the neck of the bottles and around the whole cap;
4)	After bottle sampling, the silicon hose or the stainless-steel tube of the filtration systems is secured at the sample tap with adaptors or jubilee clips. For each tap, the filtration system should be arranged in a way that it is sufficiently stable and, in a manner, to allow easy draining of filtered water;
5)	Once the filtration system is secured, switching valve B is opened together with the sampling tap. Thus, water passes in the first section of the sampling system without enter into the filtration units;
6)	Switching valves A (A1, A2 and A3) are opened while switching valve B is closed. At the same time, time is recorded while observing water flowing through the water meter. Water meter, especially for surface water, should be monitored every 10 minutes in order to check for clogging;
7)	After one hour and 20 minutes of sampling, sampling tap is closed together with switching valves A (A1, A2 and A3). Volume of filtered water is recorded through the difference in values observed on the water meter after and before sampling;
8)	The second filtration unit is easily detached from the body of the filtration system thanks to “key connectors”, sealed with aluminium foil and replaced with a new 5-µm filtration unit. Residual water from each filtration water were not released;
9)	Switching valves A (A1, A2 and A3) are opened again and filtration continues for another hour and 20 minutes, when the unit is going to be replaced again;
10)	After two replacements for each filtration system (4 hours), sampling is over. Filtration system sample then consists in three separate filtration units, which will be merged in the laboratory into a single sample, one for each type of water. The amount of water globally filtered for each filtration system is determined by adding up the volume of water filtered for each individual unit. In summary, for each sampling site there will be 6 bottles and 3 filtration units per water type.
11)	Once sampling is complete, the silicon hose or the stainless-steel tube is disconnected from the sampling tap. Samples and the remaining parts of filtration systems are moved to the laboratory for analysis and cleaning.

Figure 18. Sampling Protocol.

Sampling campaign

Sampling campaign took place in the summer of 2021, between 15th of July to the 17th of September. Originally, two sampling campaigns were planned, one in summer and one in winter, with the aim of observing any fluctuations in the type and in the number of microplastics observed caused by any seasonal variables. However, the long analysis time and pandemic-related issues forced us to eliminate this second series of samplings. Prior to any field activities, a series of conference calls with drinking water treatment plant operators took place to better understand the system's characteristics and tap connections in sampling points, in order to adapt the filtration system to the specified sampling. For each DWTP, two sampling point were then identified: one for surface water and one for drinking water, each one with different tap characteristics. Water quality data retrieved from drinking water treatment plant operators showed notable differences in water sampled (Table 11), as a direct consequence of varieties in weather conditions and in the water source sampled. Unfortunately, some values are missing because (i) some parameters are not usually monitored for certain types of water in some DWTP and (ii) the measurements were not carried out close to the sampling day.

	DWTP #1		DWTP #2		DWTP #3	
Sampling date	July 15th 2021		July 27th 2021		September 17th 2021	
Weather Condition	Sunny		Light Rain		Cloudy	
Water type	Surface Water	Drinking Water	Surface Water	Drinking Water	Surface Water	Drinking Water
Turbidity (NTU)	15	0.2	55	0.2	25	0.2
Hardness (°F)	42.5	42.5	11	12	-	23
Conductivity (µS/cm 20°C)	-	-	213	259	447	492
pH	7.5	7.5	7.9	7.3	8.1	7,6

Table 11. Weather conditions and water parameters on sampling days.

Sampling in DWTP #1 took place on July 15th morning. It was a sunny day, without any atmospheric disturbance. Furthermore, no rainfall was observed in the two weeks prior to sampling in the areas surrounding the sampling site. Sampling taps for drinking water and surface water were located in different places, one outside and one sheltered in an underground room. The two sites have located some distance from each other, requiring numerous transfers by car in order to observe any possible issues when sampling with the filtration system. Sampling with bottles (Figure 19), on the other hand, did not require any special arrangements and was completed without any problems; it required only 10 minutes for each site.

Figure 19. Bottle Sampling (Drinking Water) at DWTP #1.

For surface water, a ¾''/ ¾'' stainless steel adaptor was necessary to connect the tap to the stainless-steel tube of the filtration system. Stainless steel tube was preferred over silicon tube due to high inlet pressures. A longer silicon tube was connected instead to the discharging hose to allow water flowing into a distant manhole. Filtration was carried out without clogging for all 4 hours of sampling. Changing of the 300-µm filter was also not necessary. Approximately 2,810 m^3 of surface water was filtered among the three filters (0,968 m^3+ 0,757 m^3+ 1,089 m^3). For drinking water, tap characteristics made it mandatory to use of a short piece of silicone hose and a jubilee clip; silicone hose was also necessary to lengthen the inlet pipe due to the positioning of the tap in relation to the ground. As for surface water, a longer silicon hose was attached to the discharging hose to ensure the flowing of the filtered water into a distant manhole. As expected, filtration was carried out without clogging for all 4 hours of sampling. Approximately 1,798 m^3 of surface water was filtered among the three filters (0,708 m^3+ 0,490 m^3+ 0,600 m^3). The filtration system setup for both surface water and drinking water is shown in Figure 20.

Figure 20. Sampling setup for the filtration system in DWTP #1: surface water (up) and drinking water (down).

Sampling in DWTP #2 took place on July 27th morning. It was a rainy day, especially in the morning. However, during sampling, there was minimal rainfall and no rainfall had been observed in the previous week. This however led to the highest turbidity observed during the entire sampling campaign (55 NTU). Unlike the DWTP #1, sampling spots were located in the same place, at a short distance, in an indoor room where it was also easy to change filters and tighten bolts. As for Rome DWTP, sampling with

bottles (Figure 21) did not require any special arrangements and was completed without any problems in 10 minutes.

Figure 21. Bottle Sampling (Surface Water) at DWTP #2.

Tap design in sampling site allowed the use of only a small piece of silicone tube with a jubilee clip to connect the filtration system. Filtration (Figure 22) was carried out without clogging for all 4 hours of sampling. Changing of the 300-µm filter was also not necessary, even though it appeared full of debris when opened at laboratory. Approximately 0,661 m^3 of surface water and approximately 1,258 m^3 of drinking water was filtered among the six filters (0,236 m^3+ 0,227 m^3+ 0,198 m^3 for surface water and 0,419 m^3+ 0,418 m^3+ 0,421 m^3 for drinking water). Only a slight flow reduction was observed during surface water sampling.

Figure 22. Sampling setup for the filtration system in DWTP #2: surface water (up) and drinking water (down).

Sampling in DWTP #3 took place on September 17th, 2021, in the morning; it was a cloudy day. Furthermore, no rainfall was reported in the two weeks prior to sampling. As happened during sampling in DWTP #1, also in DWTP #3 sampling sites were located at a remarkable distance from each other so numerous transfers were required. Both sampling spots were located outside but drinking water site was inside a covered box. Once again, sampling with bottles proceeded without any problems. Surface water and drinking water taps allowed direct connection with the flexible silicon hose and the jubilee clip. As for DWTP #2 samplings, for surface water an extension in the discharging hose was not necessary due

to the proximity of the manhole. On the contrary, for drinking water, the silicon hose extension was useful. Filtration was carried out without clogging for all 4 hours of sampling. Changing of the 300 µm filter was also not necessary. Approximately 1,940 m^3 of surface water was filtered among the three filters (0,396 m^3+ 0,585 m^3+ 0,959 m^3). For drinking water, the situation was different: 5,322 m^3 spliced into the three analytical filters (2,071 m^3+ 2,084 m^3+ 1,167m^3). Filtration system setup in DWTP #3 is shown in Figure 23. Collected volumes from the three DWTP is shown in Table 12.

Figure 23. Sampling setup for the filtration system in DWTP #3: surface water (left) and drinking water (right).

	FILTRATION SYSTEM						BOTTLE SAMPLING	
Water type	**Surface Water**			**Drinking Water**			**Surface Water**	**Drinking Water**
DWTP #1	968L	757L	1089L	708L	490L	600L	12 L	12 L
	2810 L			1798 L				
DWTP #2	236L	227L	198L	419L	418L	421L	12 L	12 L
	661 L			1258 L				
DWTP #3	396L	585L	959L	2071L	2084L	1167L	12 L	12 L
	1940 L			5322 L				

Table 12. Volumes of water sampled in litres.

Storage and transport of equipment

Immediately after each sampling, with the exception of DWTP #1 samples, bottles, filtration units and the remaining of filtration systems were taken by car to the CNR-ISP in Venice in order to perform the analysis and restore the filtration system for a new sampling. Because of the distance between Rome and Venice, DWTP #1 samples were brought to the CNR only the following day. Bottles have been kept refrigerated at 4 °C and, when travelling by car, in portable coolers (Mobicool, Sweden) at the same temperature. Instead, filtration units, given their weight and size, were only kept far from light and heat in a big plastic box with castors covered by a dark cotton towel. This concept was particularly useful, especially when travelling, as it served to minimise contact with the atmosphere and prevented water leaks inside the car. No fixatives were used.

3.2.3 Sample Pre-Treatment

Sample treatment rationale

One of the purposes of the research was to study pre-treatment methods for surface and drinking waters understanding their suitability for both matrices following the classical analysis of microplastics through microscopy coupled to spectroscopy framework: digestion, extraction and filtration. The aim was looking for a new low-temperature pre-treatment method capable of preserving the structural integrity of plastics while they are separated from the matrix. Several pre-treatment methods are indeed temperature-assisted or based on reagents affecting the number, morphology and colour of microplastics (Martellone et al., 2021). Additionally, a method for resuspension of particles for filtration-system samples consistent with this philosophy needed to be developed together with a study on the filtration substrate. Following these prerogatives, sample treatment method development was divided for matrix type and studied in separate times. The only exception to this pattern is the first stage of filtration-system-samples treatment, as it needed to be performed at the same time in order to restore the filtration system for subsequent sampling. Therefore, sample treatment methods development was divided into three phases following the subject studied. Each phase was developed at Venice ISP-CNR (Table 13) (Corami et al., 2021).

PHASE	LAB ACTIVITY	PERIOD
I	Surface water and drinking water filtration-system samples: *resuspension and homogenisation*	Summer 2021
II	Surface water samples: *digestion, extraction, filtration*	Spring 2022
III	Drinking water samples: *digestion, extraction, filtration*	Autumn 2022

Table 13. Phases of sample treatment methods development.

Surface water and Drinking water filtration-system samples: resuspension and homogenisation

Particles recovered on 5 µm filters need to be resuspended in a liquid carrier in order to subsequently convey them onto a suitable substrate for microscopy-spectroscopy analysis. Some authors (e.g., Mintenig et al., 2019; Drinking Water Inspectorate, 2019; Primpke et al., 2020) studied resuspension methods for this type of sampling. Sonication followed by some kind of chemical treatments (e.g., SDS) to facilitate the separation of particles from the filter was usually performed. In order to follow the philosophy of preserving as much as possible the structural integrity of plastics, shorter sonication times together with no reagents were employed. It was also decided to keep the residual water inside the filter holders, in order to avoid particle losses. Furthermore, because of the choice to keep residual water and for logistical reasons, a homogenization step was required. Briefly, filtration units were opened under fume hood in ISO 7 Clean Room plastic-free. Cartridge filters, together with residual water (at least 1.5 L per sample), were removed using stainless-steel tweezers and inserted in a 5 L cleaned and decontaminated glass beaker. Filter housings were rinsed with ultrapure water (UW; Elga Lab Water, Veolia, High Wycombe, UK) and the water was collected into the beaker; cartridge filters inside the beaker were also rinsed with ultrapure water. The final volume of water for each beaker, including rinsing water and the residue left inside the filter holder, was set at 2.4 L. This volume covered the filter by half. Therefore, the beaker was covered with an aluminium foil and 3 minutes of sonication (FALC INSTRUMENTS, Italy) at room temperature for each cartridge side was performed (Figure 24). After that, 800 mL of the solution were transferred into a clean and decontaminated 2.5 L glass bottle. This procedure was repeated three time, one for each cartridge filter of a single sampling site, allowing the collection of 2.4 L per sampling site for each type of water (resuspension + homogenization). The same workflow was employed also for each 300-µm filtration unit, but the resulting samples were not

analysed due to time limitations. Each bottle was then covered in the same way as the bottled water samples and stored at 4 °C for further pre-treatment procedures.

Figure 24. Resuspension of particles for a surface water sample (left) and a drinking water sample (right).

Surface water samples: digestion, extraction and filtration

Surface water samples, of both types, pose a unique challenge given the extreme heterogeneity of the matrix under investigation and differences in weather conditions during sampling. The choice to carry out a light room-temperature pre-treatment method could have resulted in the inability of reading samples due to the high content of interferers that surface water is usually full. In addition, due to time restrictions, it was impossible to carry out preliminary tests, which would have required a test surface water with similar properties to those of the sampled waters. Therefore, a detailed study for digestion, extraction and filtration based on previous experiences and literature search was performed before using sampled water. For digestion, H_2O_2 is recognised as of the most effective digestive for microplastics analysis and it is the safer for microplastics integrity when used at room temperature (Martellone et al., 2021). In order to achieve organic matter decomposition, sediments, samples from wastewater treatment plants and surface waters are usually treated with H_2O_2 at high temperatures, sometimes with the aid of a Fe (III) catalyst, to ensure a high decomposition rate. Since this path was not viable, research mainly focused on an extraction method allowing almost complete removal of the water in order to mitigate the matrix effect. Hypersaline extraction was immediately discarded due to: (i) high percentage of water and thus impurities retained in the hypersaline phase (ii) inconsistent results for small microplastics analysis (iii) unavailability of salts ensuring a good density separation (Corami et al., 2021). For the extraction of microplastic in environmental matrices, one of the few alternatives to density separation is

oil extraction. In a recent work on seawater and sediments (Corami et al., 2021), organic cold-pressed sunflower seed oil (SSO) extraction allowed a clear separation of microplastics from matrix and the analysis through FTIR microscopy. Oil extraction could also allow the extraction of high-density microplastics (e.g., fluorinated polymers) that is impossible with a hypersaline solution. Organic cold-pressed SSO (Consilia, Italy) was chosen because it was certified solvent-free; besides, it had a minor content of pigments (e.g., chlorophyll, carotenoids, etc.) compared to other oils (e.g., canola oil, castor oil, extra virgin olive oil, rapeseed oil, etc.) (Mani et al., 2019; Lechthaler et al., 2020; Scopetani et al., 2020). Given these prerogatives, it was decided to apply the oil-extraction method to these samples as well, by intensively working on the volumes of water to be employed (Figure 25). In the previous work, 200 mL of seawater was diluted with UW in a 500 mL cleaned and decontaminated separating funnel (Funnels "A") to diminish salinity and avoid spectral interferences (1:1 ratio, 400 mL in total). Given the lower salinity of surface water, a first attempt with 450 mL was made. Due the problems related to specific samples, a second attempt following the same procedure of seawater (200 mL of sample + 200 mL of UW) was also made. It was also decided to follow the two-digestion-extraction scheme proposed by Corami et al. (2021) in order to ensure higher particles recoveries (Figure 26). Assuming a higher content of suspended solids in surface water, especially for filtration system samples, a higher volume of H_2O_2 30% solution (ACS reagent, Sigma Aldrich, Merck, Darmstadt, Germany) and oil for particles recovery was used for both extractions (7 mL of H_2O_2 and 7 mL of SSO over 5 mL of H_2O_2 and 5 mL of SSO in previous seawater tests). In order to achieve a complete separation of phases, separating funnels were left to rest for 24 hours after the first extraction and 3 hours after the second extraction, following a sort of compromise between 72 hours/24hours for sediments and 3 hours/3 hours for seawater in the previous work. Given the choice of performing a double digestion-extraction, an expedient had to be found to prevent the residual aqueous phase from interacting with the sediment potentially formed at the bottom of the funnel just after the first extraction. Therefore, UW was slowly added to bring the oil phase to the top of the funnel. After 15 minutes, the oil together with approximately half of the water contained in the funnel was moved into a new funnel (Funnels "B"). Residual water and sediment in Funnels "A" have been subjected to the second digestion-extraction following the scheme previously described. As in some cases sediment was still observed in the bottom of second-series funnels, pouring oil from the top of the funnels with the aid of UW in a new series of funnels (Funnels "C") was necessary.

Figure 25. Microplastics digestion/extraction for surface water DWTP #3 samples

After the extraction and/or the transfer, residual water was discharged from the bottom of the funnel. To ensure appropriate filtration due to viscosity, oil needed to be solubilised in an organic medium. Following a similar scheme to those proposed by Corami et al. (2021), each oil phase was recovered with 20 mL of n-Hexane (95%, Romil, Leicestershire UK) and 20 mL of ethanol (absolute, for HPLC, ≥99.8%, Sigma Aldrich, Merck, Darmstadt Germany)/methanol (Superpurity Solvent >99.9%, Romil, Cambridge UK) 50:50 solution and placed in the same Erlenmeyer flask. Filtrations were in most cases conducted immediately after extraction. As it was planned to analyse the samples with both FTIR microscopy (in transmission mode) and Raman microscopy, a suitable medium needed to be chosen. Following the literature data, only aluminium oxide filters and silicon filters produced adequate results for both techniques (Martellone et al., 2021). However, as silicon filters have a smaller surface area and are more susceptible to clogging and overlapping of particles, for surface water sample set it was decided to employ alumina filters (0.2 μm, 47 mm diameter, ANODISC (Anopore Inorganic Membrane), Whatman, Merck Darmstadt Germany). Forty-seven mm filter diameter was chosen based on previous experiences and since a high number of particles, both microplastics and interfering substances, was expected. Therefore, after the decontamination of filter and flasks manual shaking, samples were slowly vacuum filtered using hexane to solubilise the oil even more and ethanol/methanol 50:50 solution for cleaning the filter after a portion of the sample was passed through the filtration apparatus (VWR International, Milan, Italy). All filters were stored in cleaned and decontaminated glass Petri dishes

coated with aluminium foil in the clean room and left to dry at room temperature waiting for the analysis. The entire sample pre-treatment protocol is described below.

1) Each bottle was manually stirred 300 times before 450 mL of water/200 mL of sample + 200 mL of UW was poured into cleaned and decontaminated 500 mL glass separating funnels (A). In order to measure volumes, water in bottles was firstly poured into a graduated 1 L cleaned and decontaminated glass beaker. Each sample is processed in triplicate under fume hood in ISO 7 Clean Room.

2) In each (A) separating funnel 7 mL of H_2O_2 30% solution and 7 mL of SSO were added with the aid of cleaned and decontaminated glass measuring cylinders. After decontaminating the caps, funnels (A) were vigorously and manually stirred. The content of the funnels was manually stirred again after 3 and 6 hours. The mixture is then left to rest until the following morning (24 hours in total) as the separation occurs.

3) The following morning measured UW water was slowly poured into the separating funnels (A) until oil reaches the top (neck) of the funnel. At this point, after 15 minutes, oil with approximately half of the water contained in the funnel, was moved into a new set of 500 mL cleaned and decontaminated glass funnels (funnels "B"). After 15 minutes, residual water contained in the funnels (B) was discharged from the bottom of the funnel. The oil phase was recovered with 20 mL of hexane and 20 mL of ethanol/methanol 50:50 solution and placed in a cleaned and decontaminated Erlenmeyer flask. Hexane and ethanol/methanol solution were added with the aid of cleaned and decontaminated glass measuring cylinders.

4) Residual water and sediment in funnels "A" were subjected to the second digestion-extraction. Briefly, 7 mL of H_2O_2 and 7 mL of SSO were added in funnels "A" with the aid of cleaned and decontaminated glass measuring cylinders. After decontaminating the caps, funnels (A) were vigorously and manually stirred again. Then the mixture is left to rest for 3 hours as the separation occurs.

5) After 3 hours, if sediment in funnels (A) persisted despite the second digestion-extraction, measured UW water is slowly poured into the funnels until oil reaches the top (neck) of the funnel. At this point, after 15 minutes, oil with approximately half of the water contained in the funnel is moved into a new set of 500 mL cleaned and decontaminated glass funnels (funnels "C") (Step 6). Otherwise, residual water contained in the funnels (A) was discharged from the bottom of the funnel (Step 7).

6) After 15 minutes, residual water contained in the funnels (C) was discharged from the bottom of the funnel.

7) The oil phase of funnels (A) or (C) is recovered with 20 mL of hexane and 20 mL of ethanol/methanol 50:50 solution and placed in a cleaned and decontaminated Erlenmeyer flask, the same one into which the oil from the corresponding (B) funnel was poured.

8) The cumulative oil/Hexane/Methanol: Ethanol sample (B+A or B+C) was immediately subject to filtration or stored in the Clean Room away from light and heat sources.

9) After cleaning and decontamination, the filtration apparatus was assembled; the new ANODISC filter is picked up from the supporting box with the help of decontaminated steel tweezers and placed on the filtration membrane before the block with the stainless-steel clamp.

10) Before the filtration, the ANODISC filter was cleaned by passing about half the volume of the glass bell of ethanol/methanol 50:50 solution.

11) When the vacuum pump was turned on, a small sample amount (about 1/3 of the sample) was manually poured into the glass bell from the Erlenmeyer flask, immediately followed by a small amount of hexane to ensure oil solubilisation. Between each portion of sample, a cleaning step with ethanol/methanol 50:50 solution was performed.

12) At the end of each filtration, the apparatus was disassembled, and the filter is placed, with the aid of decontaminated steel tweezers, in a cleaned and decontaminated glass Petri dishes. Petri dishes are labelled, covered with aluminium foil and stored in the Clean Room until the analysis.

The protocol was applied in triplicate to each surface water sample. A single surface water sample required 6 funnels (or 9 funnels if sedimentation blocked discharging of water from the bottom) and in medium 3 days for digestion, extraction and filtration (including cleaning and decontamination procedures). In the first test (450 mL of sample), given 6 samples (3 for the bottles and 3 for the filtration system samples), were produced a total of 18 replicas which have been labelled in Petri Dishes as described in Table 14. The second test (200 mL of sample + 200 mL of UW) needed to be conducted only for those samples that proved to be unreadable through FTIR microscopy. Unfortunately, due to time constraints, it was only conducted on one of the two samples concerned on only one of the two samples involved. Samples involved in the issue were DWTP#1 SF5 and DWTP #2 SF5, but a test was only conducted on DWTP #2 SF5 (Table 14).

FIRST TEST (450 mL of the sample)		
DWTP #1 **Raw Water Samples** **(GRZ DWTP #1)**	Filtration system (GRZ DWTP #1 SF5)	GRZ DWTP#1 SF5 #1 GRZ DWTP#1 SF5 #2 GRZ DWTP#1 SF5 #3
	Bottles (GRZ DWTP#1 TQ)	GRZ DWTP#1 TQ #1 GRZ DWTP#1 TQ #2 GRZ DWTP#1 TQ #3
DWTP #2 **Raw Water Samples** **(GRZ DWTP #2)**	Filtration system (GRZ DWTP#2 SF5)	GRZ DWTP#2 SF5 #1 GRZ DWTP#2 SF5 #2 GRZ DWTP#2 SF5 #3
	Bottles (GRZ DWTP#2 TQ)	GRZ DWTP#2 TQ #1 GRZ DWTP#2 TQ #2 GRZ DWTP#2 TQ #3
DWTP #3 **Raw Water Samples** **(GRZ DWTP #3)**	Filtration system (GRZ DWTP#3 SF5)	GRZ DWTP#3 SF5 #1 GRZ DWTP#3 SF5 #2 GRZ DWTP#3 SF5 #3
	Bottles (GRZ DWTP#3 TQ)	GRZ DWTP#3 TQ #1 GRZ DWTP#3 TQ #2 GRZ DWTP#3 TQ #3
SECOND TEST (200 mL of sample + 200 mL of UW)		
DWTP #2 **Raw Water Samples** **(GRZ DWTP #2 BIS)**	Filtration system repeated (GRZ DWTP#2 SF5 BIS)	GRZ DWTP#2 SF5 #1 BIS GRZ DWTP#2 SF5 #2 BIS GRZ DWTP#2 SF5 #3 BIS

Table 14. Filters produced by filtering surface water samples. SF5 ("Sistema Filtrazione 5 µm") samples refers to samples from the filtration system while TQ ("Tal Quale") samples refers to discrete samples.

Drinking water samples: digestion, extraction and filtration

For Drinking water, a similar workflow was observed. As it is generally considered a clear medium in relation to matrix-effect, compared to surface water, fewer complications were assumed. However, although drinking water samples generally undergo minimal preliminary treatments, a digestion step is usually required in order to dissolve inorganic precipitates related to water hardness prior to FTIR and Raman microscopy inspection (Mintenig et al., 2019; Weber et al., 2021). Inorganic precipitates, in addition to hindering the analysis, could form a salt layer on the filter which, once dry, could result in breakages due to its relative fragility. After the digestion step, the sample is usually filtered as such on an appropriate substrate and undergoes instrumental analysis. In contrast to surface waters, it was possible to carry out a preliminary experimental study on a test drinking water. Test drinking water was collected in 2 L decontaminated glass bottles from a tap located in CNR-ISP ISO-7 Clean room. Drinking water in Mestre (Venice) is processed by Quarto d'Altino ("Ca' Solaro") DWTP. This DWTP

treats water from Sile, a medium-length river that passes across the Veneto region and flowing into the Venice lagoon. Surface water undergoes flocculation, sand-filtration and disinfection before entering the water supply network. Before preliminary tests, an approximate measure of water hardness was carried out with a water hardness rapid test (Aquakaiser, Germany). As expected, Ca' Solaro drinking water proved to be very hard water (35-40 °F), giving an ideal test water to verify problems related to water hardness. Once data on water hardness was retrieved, an experimental scheme was drawn up. Regarding water volumes to be analyzed, 700 mL of drinking water was poured into 1L separating funnel. The choice of working with higher volumes compared to surface water was taken given the lower number of microplastics expected. For the digestion step, it was followed the scheme used for surface water, adding in separating funnels a total of 14 mL of H_2O_2 30% solution (ACS reagent, Sigma Aldrich, Merck, Darmstadt, Germany) as other paths were not practicable. For the extraction step and filtration step, different approaches were attempted:

A. In the first separating funnels series, water was treated with disodium ethylenediaminetetraacetic acid (disodium EDTA). Briefly, a solution 0.20 M of disodium EDTA in test water was prepared. 700 mL of the solution were poured into the funnels and treated with 14 mL of H_2O_2 30% solution (ACS reagent, Sigma Aldrich, Merck, Darmstadt, Germany). Funnels were manually stirred and after 3 hours the water was directly filtered into ANODISC filters. Despite chelation of Ca^{2+} and Mg^{2+} with EDTA works better with pH > 8 an ammonium buffer was not used as 1) pH of test water was alkaline 2) an ammonium buffer had to be tested for the presence of microplastics 3) the objective was not the complete removal of water hardness but only a reduction.

B. In the second separating funnels series, water was treated with ethylenediaminetetraacetic acid (EDTA). Briefly, a solution 0.016 M of acid EDTA in test water was prepared. 700 mL of the solution were poured into the funnels and treated with 14 mL of H_2O_2 30% solution (ACS reagent, Sigma Aldrich, Merck, Darmstadt, Germany). Funnels were manually stirred and after 3 hours the water was directly filtered into ANODISC filters. An attempt with acid EDTA was made given the results of disodium EDTA treatment. Acid EDTA actually does not provide Na^+ ions that combined with other drinking water components could result in salt depositions on the filter.

C. In the third separating funnels series, a two steps oil-extraction similar to those carried out for surface water was performed. Briefly, 7 mL of H_2O_2 and 7 mL of SSO were poured into separating funnels. The content of the funnels was manually stirred and left to rest for 3 hours. After this period of time, water was discharged in a second series of 1 L separating funnels while the oil was recovered with 20 mL of hexane and 20 mL of ethanol/methanol 50:50 solution in a previously decontaminated Erlenmeyer flask. The water was treated again with 7 mL of H_2O_2 and 7 mL of SSO, manually stirred and left to rest for a further 3 hours. The oil was once again recovered in the same Erlenmeyer flask with 20 mL of n-Hexane and 20 mL of ethanol/methanol 50:50 solution while the water was discharged to waste. After that, oil was vacuum filtered on ANODISC filters.

Given the results of these tests, drinking water samples were subjected to the two-digestion- oil extraction (Figure 26) scheme studied for surface water.

1) Each bottle was manually stirred 300 times before pouring 700 mL of water sample into cleaned and decontaminated 1 L glass separating funnels (A). In order to measure volumes, water in bottles was firstly poured into a graduated 1 L cleaned and decontaminated glass beaker. Each sample was processed in triplicate under fume hood in ISO 7 Clean Room.

2) In each (A) separating funnel 7 mL of H_2O_2 30% solution and 7 mL of SSO were added with the aid of cleaned and decontaminated glass measuring cylinders. After decontaminating the caps, funnels (A) were vigorously and manually stirred. The content of the funnels was manually stirred again after 1.5 hours. Then the mixture is left to rest for 1.5 hours more (3 hours in total) as the separation occurs.

3) Water contained in the funnels (A) was discharged from the bottom of the funnels in a news set of 1 L funnels (B). The oil phase was recovered with 20 mL of hexane and 20 mL of ethanol/methanol 50:50 solution and placed in a cleaned and decontaminated Erlenmeyer flask. Hexane and ethanol/methanol solution were added with the aid of cleaned and decontaminated glass measuring cylinders.

4) Water in funnels (B) was then subjected to the second digestion-extraction. Briefly, 7 mL of H_2O_2 and 7 mL of SSO are added in funnels (B) with the aid of cleaned and decontaminated glass measuring cylinders. After decontaminating the caps, funnels (B) were vigorously and manually stirred again. Then the mixture is left to rest for 3 hours as the separation occurs. After 15 minutes, residual water contained in the funnel (B) was discharged from the bottom of the funnels while the oil phase was recovered with 20 mL of hexane and 20 mL of ethanol/methanol 50:50 solution and placed in a cleaned and decontaminated Erlenmeyer flask, the same one into which the oil from the corresponding (A) funnel was poured.

5) The cumulative oil/Hexane/Methanol: Ethanol sample (A+B) was immediately subject to filtration or stored in the Clean Room away from light and heat sources.

6) After cleaning and decontamination, the filtration apparatus is assembled; the new ANODISC filter was picked up from the supporting box with the help of decontaminated steel tweezers and placed on the filtration membrane before the block with the stainless-steel clamp. Before the filtration, the ANODISC filter was decontaminated by passing about half the volume of the glass bell of ethanol/methanol 50:50 solution.

7) When the vacuum pump was turned on, a small sample amount (about 1/3 of the sample) was manually poured into the glass bell from the Erlenmeyer flask, immediately followed by a small amount of hexane to ensure oil solubilisation. Between each portion of sample, a cleaning step with ethanol/methanol 50:50 solution.

8) At the end of each filtration, the apparatus was disassembled, and the filter was placed, with the aid of decontaminated steel tweezers, in a cleaned and decontaminated glass Petri dishes. Petri dishes are labelled, covered with aluminium foil and stored in the Clean Room until the analysis.

Figure 26. Digestion/Extraction for Drinking Water DWTP #3 Samples.

During Drinking Water processing (700 mL of sample), given 6 samples (3 for the bottles and 3 for the filtration system samples), were produced a total of 18 replicas, labelled in Petri Dishes as described in Table 15.

SINGLE TEST (700 mL of the sample)		
DWTP #1 Drinking Water Samples (POT DWTP #1)	Filtration system (POT DWTP #1 SF5)	POT DWTP#1 SF5 #1 POT DWTP#1 SF5 #2 POT DWTP#1 SF5 #3
	Bottles (POT DWTP#1 TQ)	POT DWTP#1 TQ #1 POT DWTP#1 TQ #2 POT DWTP#1 TQ #3
DWTP #2 Drinking Water Samples (POT DWTP #2)	Filtration system (POT DWTP#2 SF5)	POT DWTP#2 SF5 #1 POT DWTP#2 SF5 #2 POT DWTP#2 SF5 #3
	Bottles (POT DWTP#2 TQ)	POT DWTP#2 TQ #1 POT DWTP#2 TQ #2 POT DWTP#2 TQ #3
DWTP #3 Drinking Water Samples (POT DWTP #3)	Filtration system (POT DWTP#3 SF5)	POT DWTP#3 SF5 #1 POT DWTP#3 SF5 #2 POT DWTP#3 SF5 #3
	Bottles (POT DWTP#3 TQ)	POT DWTP#3 TQ #1 POT DWTP#3 TQ #2 POT DWTP#3 TQ #3

Table 15. Filters produced by filtering drinking water samples.

FTIR Microscopy Analysis and Data Acquisition

Fourier Transform Infrared microscopy analysis, separately for surface water and drinking water, were carried out using a Micro-FTIR Nicolet™ iN™ 10 (Thermo Fisher Scientific) equipped with an ultra-fast motorized stage and liquid nitrogen cooled MCT (mercury cadmium telluride) detector. Abundance and polymer identifications were evaluated according to Corami et al. (2020a, 2021), following the *"semi-automated analysis: particle measuring"* and "*subsampling*" approaches (Figure 27). Briefly, an optical image of 20 known-sized areas (i.e., count fields) was chosen in each filter before the microscopic count. Each count area (2000 µm x 1200 µm, 2,4 mm^2) was randomly selected with no overlapping ("*box-based subsampling*" in "*random*" mode) by employing the WIZARDS section of the Omnic™ Picta™ software. 64 co-scans were collected (spatial resolution 100 µm, aperture 100 µm × 100 µm, spectral range 4000–1200 cm^{-1}) on transmittance mode for each particle. Each IR transmittance spectrum of suspected plastics was then compared with specific microplastics reference libraries (Appendix 2). Microplastics were counted and identified only when the identification match percentage was ≥ 65%. The abundance of microplastics in surface water and drinking water was then evaluated (N_{SMP}/L) according to the following equation (Corami et al., 2021):

$$N_{SMP}/L = \frac{n*1000*F}{V}$$

Where n = microplastics counted on every field, V = volume of filtered water (L) and F is the count optical factor, necessary to relate the values to the entire filter area and calculated as follows:

$$F = \frac{Filter\ area}{Count\ Field\ area * n\ of\ count\ fields} = 36{,}13$$

Where Filter area is 1734 mm^2, Count Field area is 2,4 mm^2 and n of Count Fields, as previously described, are 20. Microplastics were counted as total per filter and by type. As Omnic™ Picta™ software also provides particle length and width, microplastics size was also retrieved, calculated by the longest particle side and related the total number of particles to the entire filter using the same formula described before. According to SMPs common definition, particles were divided into three different size clusters: microplastics > 100 µm (conventional and bigger microplastics), microplastics between 20 and 100 µm and microplastics < 20 µm. For particles between 20 and 100 µm a size range distribution was performed in relation to their size and abundance. Furthermore, it was also possible to distinguish between elongated and non-elongated particles calculating particles aspect ratio (AR). The aspect ratio or elongation ratio is the ratio (E) between the maximum length (L) and the maximum width (W) of the smallest rectangle (bounding box) enclosing the shape (Adachi and Buseck, 2015). When AR < 2 particles were considered non-elongated with a pseudo-spherical shape. Instead, when AR ≥ 2 particles were considered elongated. Data analyses were performed using Excel software. Microplastic abundance data are count data and follow a Poisson distribution (Filella, 2015; Courtene-Jones et al., 2017a; Karlsson et al., 2020); Poisson's confidence interval was calculated accordingly. The homogeneity of the variances of abundances of SMPs was tested (F-Test, $\alpha = 0.05$). After invalidation of homogeneity of variances, non-parametric statistical tests were performed on collected data to assess significant differences in the polymer abundance of SMPs among different surface water samples. While the Kruskal–Wallis test ($p < 0.05$) was employed for multiples comparison, the Mann–Whitney U test ($p < 0.05$) was performed for pairwise comparisons.

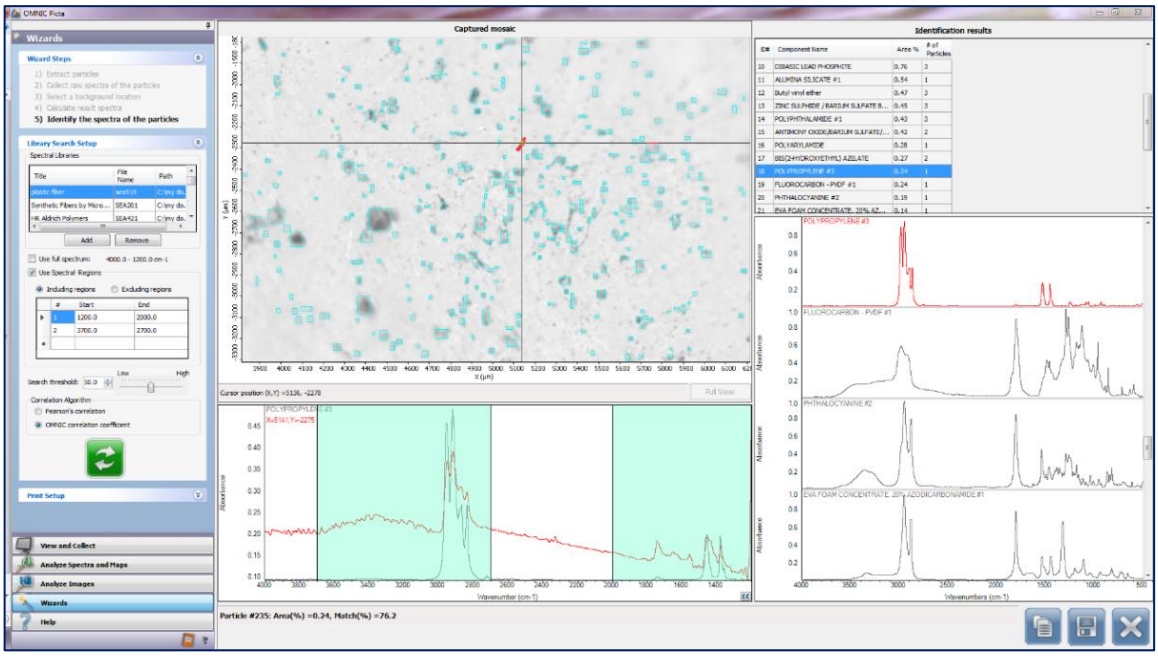


Figure 27. A screenshot of the OMNIC Picta software on a drinking water sample count field.

Raman Microscopy Analysis and Data Acquisition

Raman microscopy analysis was carried out on two selected and different surface water filters (GRZ DWTP #2 TQ #1 and GRZ DWTP #3 TQ #1) once FTIR microscopy analysis was complete. This choice was made in order to avoid the possibility of laser-induced particle and filter degradation. Raman microscope analysis were carried out using Leica microscope coupled to a Raman Renishaw in VIA RM2000 spectrometer (Objective 20x and 100x Leica Fluotar/Darkfield, 633 nm Laser, Aperture 2 µm × 2 µm, Raman Shift 0-3500 cm^{-1}). The rationale of analysis with Raman spectroscopy was to investigate about different microplastics analysis approaches other than, in particular *Point-and-shoot* approach and "*Semi-automated analysis: Imaging* approach. *Point-and-shoot* analysis with Raman microscopy would have allowed investigating the filter with a higher magnification in order to better emphasise the particle shape while imaging analysis would have allowed investigating extremely restricted areas possibly identifying particles under the size limit of FTIR microscopy. Therefore, the surface area of the two filters was investigated in two working days. Several *Point-and-shoot* analysis and Raman *Imaging* protocols in random filter areas were performed (50x50 µm, 0,0025 mm^2 mapped surface, points spaced by 2 µm). Each Raman spectrum obtained was manually compared to those in reference libraries. However, microplastic specific reference spectra were not available through the software so the assignment of spectra was more complex and arduous requiring a lot of time.

Quality Assurance/Quality Control (QA/QC)

Microplastics analysis requires dedicated measures in order to mitigate contamination during the analysis and provide quality data (Table 16). Therefore, a combination of general and specific measures

was implemented during each phase of the analysis. In the general measures group are included: a) working in ISO 7 Clean Room, b) operators' equipment, c) laboratory equipment, d) surface cleaning and e) decontamination just after and before operations. A Clean Room, described as a *"room within which the concentration of airborne particles is controlled and classified, and which is designed, constructed and operated in a manner to control the introduction, generation and retention of particles inside the room"* (ISO 14644 Standards) was employed during each phase of the analysis, except for sampling. Clean Room ISO 7 Class ensured that *"the air contains less than 352 000 particles equal to or greater than 0.5 micron per cubic meter and 2,930 particles equal or greater than 5 micron per cubic meter"* (ISO 14644-1:2015) (International Organization for Standardization, 2016). The ISO 7 requirements, together with the construction of the room itself (stainless steel walls and surfaces) allowed to minimize microplastics airborne contamination. The ISO 7 ISP-CNR Clean Room (Figure 28) has already been used for a long time in Venice ISP-CNR for studying microplastics (Corami et al., 2020b; 2021)

Figure 28. ISO 7 Clean Room at Venice ISP-CNR.

Operators working in ISO 7 Clean Room dressed 100% cotton lab coats with hoods capable of covering hair, nitrile gloves and left their shoes outside the room. For the operations, non-stainless steel and non-glass equipment was avoided as far as possible; equipment was wrapped or covered by aluminium foil when not in use. Decontamination step was introduced in order to ensure that every laboratory equipment had resulted plastic-free. Briefly, just before the deployment of any tools (after the cleaning step) these were firstly rinsed three times with ultrapure water (UW; Elga Lab Water, Veolia, High Wycombe, UK) and then rinsed with a 50:50 methanol (Superpurity Solvent >99.9%, Romil, Cambridge UK) and ethanol (absolute, for HPLC, ≥99.8%, Sigma Aldrich, Merck, Darmstadt Germany) solution. Specific measures mainly included cleaning protocols and blanks. Cleaning protocols development depended on the equipment's nature and its specific role in the analysis. For the sampling step, a distinction has been made between cylindrical filters and the other equipment. Bottles, caps and filtration

system parts except stainless-steel filters were manually cleaned with a surfactant alkaline detergent previously tested plastic-free (Contrad® 2000, Thermo Fischer, Germany), followed by rinsing several times with 60° ultrapure water (UW; Elga Lab Water, Veolia, High Wycombe, UK). Instead, stainless steel filters and filter housing gaskets were first sonicated 30 minutes in an ultrasonic stainless-steel bath filled with ultrapure water (UW; Elga Lab Water, Veolia, High Wycombe, UK) before cleaning with detergent and ultrapure water. For the sample treatment step, a targeted cleaning protocol was developed for glass as funnels, beakers, Erlenmeyer flask and Petri dishes. Glass was manually cleaned with a surfactant alkaline detergent previously tested plastic-free (Contrad® 2000, Thermo Fischer, Germany), followed by rinsing several times with 60° ultrapure water (UW; Elga Lab Water, Veolia, High Wycombe, UK). Between ultrapure water rinsing and decontamination, glass was treated with 20 mL a 0.25 M NaOH (≥ 98%, Sigma-Aldrich, Merck, Darmstadt, Germany) solution prepared with ultrapure water (UW; Elga Lab Water, Veolia, High Wycombe, UK). In order to understand, respectively, reagent and procedural contamination reagent blanks and procedural blanks were performed. Reagent blanks were performed for ultrapure water, 50:50 methanol and ethanol solution and sunflower seeds oil by filtering the amount of solvent used in the analysis on ANODISC filters; sunflower seeds also needed n-Hexane (95%, Romil, Leicestershire UK) and the 50:50 methanol and ethanol solution for solubilisation. Procedural blanks were instead performed simulating the digestion-extraction-filtration process by using ultrapure water instead of surface or drinking water. Replicate analysis can be also considered a specific QA/QC procedure. Each sample (surface water sample, drinking water sample, bottle sample or filtration system sample) and blanks was processed in triplicate.

QA/QC	
GENERAL PROCEDURES	- ISO 7 Clean Room - Non-plastic operators' equipment - Stainless steel and glass equipment - Aluminium foil wrapping and covering equipment - Surface cleaning prior and post analysis - Decontamination of equipment before the analysis following a standard protocol
SPECIFIC PROCEDURES: SAMPLING	- Filtration system-closed construction - Cleaning protocol for filtration system equipment - Cleaning protocol for stainless steel filters
SPECIFIC PROCEDURES: SAMPLE TREATMENT	- Cleaning protocol for glass - Reagent blanks - Procedural blanks - Replicate analysis (triplicate) - Separate sample processing for Surface water and Drinking water
SPECIFIC PROCEDURES: ANALYSIS	- Replicate analysis (triplicate) - Separate analysis for Surface water and Drinking water

Table 16. QA/QC procedures

3.3 EXPERIMENTAL RESULTS

3.3.1 Development of the sampling method and application of the analytical method to the analysis of microplastics

A new sampling method, including 2-steps *in situ* filtration was developed in order to collect surface water entering the DWTP and treated water.

Assembled filtration system proved to be effective in filtering high volumes of surface water without clogging (average of 1.803,6 L). Tests to assess the goodness of this sampling method for drinking water were also carried out and an average of 2792,6 L litres was filtered without clogging. Sampling a large volume of water for MPs > 100 µm analysis is indeed highly suggested, although no standards have been set (Koelmans et al., 2019). SMPs (MPs < 100 µm) may be more plentiful than those > 100 µm, and in the environment can also be sampled with discrete sampling devices, e.g., Niskin bottles, collecting volumes between 5 and 10 L (Khalik et al., 2018; Covernton et al., 2019; Zhu et al., 2019; Corami et al, 2021). Hence, data from samples collected with the developed in *situ-filtration* method was compared with that from discrete samples collected in decontaminated glass jars. Regarding sampling with the filtration system, pressures were high enough to achieve adequate filtration volumes within the time set for sampling at drinking water treatment plant at the planning stage. In any case, clogging did not occur during sampling even with the most turbid water (Adige River water from DWTP #2, 55 NTU). The presence of coarse matter was also observed around each 300-µm surface filter and in their corresponding filter housings with a significant decrease in the 5-µm one. For drinking water, on the contrary, as it was expected, coarse matter was not observed around either of the filters or inside the corresponding housings.

Regarding sample pre-treatment, the method previously developed by Corami et al. (2021) was employed with slight modifications. The method has been proved to be efficient to extract microplastics (minimizing any interferers for the analysis via Micro-FTIR), replicable and with optimal yield (Corami et al., 2021; Rosso et al., 2022). As it was expected, filtration of surface water from bottles provided a clearer medium than those observed filtering the water containing particles from the filtration system, as these samples represent the sampling of several hundred litres.

For 18 filters produced, only 12 were analysable via Micro-FTIR. Filters derived from the filtration of samples obtained from Tevere and Adige River using the filtration system (GRZ DWTP #1 SF5 and GRZ DWTP #2 SF5) showed the presence of coarse and stratified matter that made it impossible to analyse them through the *Particle measurement* approach (Figure 29, 30). Each replica of GRZ DWTP #1 SF5 and GRZ DWTP #2 SF5 sample exhibited the same behaviour.

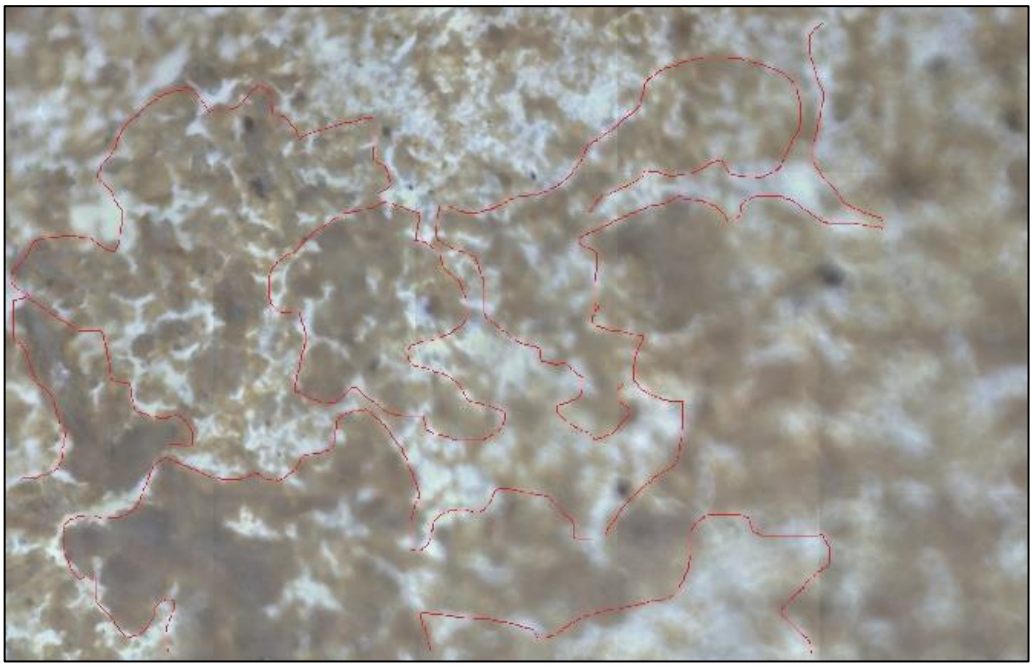


Figure 29. A count field from GRZ DWTP #1 SF5 #1 showing (in red) coarse and stratified matter.

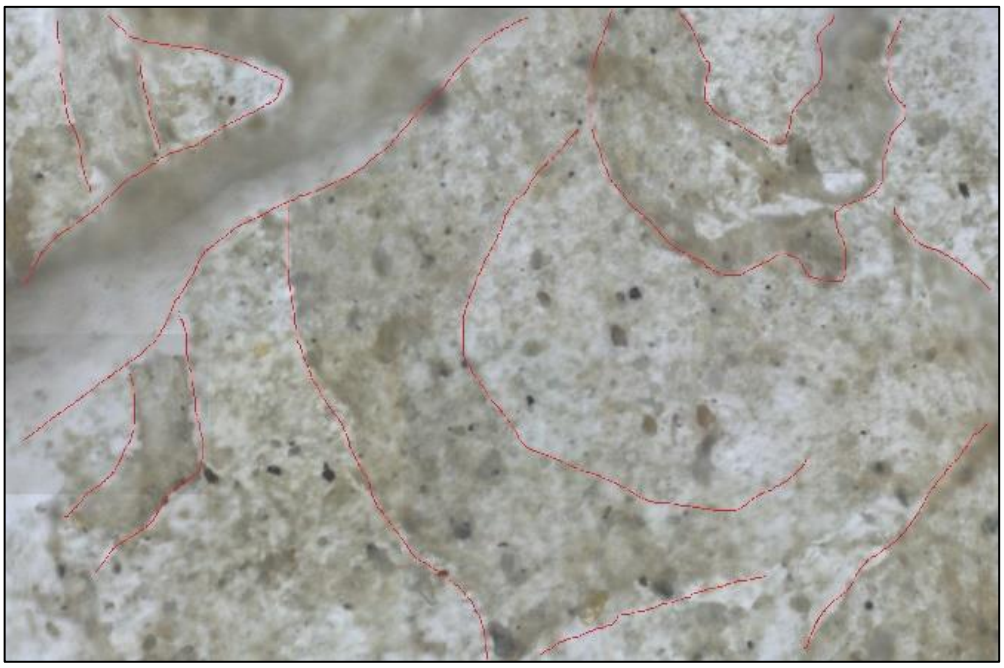


Figure 30. A count field from GRZ DWTP #2 SF5 #1 showing (in red) coarse and stratified matter.

As aforementioned, a second test with different sample volume was performed for GRZ DWTP #2 SF5 samples (GRZ DWTP #2 SF5 BIS), showing a significant decrease in term of coarse and stratified matter. Hence, the stratified interferers were successfully removed, and it was therefore possible to perform the analysis via Micro-FTIR (Figure 31).

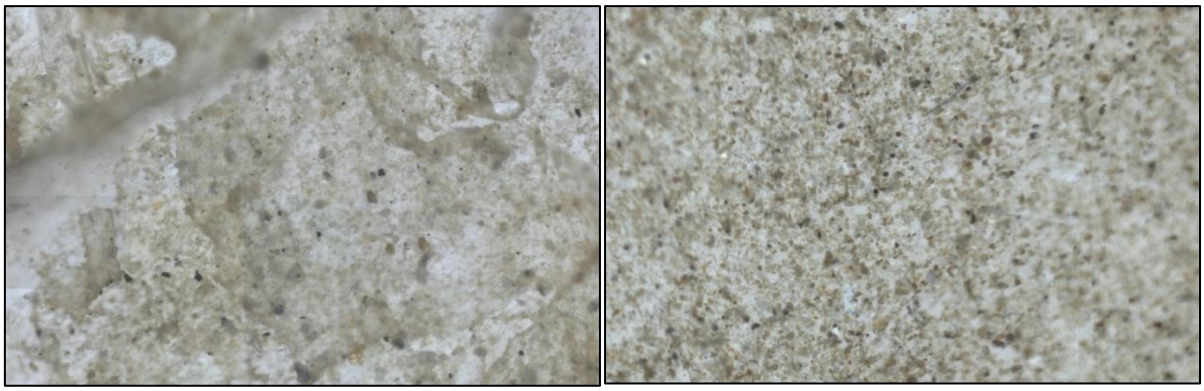


Figure 31. Count fields from GRZ DWTP #2 SF5 #1 and GRZ DWTP #2 SF5 #1 BIS. The count field appears without coarse and stratified matter even with a high number of particles.

In other samples, such high amounts of stratified matter were not observed and analysis with Micro-FTIR was possible. Some count fields from surface water samples are showed in Figure 32-36.

Figure 32. A count field from GRZ DWTP #1 TQ.

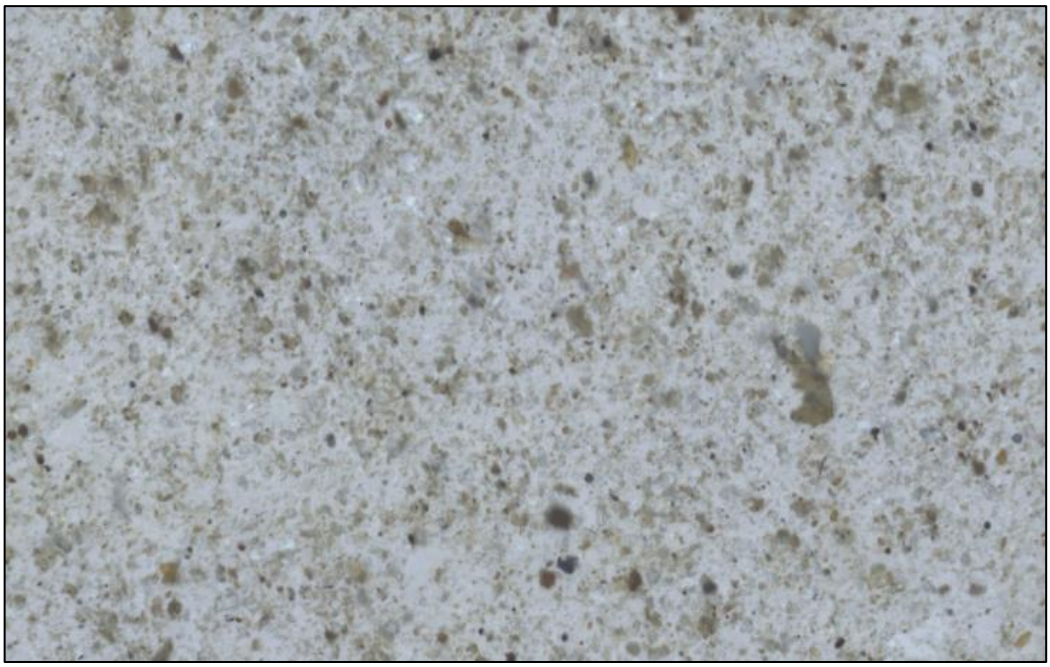


Figure 33. A count field from GRZ DWTP #2 SF5 BIS

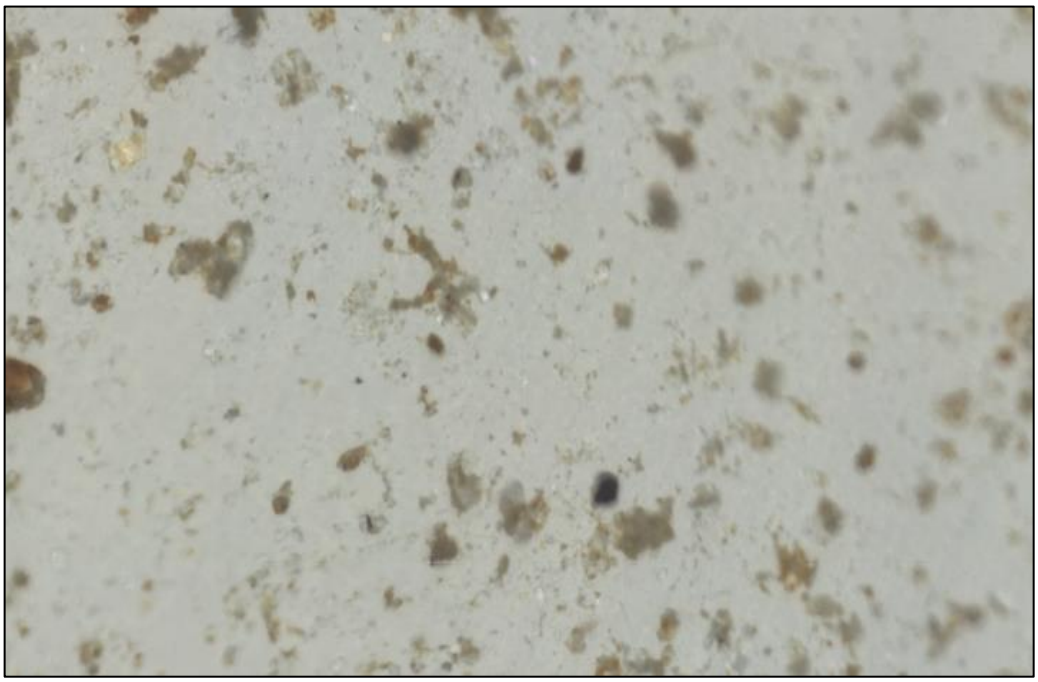


Figure 34. Count fields from GRZ DWTP #2 TQ.

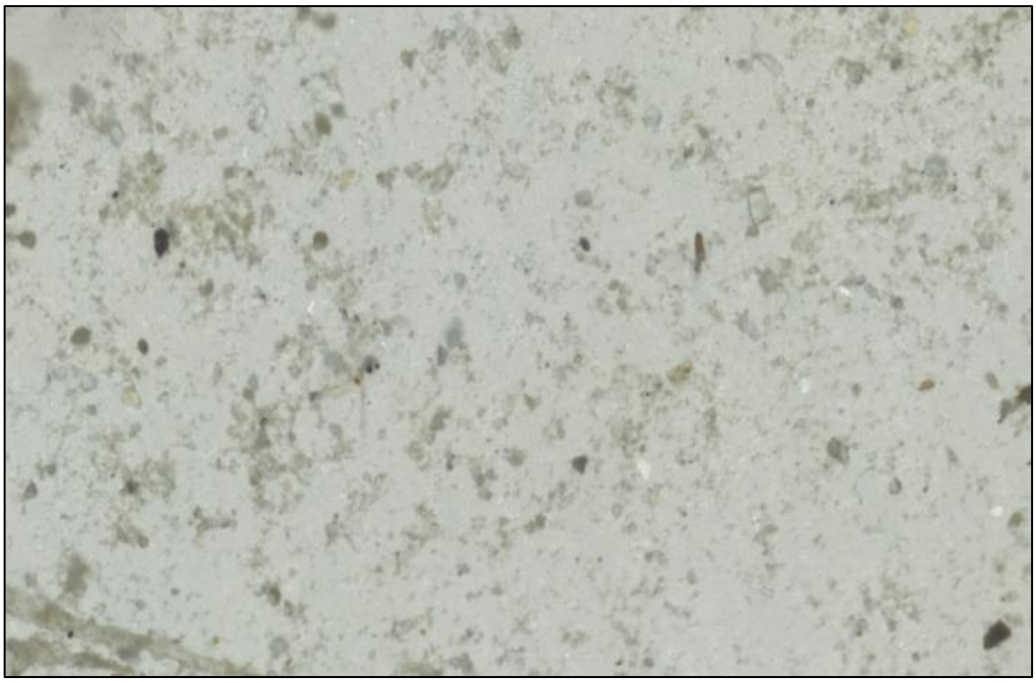


Figure 35. Count fields from GRZ DWTP #3 SF5

Figure 36. Count fields from GRZ DWTP #3 TQ.

3.3.2 Analysis via Micro-FTIR

Blanks

Reagents blanks showed no microplastics (Figure 37) in any count field while some microplastics were identified in Procedural Blanks and were then subtracted from the samples. Some count fields and *particle measurement* of procedural blanks are showed in Figure 38. Microplastics in procedural blanks were counted, classified by polymer type and by size in order to allow them to be subtracted, if applicable, from the amount found in samples (Figure 39). Nylon (PA) was the most common polymer identified in both blanks, followed by PPA (Polyphtalamide) and Epoxy resin.

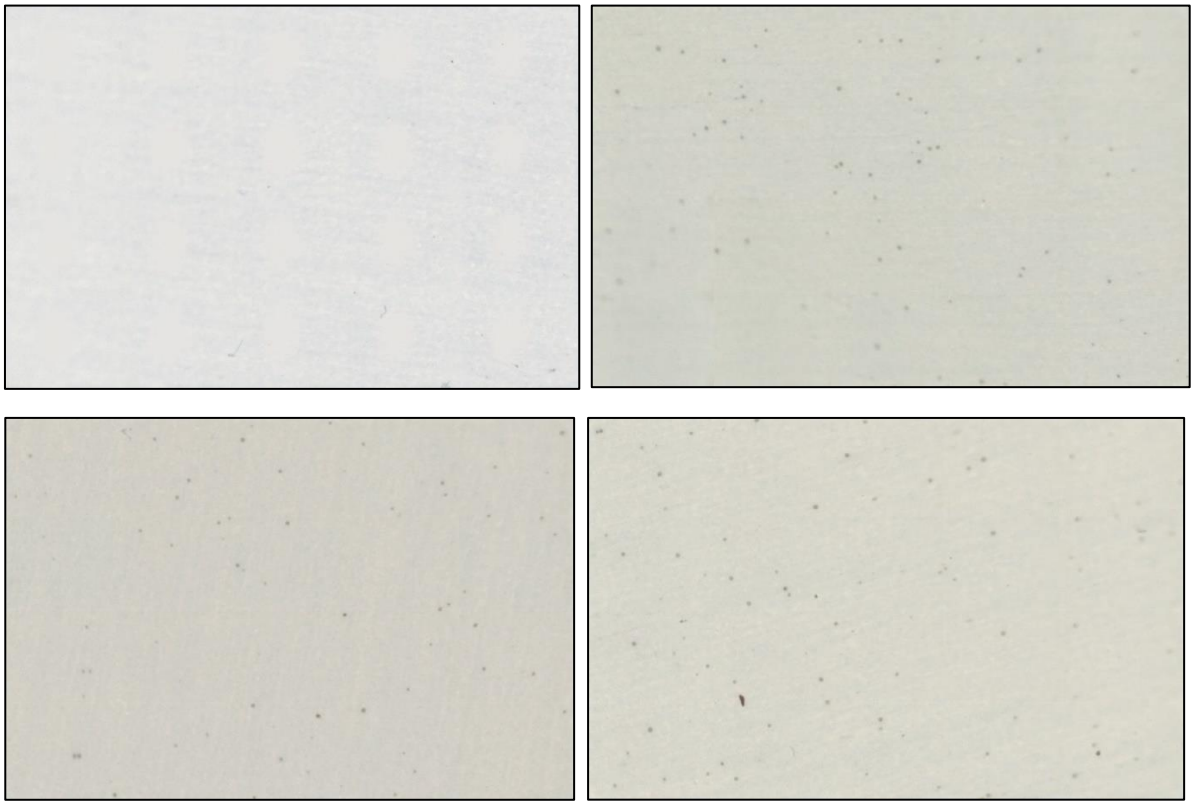


Figure 37. Count field from Reagent Blanks: ultrapure water (upper left), 50:50 methanol and ethanol solution (upper right), hydrogen peroxide solution (lower left) and sunflower seeds oil + 50:50 methanol and ethanol solution (lower right).

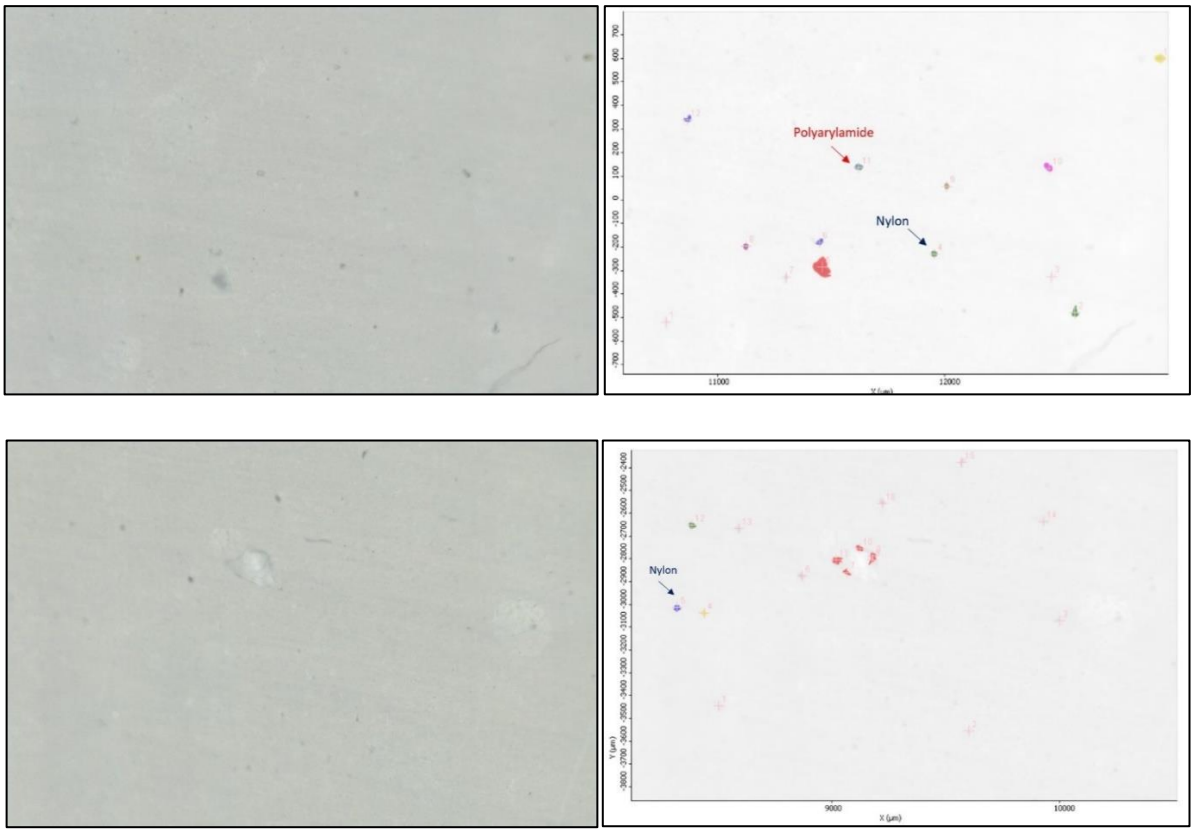


Figure 38. Some count fields and *particle measurement* from procedural blanks

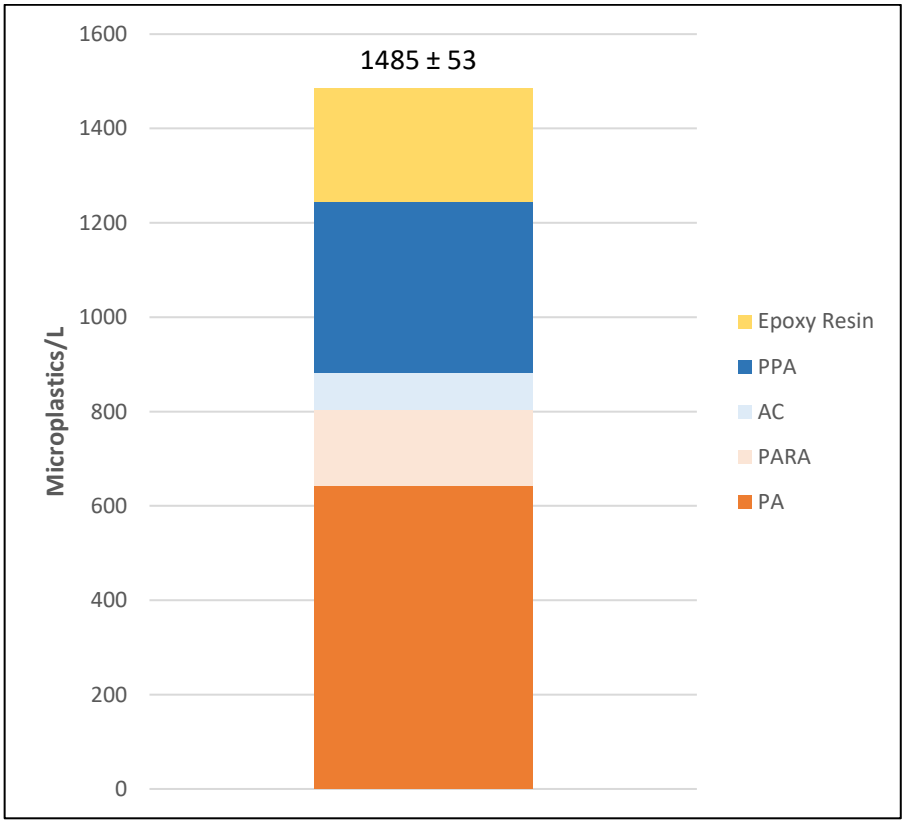


Figure 39. Microplastics number in surface water procedural blank. The abundance is reported with the confidence interval according to the Poisson distribution.

Regarding size distribution, microplastics in procedural blanks appeared to be more common in 20-30 µm and 30-40 µm size clusters (Figure 40).

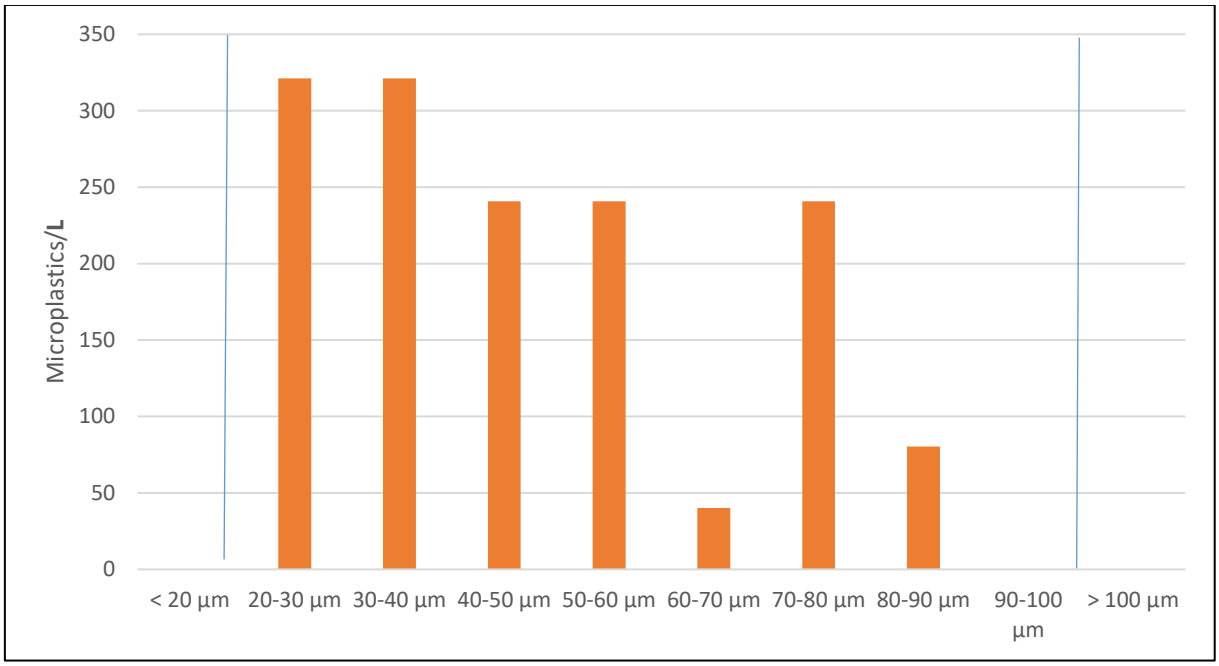


Figure 40. Microplastics size distribution in procedural blank.

Surface water

Microplastics were observed in every surface water samples collected with both sampling methods, with the exception of the one performed at DWTP #1 with the filtration system, due to inability to properly apply *particle measurement* to the filters. Microplastics found greatly differed in type and number at each sampling site, while for size distribution and shape greater homogeneity was observed. As previously stated, for DWTP #1 only data from discrete sampling (GRZ DWTP #1 TQ) were produced. For DWTP #2 and DWTP #3, also data from filtration system was available (GRZ DWTP #2 SF5 BIS and GRZ DWTP #3 SF5). In GRZ DWTP #2 SF5 BIS, an average of 1716 ± 63 microplastics were found while in GRZ DWTP #3 SF5 the number was by far higher, with an average of 21555 ± 204 microplastics (Figure 41). Regarding the chemical composition, in GRZ DWTP #2 SF5 BIS, PTFE was the most common polymer followed by Polyolefin (PO), Acrylic (AC)/Acrylonitrile-butadiene-styrene copolymer (ABS) and Polyester (PES)/ Fluorocarbon (FC)/ Polypropylene (PP). In GRZ DWTP #3 SF5 the most common polymer was PES, followed by Pefluoroalcoxy Fluorocarbon (PFA), Polytetrafluoroethylene (PTFE), AC, Nylon (PA), FC, Polyurethane (PU) and Polyethylene (PE). Differences in microplastics number and types were also observed in the corresponding samples (Figure 42,43) obtained from the discrete sampling (GRZ DWTP #2 TQ and GRZ DWTP #3 TQ). GRZ DWTP

#2 TQ showed a similar a number of microplastics to those observed with the corresponding filtration-system sample with an average of 2127 ± 61 microplastics; the typology of microplastics found is also similar with FC and PTFE as major components followed (in order) by Poly (Ethylene Vinyl Alcohol) (EVOH), PO and PP/PU. In GRZ DWTP #3 TQ there were 12604 ± 159 microplastics. Pefluoroalcoxy Fluorocarbon (PFA) was the most common microplastic, followed (in order) by PA, PES, PTFE, FC, Modacrylic/Fluorinated ethylene propylene (FEP), PO/ Ethylene-vinyl acetate (EVA). Sample from DWTP #1 from discrete sampling (GRZ DWTP #1 TQ) showed the highest number of microplastics among all bottle sampling sets, with an average of 109061 ± 458 particles/L. Vinyl ester resin (VER) and Polyester (PES) were the most common microplastics identified, followed by Fluorocarbon (FC), Polyethylene (PE) and a small percentage of Polyolefin (PO). Regarding the statistical tests, differences in the abundances and polymeric distribution observed in the surface water of the three sites for both sampling systems were statistically significant ($p < 0,03$ for filtration system sampling and $p < 0,01$ for sampling with bottles).

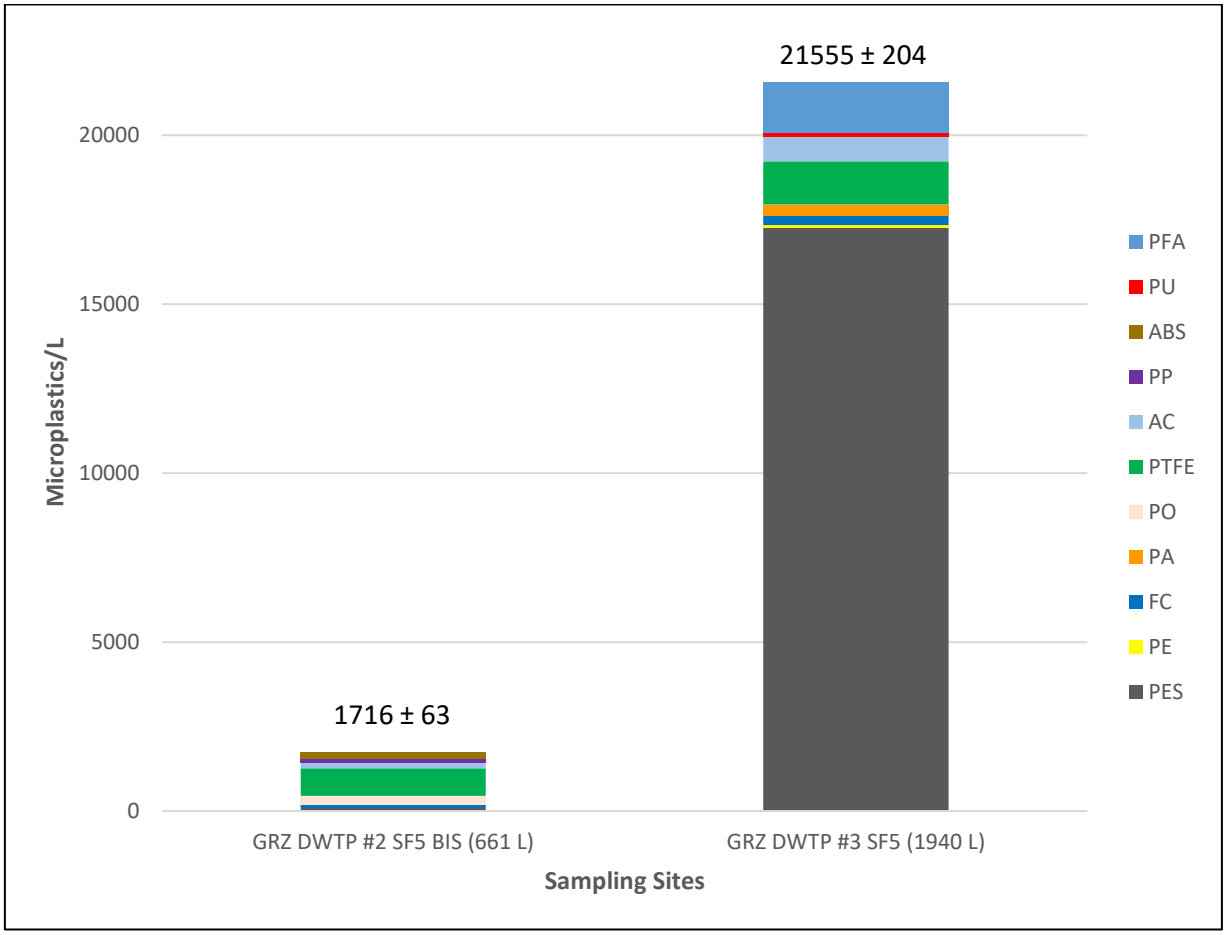


Figure 41. Microplastics number and type in surface water filtration system samples (GRZ SF5). The abundance is reported with the confidence interval according to the Poisson distribution.

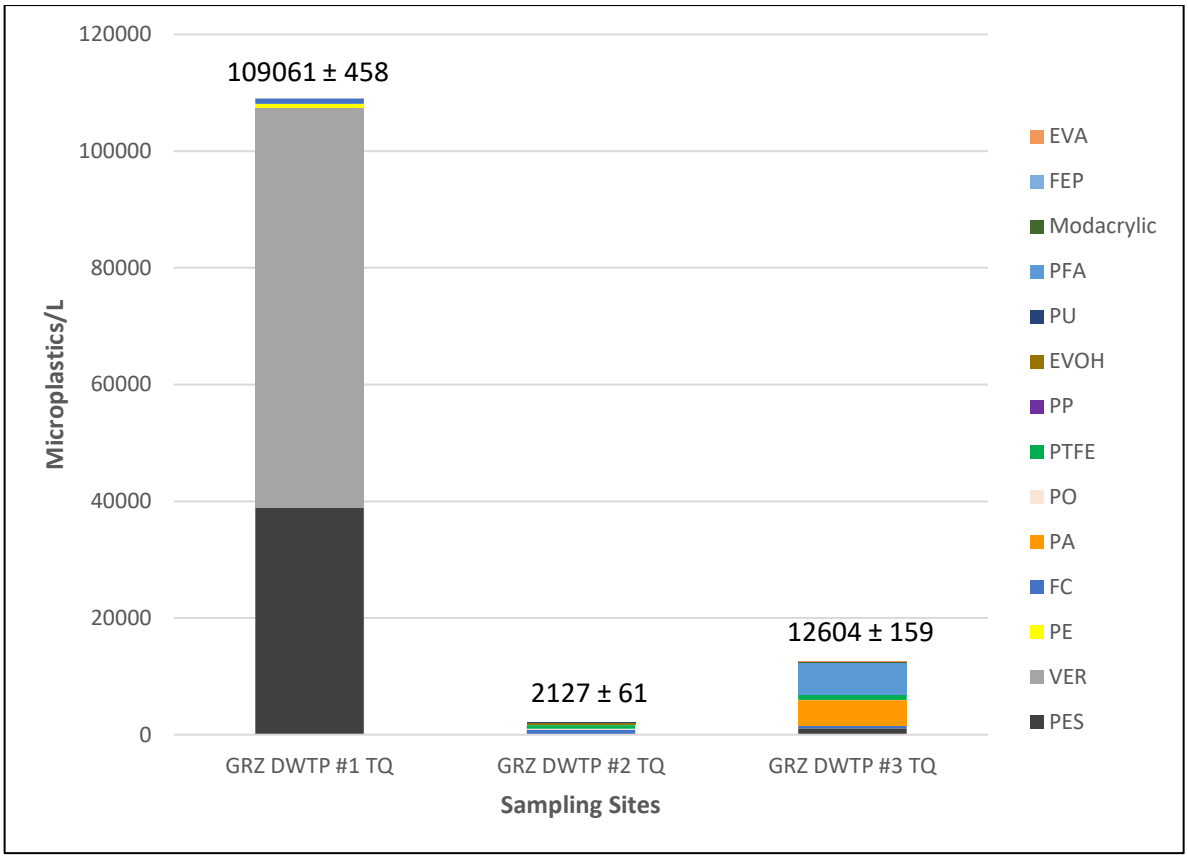


Figure 42. Microplastics number and type in discrete surface water samples (GRZ TQ). The abundance is reported with the confidence interval according to the Poisson distribution.

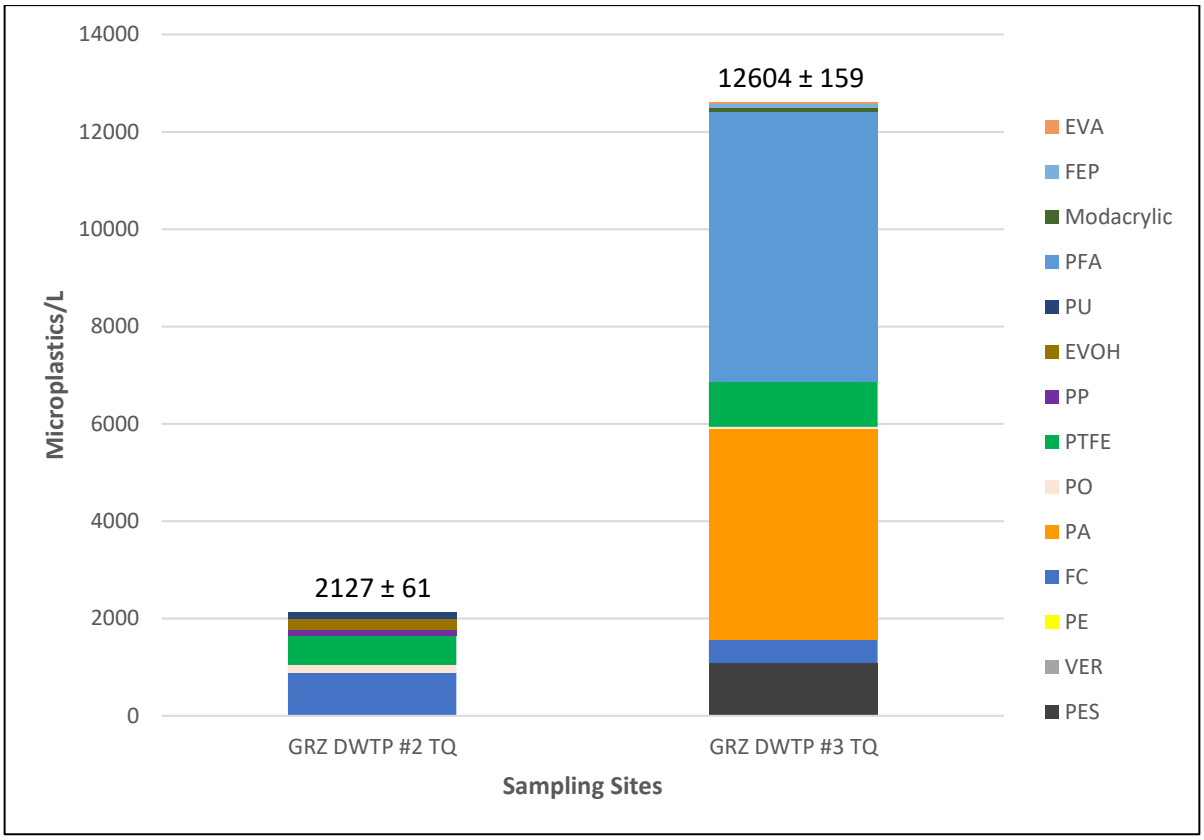


Figure 43. Microplastics number and type in surface water bottle samples (GRZ TQ) with a focus on GRZ DWTP #2 TQ and GRZ DWTP #3 TQ. The abundance is reported with the confidence interval according to the Poisson distribution.

Regarding size distribution, microplastic in surface water were found in the 24,9 µm – 515,9 µm size range. Particles in 20-100 µm range were the most abundant despite the sampling method. Particles > 100 µm were found in every sample except for GRZ DWTP #2 SF5 BIS. In the 20-100 µm range, for filtration systems samples 40-50 µm and 50-60 µm were the most populated size cluster while for discrete sampling 30-40 µm and 40-50 µm were the most populated; the third populated size cluster for bottles was 50-60 µm. However, considerations for filtration system samples do not include data from GRZ DWTP #1 SF5 that is missing.

In GRZ DWTP #1 TQ (Figure 44), the smaller particle detected was 24,9 µm in size while the biggest one was 326,7 µm long. Several particles > 100 were found (3813 ± 86) and 50-60 and 40-50 µm µm were the most populated size clusters.

In GRZ DWTP #2 SF5 BIS (Figure 45), the smallest particle detected was 26,2 µm in size while the biggest one was 99,5 µm long. Therefore, no particles > 100 µm were detected in this sample. In the 20-100 µm range, 40-50 µm and 50-60 µm were the most populated size clusters. In GRZ DWTP #2 TQ (Figure 46) the smallest particle detected was 27,7 µm in size while the biggest one was 135,8 µm long. Several particles > 100 µm were detected in this sample. In the 20-100 µm range, 40-50 µm and 50-60 µm were the most populated size clusters as observed in the corresponding filtration system sample.

In GRZ DWTP #3 SF5 (Figure 47), the smaller particle detected was 25,9 µm in size while the biggest one was 515,9 µm long. Several particles > 100 were found (6222 ± 109) and 40-50 µm and 50-60 µm were the most populated size clusters. In GRZ DWTP #3 TQ (Figure 48), the smaller particle detected was 26,4 µm in size while the biggest one was 285,6 µm long. Several particles > 100 were found (an average of 1766 ± 58 particles) and 40-50 µm and 50-60 µm were the most populated size clusters.

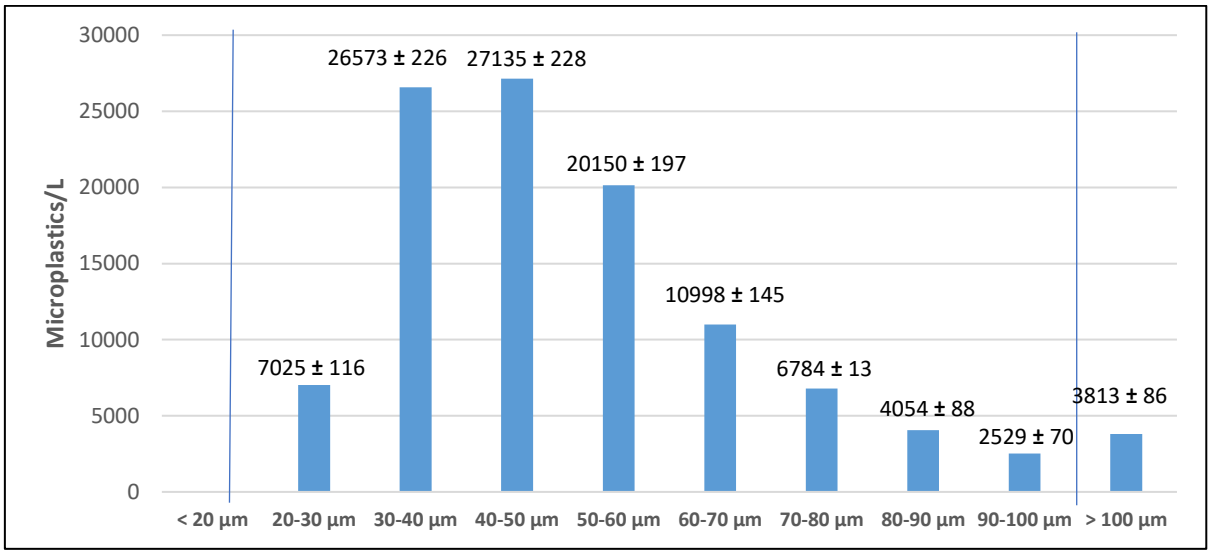


Figure 44. Microplastics size distribution in GRZ DWTP #1 TQ. The abundance is reported with the confidence interval according to the Poisson distribution.

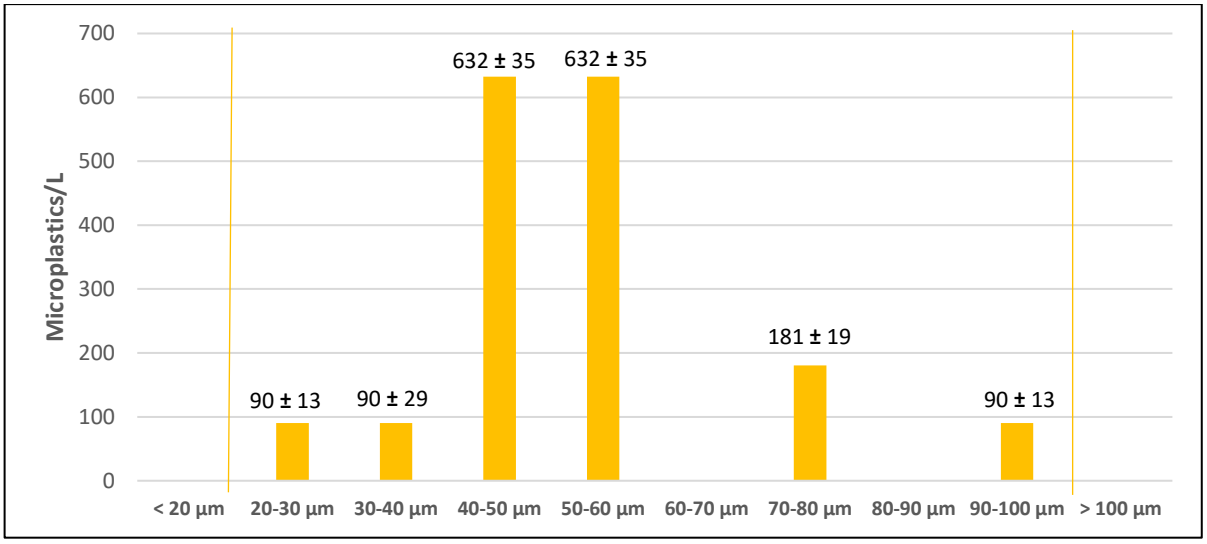


Figure 45. Microplastics size distribution in GRZ DWTP #2 SF5 BIS (661 L). The abundance is reported with the confidence interval according to the Poisson distribution.

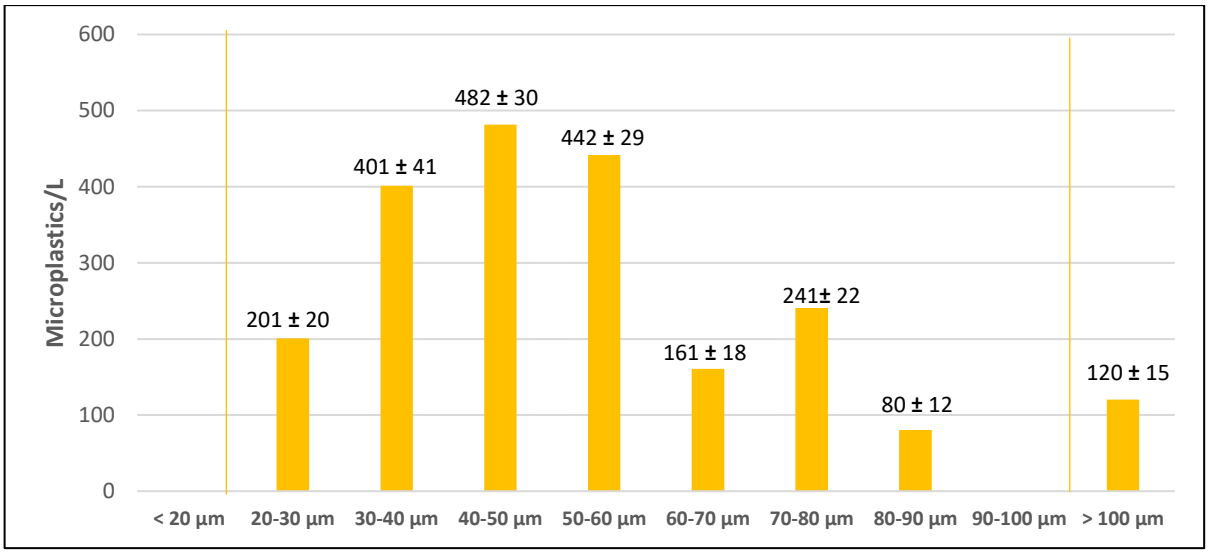


Figure 46. Microplastics size distribution in GRZ DWTP #2 TQ. The abundance is reported with the confidence interval according to the Poisson distribution.

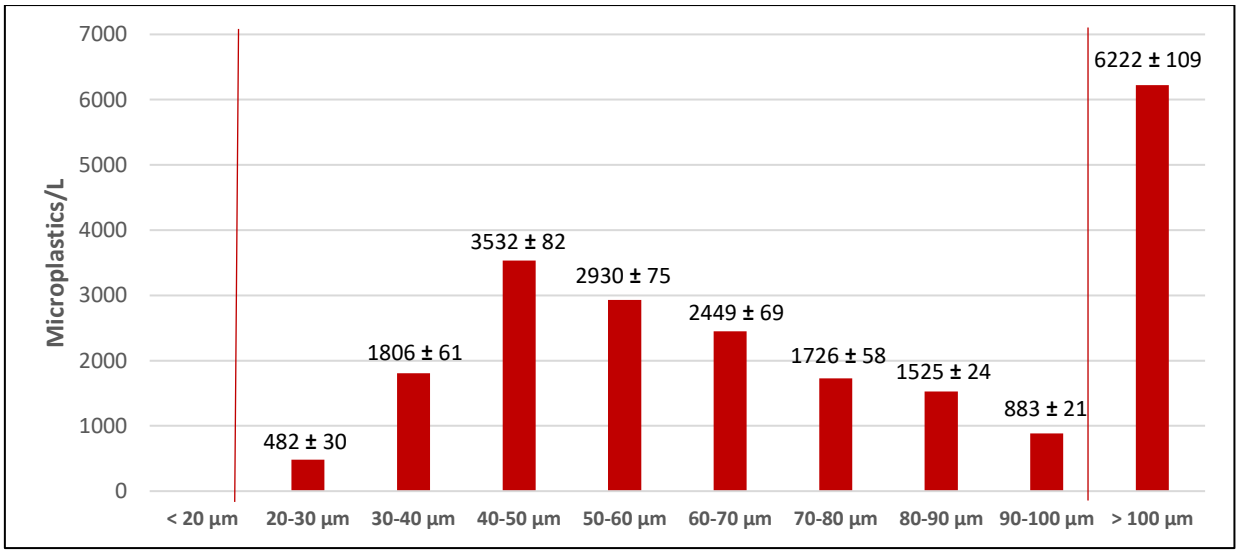


Figure 47. Microplastics size distribution in GRZ DWTP #3 SF5 (1940 L). The abundance is reported with the confidence interval according to the Poisson distribution.

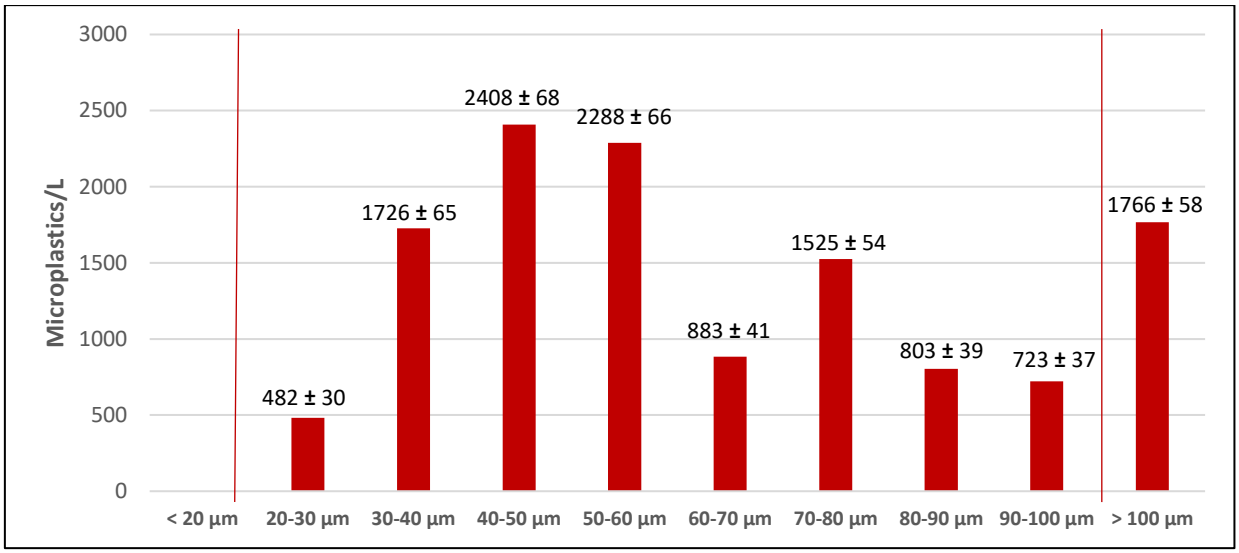


Figure 48. Microplastics size distribution in GRZ DWTP #3 TQ. The abundance is reported with the confidence interval according to the Poisson distribution.

Regarding shape, a high degree of homogeneity was observed. In each sample, regardless of sampling type, non-elongated particles (AR < 2) were higher in number than elongated particles (AR ≥ 2). Similar results were observed for GRZ DWTP #2 samples (Figure 49) and GRZ DWTP #3 TQ (Figure 50) while GRZ DWTP #3 SF5 (Figure 50) and GRZ DWTP #1 TQ (Figure 51) exhibited the highest percentage of elongated particles.

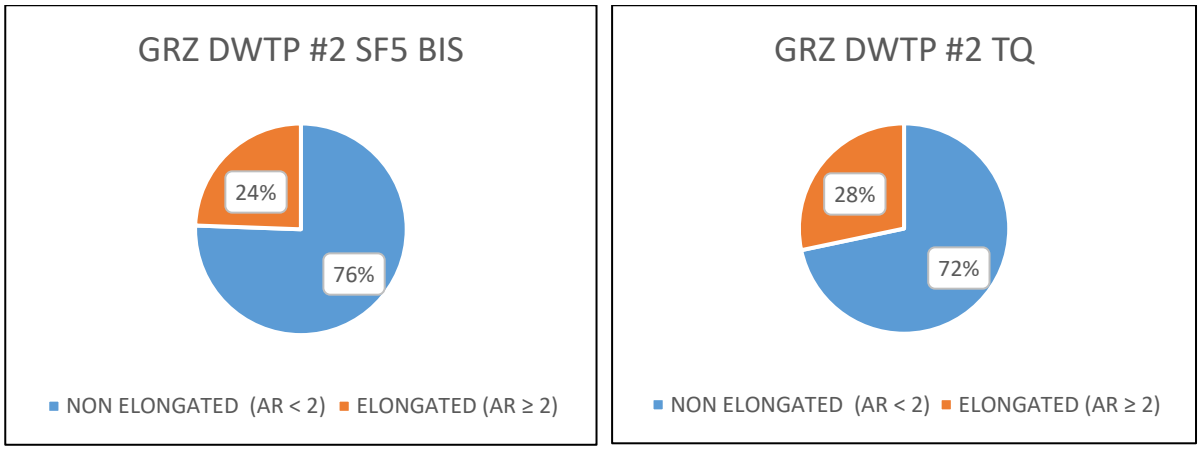


Figure 49. Microplastics shape in GRZ DWTP #2 SF5 BIS and GRZ DWTP #2 TQ

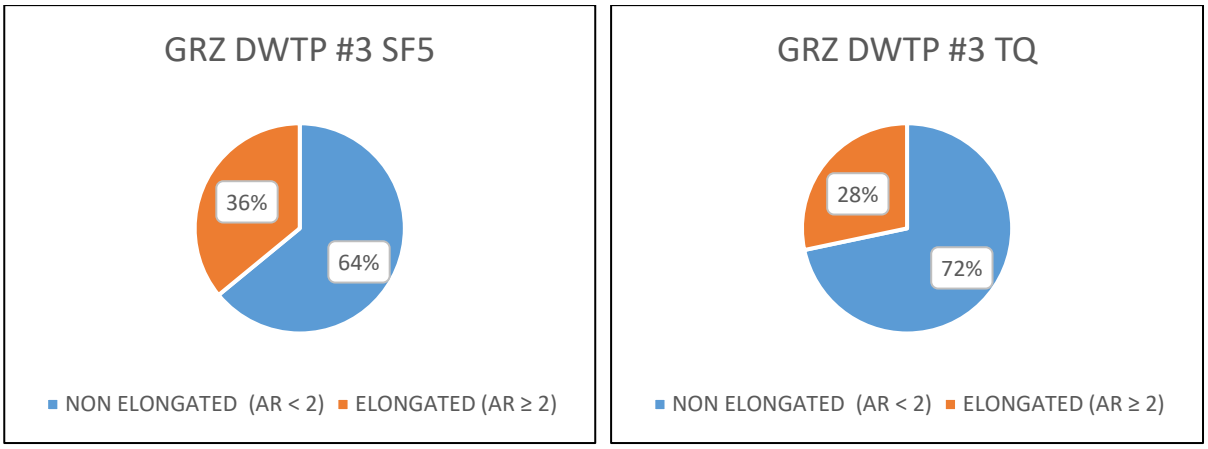


Figure 50. Microplastics shape in GRZ DWTP #3 SF5 and GRZ DWTP #3 TQ

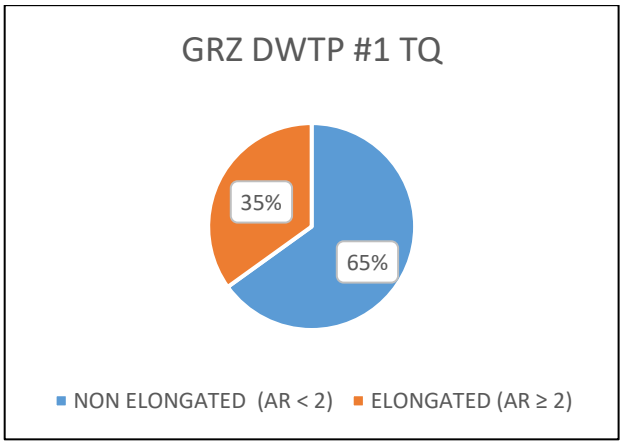


Figure 51. Microplastics shape in GRZ DWTP #1 TQ

Regarding samples obtained from the filtration system, a direct comparison between the different sampling sites is possible only if volumes are standardized. Assuming, for volumes similar to those sampled, a direct proportionality relationship between filtered water and particle number, a confrontation can be made for number and size. For this purpose, 1000 L were used as a guideline as they were required in Koelmans et al. (2019) requirements for drinking water. As can be seen from bar graph (Figure 52), standardizing volumes to 1000 L decreases the difference in terms of microplastics number between GRZ DWTP #2 SF5 BIS and GRZ DWTP #3 SF5. Bar graph related to size for filtration system samples are showed in Figure 53 while bar graph for samples obtained from filling bottles are showed in Figure 54 (additional extrapolation not needed).

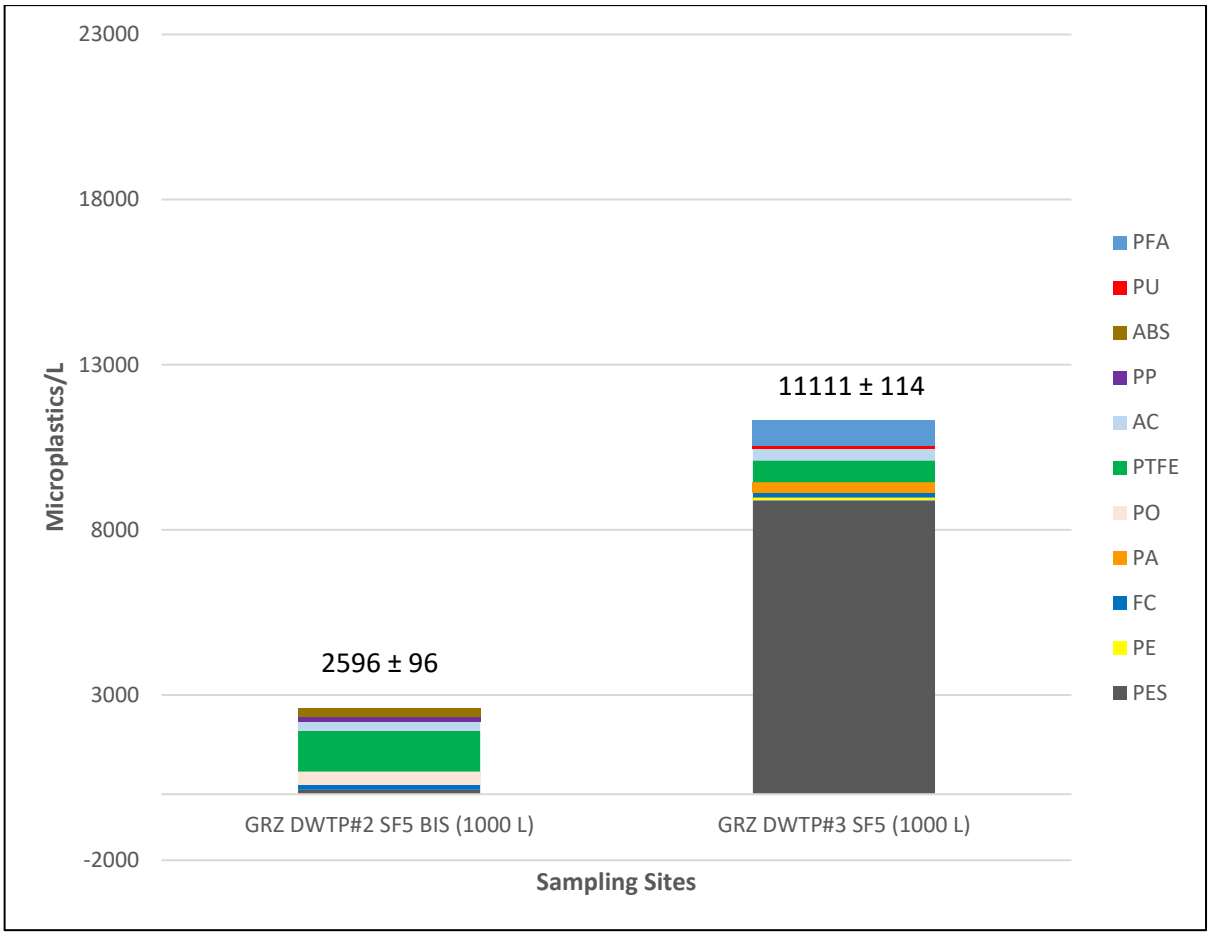


Figure 52. Microplastics number and type in surface water filtration system samples (GRZ SF5) standardizing volumes to 1000 L. The abundance is reported with the confidence interval according to the Poisson distribution.

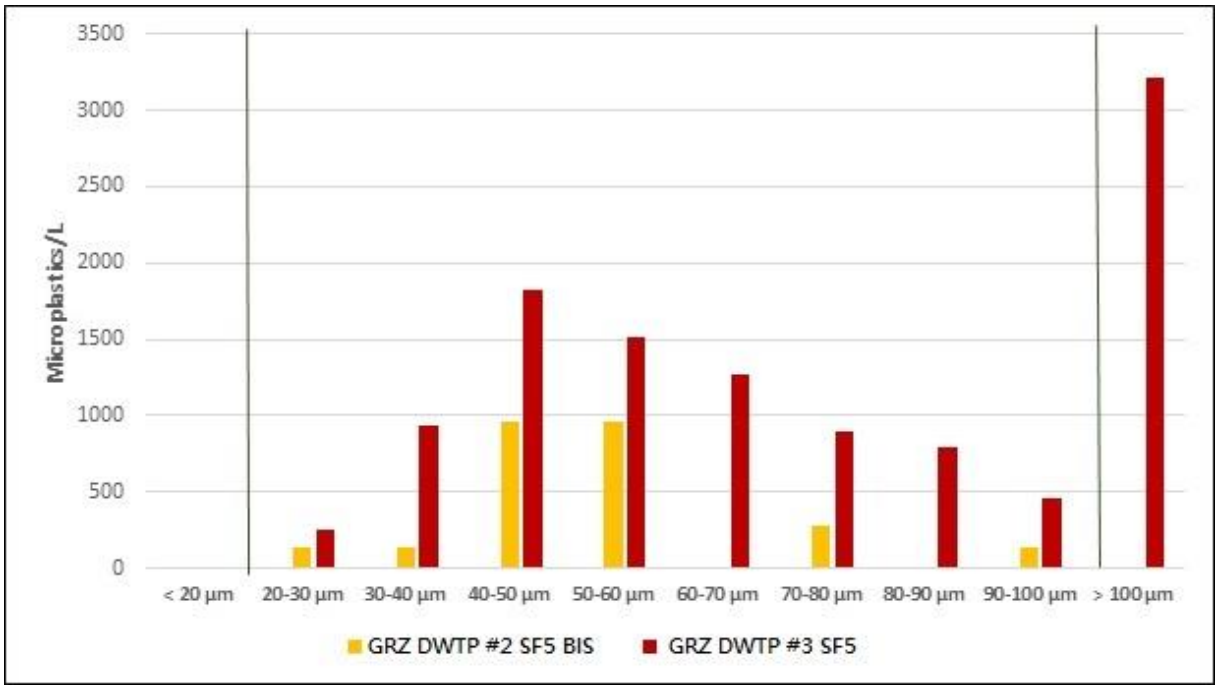


Figure 53. Bar graph showing differences in size distribution of microplastics in GRZ DWTP #2 SF5 BIS and GRZ DWTP #3 SF5 assuming 1000 L as a reference volume.

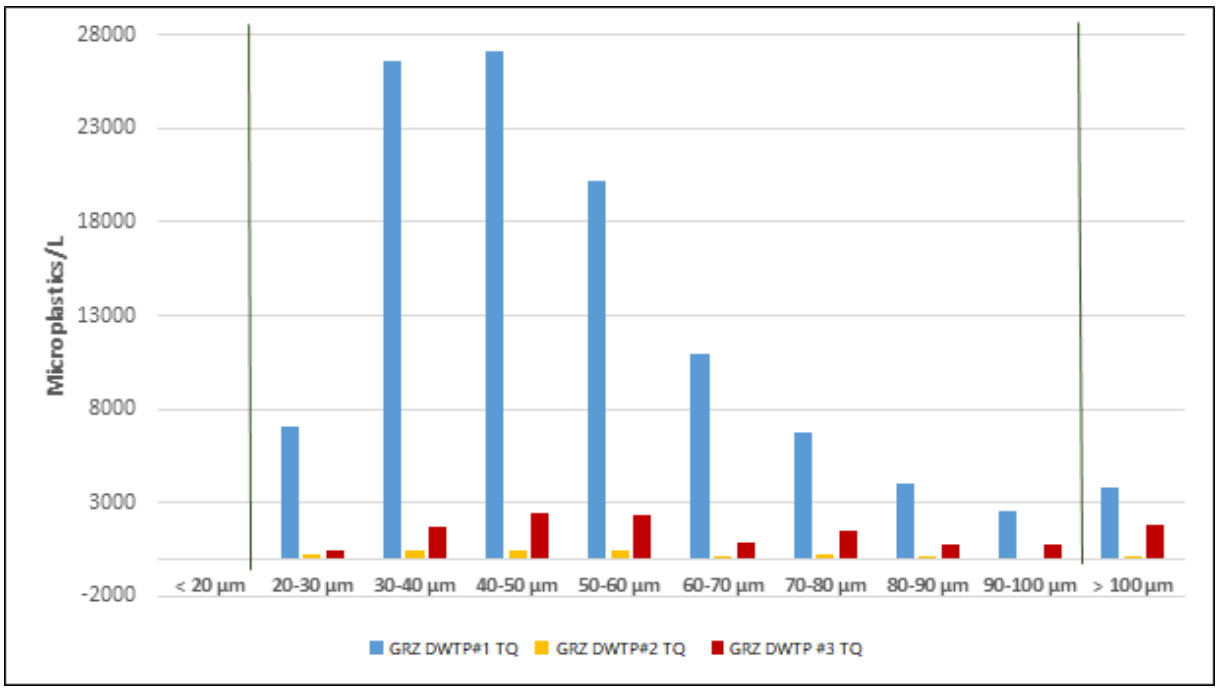


Figure 54. Bar graph showing differences in size distribution of microplastics in GRZ DWTP #1 TQ, GRZ DWTP #2 TQ and GRZ DWTP #3 TQ.

Several tests regarding sample treatment and particle analysis have been carried out also for drinking water, but results are still under in-deep investigation, and they will be sent to the JRC as part of the agreement with the European project.

3.3.3 Analysis via Raman Microscopy

Raman microscopy analysis was performed on GRZ DWTP #2 TQ #1 and GRZ DWTP #3 TQ #1 samples with *"Point and shoot"* and *"Imaging"* mode. *"Point and shoot analysis"* allowed the identification of some microplastics particles. In particular, a PU (Pearson's R = 0.8614) non-elongated particle was identified in GRZ DWTP #2 TQ #1 (Figure 55).

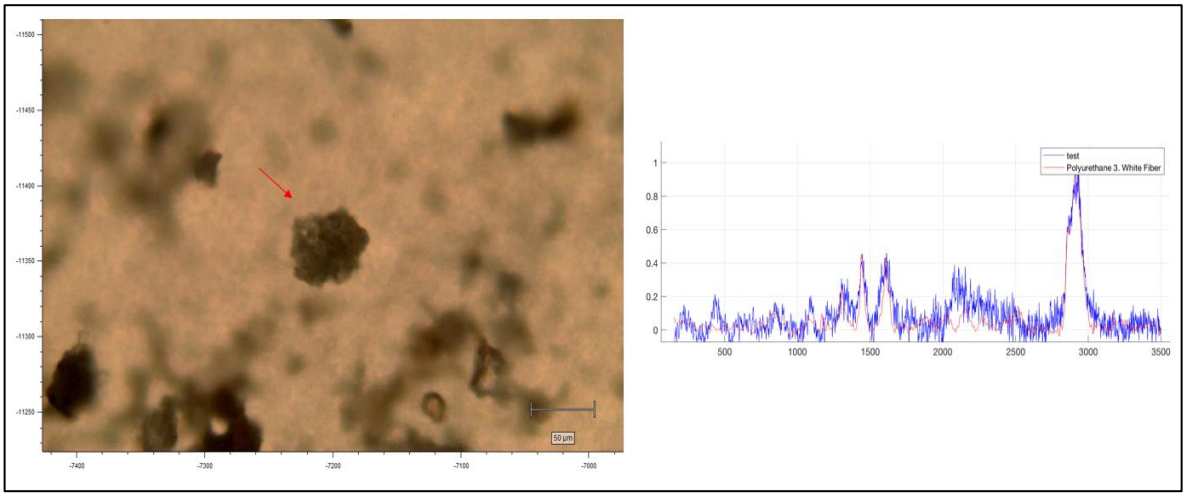


Figure 55. PU Particle #1 in GRZ DWTP #2 TQ #1 with the corresponding Raman spectrum

A PU particle, consistent with its size (approx. 60 µm), was also identified with FTIR microscopy in the same sample (count field #2) but the match was below (56.53%) the predetermined threshold, so it was not counted, and PU does not appear in the final data of GRZ DWTP #2. Another PU (Pearson's R = 0.7673) particle was identified in GRZ DWTP #2 TQ #1 (Figure 56), but the size did not match with any particle observed in the same filter with FTIR microscopy.

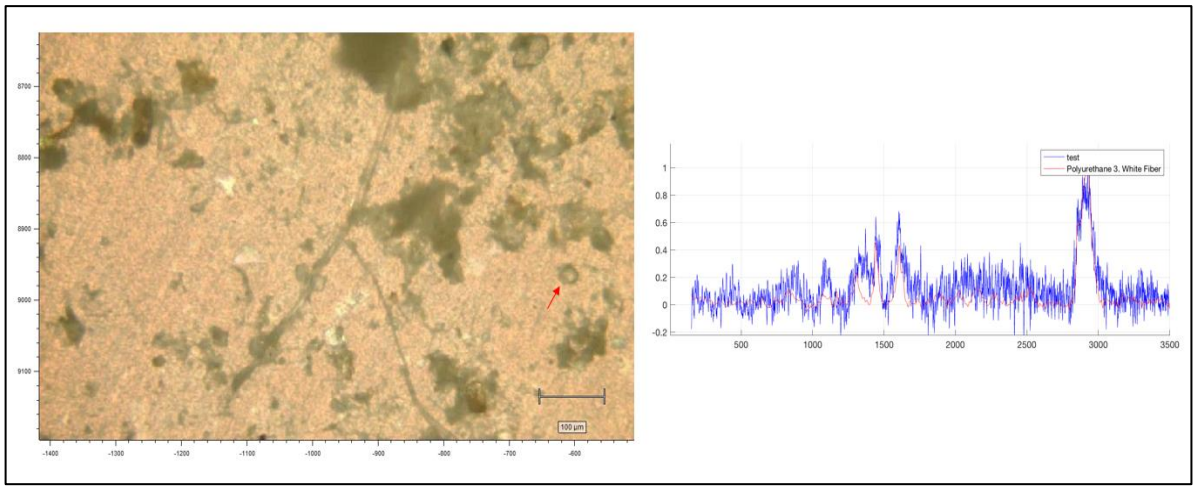


Figure 56. PU Particle #2 in GRZ DWTP #2 TQ #1 with the corresponding Raman spectrum

In GRZ DWTP #3 TQ #1 no microplastic was clearly identified with *"Point and shoot"* mode. A suspected PU particle in GRZ DWTP #3 TQ #1 with the corresponding Raman spectrum is illustrated in Figure 57. However, Pearson's R (0.46) indicated a low correlation with PU.

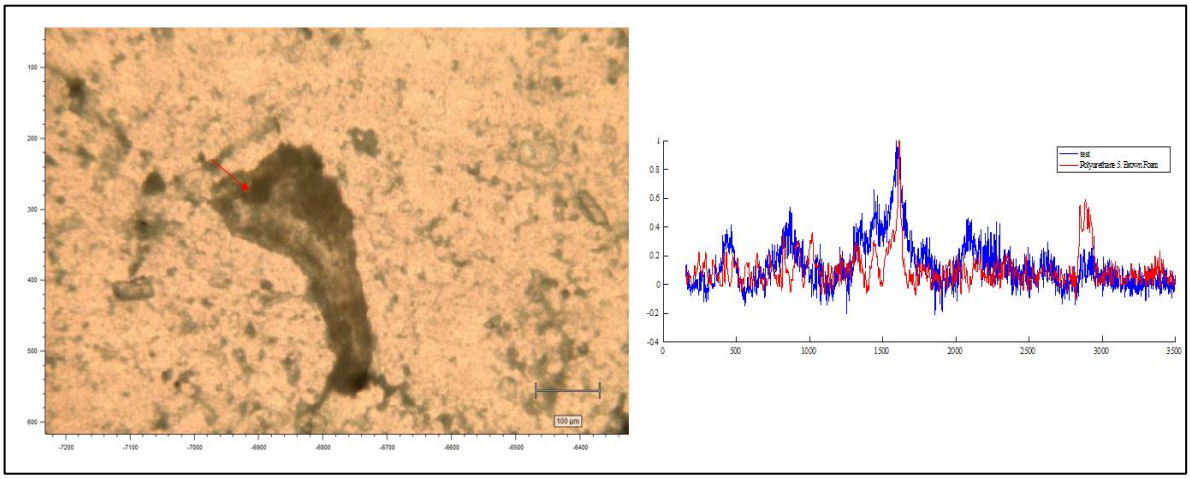


Figure 57. PU Particle #2 in GRZ DWTP #3 TQ #1 with the corresponding Raman spectrum

"Point and shoot analysis" also allow to understand specific issues related to Raman Microscopy for microplastics analysis. During the analysis of a suspected microplastics fiber, the 633 nm Laser damaged (Figure 58) the fiber while switching to a higher intensity. The particle proved later that it was not plastic. This issue was observed also later with other suspected particles.

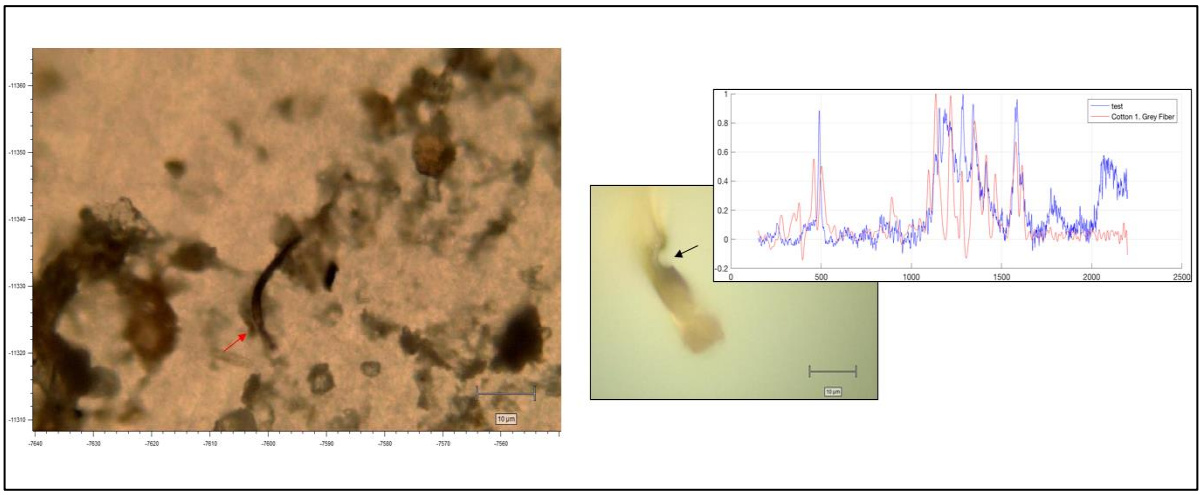


Figure 58. Suspected microplastics particle damaged by 633 nm laser.

Three different *"imaging"* investigations were performed. Only the second test, performed on GRZ DWTP #3 TQ #1, showed remarkable results. In this test, a 50x50 µm surface with a 2 µm-spaced grid was mapped. The experiment resulted in the acquisition of 676 Raman spectra in approximately two hours (10 seconds for each spectrum). Polyvinyl chloride (PVC) and PE signals (R = 0.53 and R = 0,69), were found in two different points, without any similar signals in the surrounding area (Figure 59). Thus, two suspected microplastics < 2 µm were detected in the sample.

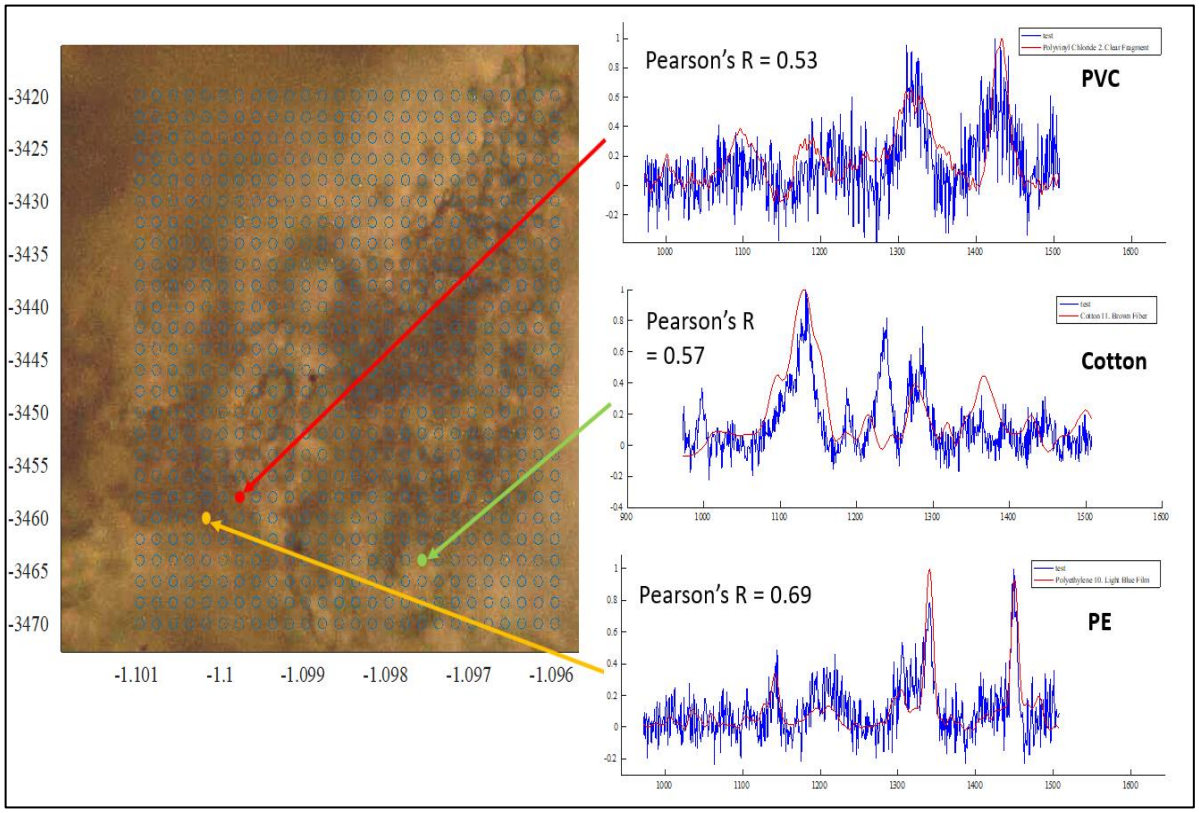


Figure 59. Imaging test #2 (GRZ DWTP #3 TQ #1) and results.

PART IV: DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH NEEDS

4.1 DISCUSSION

4.1.1 Institutional Activities

The challenges experienced in proposing analytical methodologies and performing toxicological evaluations for microplastics have so far been due to the lack of a common definition and their chemical and physical properties, essentially different from those of other emerging pollutants. Another critical issue related to microplastics is that the topic requires considerable study often involving adequate *preparedness* of those approaching it, especially in the analytical field. In the JRC survey (JRC, 2022), from N = 38 entities attending the survey, half of them declared to have "low" or "very low" *preparedness* for microplastics analysis. Only 4 entities out of 17 that answered the question regarding sampling applied *in situ* filtration while discrete sampling was performed by 6 entities out of 17. For the remaining entities, the sampling method a) is under development (a small percentage) b) is unclear or has not been reported. A different scenario was observed from data retrieved from ISS survey (Martellone et al., 2022a/b) as Italian National Working Group was established with the purpose of assembling Italian excellence in the research area to support JRC activities. At the time of the survey, 5 group out of 23 were in the "instrument acquisition" phase, a signal that experts are flexibly adapting to new innovations in microplastics analysis. For SNPA members, this represents a transition from older microplastics campaign in large water bodies (net sampling) to more restricted areas (discrete sampling/filtration system sampling) and to visual inspection analysis (Light Microscopy) to visual inspection/chemical identification analysis (FTIR and Raman microscopy). Regarding sample treatment, in both surveys greater heterogeneity was observed, reflecting the different viewpoints on the topic in the scientific literature. Following data from the JRC survey (JRC, 2022) and not considering filtration, digestion is the most widely used (9 entities out of 21) pretreatment methodology. A similar situation was observed for the National Working Group, in which digestion represented the majority of pre-treatment methods. However, should also be considered that data on pre-treatment methods also refer to matrices different from surface water and drinking water (e.g., sea water, sediments) which often still follow different analytical patterns (e.g., particles > 300 µm sampled with nets). Experts, as noted during the first meeting, were in any case opened to new solutions as oil extraction and other mild pre-treatment methods. Mild pre-treatments, especially when applied to water rich in organic content, as can be seen from the experimental results, can help in lowering matrix effect facilitating the visual inspection through microscopy-spectroscopy techniques. From the JRC survey (JRC, 2022) and National Working Group survey (Martellone et al., 2022a, 2922b), confirming literature data (Wang et al., 2021; Yusuf et al., 2022), also emerged that FTIR microscopy and Raman microscopy were actually the most common analytical techniques for microplastics analysis. Coupled techniques are currently the only ones allowing

the simultaneous assessment of abundance, type and shape of microplastics in environmental matrices and the fact that it is well understood is certainly a good indication. However, optical microscopy still remains a widely used technique, but this is about to change given the recent interest of many groups in drinking water. The expertise of the Italian working group members will form the basis of a soon-to-be-published technical report on analytical techniques.

4.1.2 Experimental Activities

Experimental activities allowed to understand and confirm different aspects related to the analysis of microplastics in freshwater environments. Regarding sampling, experience acquired during the experimental part PhD project allowed to better understand differences on a practical level between *"in situ filtration"* sampling and "*discrete sampling*". The design, assembling and testing of the filtration system required several months but it allowed, thanks to the sequence of stainless-steel filters, the filtration of a considerable volume of surface water (an average of 1.803,6 L for surface water) without clogging. Clogging was frequently reported by researchers (Ziajahromi et al., 2017; DWI, 2019; Mintenig et al., 2019) but in this case was not observed. This was probably due to the inclusion of the 300-micron filter before the analytical one, which blocked most of the heavier particulate matter allowing a longer sampling time. The easy of disassembling is another advantage of the filtration system proposed in this PhD project, compared to those found in the literature. Filtration units, because of switching valves, can be easily changed without open it, minimizing possible contamination. However, additional tests with a water with high turbidity (> 55 NTU) are required to confirm these findings and verify the absence of blockages with representative volumes. Sampling volumes also strongly depended on pressures at the tap level so reaching representative volumes could be possible also in timescales < 6 hours. It is also possible, by working with pressures (e.g., employing pressure reducers), to normalize sampling volumes and sampling times. In case of low pressures or absence of pressurized systems, the solution could be employing a pumping system. Due to the possibility of adjusting the sampling parameters in a flexible way, sampling with a filtration system appears to be complementary approach to the *"discrete sampling"*, as provides an evaluation of microplastic content in a given time period. Regarding *"discrete sampling"* a certain grade of contamination by PBT red caps was expected, but PBT was not found in any samples. Covering bottles with a double layer of aluminium foil may have contributed to prevent the contamination. However, is also possible to theorise that PBT does not actually release microplastics compared to what has been assumed for PP caps (WHO, 2019). Data retrieved from the PhD experimental part strongly supported the feasibility of oil extraction as one of the pre-treatment methods for the analysis of microplastics in water matrix, especially when there is a need to compare results between drinking water and the water with which is produced (which requires a strong suppression of the matrix effect) or when the analysis need to be performed at room temperature to preserve microplastics' integrity (e.g., when focusing on SMPs). Only 2 surface water samples proved

to be unprocessable through FTIR microscopy and, with one of them, lowering the processed volume resulted in in the possibility of performing analysis without further problems, even with turbid water. Oil extraction also allowed retrieving a high number of fluorinated microplastics compared to other available pre-treatments, as later will be discussed. Regarding filtration, as expected, good results in term of visual inspection and spectra quality in FTIR analysis were obtained from Anodisc filters. Thanks to their diameter (47 mm), Anodisc filters allowed a reduction in particle and interferers overlapping. Particle overlapping, as can be seen in GRZ DWTP #1 SF5 and GRZ DWTP #2 SF5 can hinder visual inspection. However, if the filter has adequate dimensions, the chance of overlapping can be minimised by simply reducing the volume of filtered water (GRZ DWTP #2 SF5 BIS). From surveys (JRC, 2022; Martellone et al., 2022) and literature data (Wang et al., 2021; Yusuf et al., 2022) emerged that analytical techniques of choice for microplastics in water environments are FTIR and Raman microscopy. These techniques, employing *Particle measurement* or *Imaging* approach both allow an easy identification of particles along with particle count and shape visualization. However, *Imaging* without a dedicated software for particle reconstruction is often reported to be more complex and time-consuming than *Particle measurement*, given a) the amount of redundant data produced, b) the need for specific detector allowing faster analysis 3) the know-how needed in data processing unless dedicated software is available. The comparison carried out during the PhD project confirmed the assumptions found in literature (Primpke et al., 2020; Ivleva et al., 2021; JRC, 2022). A surface water filter (Micro-FTIR Nicolet™ iN™ 10, 64 co-scans, 20 count fields = 48 mm^2) can be fully analysed (count field approach) within one day while a very restricted area (e.g., a single area of 0,0025 mm^2, points spaced by 2 µm) with Raman microscopy (Raman Renishaw in VIA RM2000 spectrometer, Objective 100x Leica Fluotar/Darkfield, 633 nm Laser) in *Imaging* mode required more than 2 hours without considering data processing. A larger point-by-point distance could have been selected in experimental with Raman microscopy, but the risk of particles not being count between points is considerably greater this way as *Imaging* is not particle-based. A quantification without dedicated software with *Imaging* appeared, for this reason, impossible even if employing a filter with a more restricted area. Regarding Micro-FTIR analysis, data retrieved from the experimental showed that microplastics are widespread in surface water and can reach drinking water. Regarding abundance of microplastics in the three different sites, some consideration can be made. The abundance at DWTP #1 site greatly differed from that observed at the other sites. The high number of microplastics detected in DWTP #1 could be implying that this site is simply more contaminated than the others are, but as the sample GRZ DWTP #1 SF5 is missing and each site is very different from the others nothing else can be assumed. However, GRZ DWTP #1 samples (both SF5 and TQ) proved to be particularly difficult to handle, as a lot of suspended substance was observed. This could be the reason behind the inability to analyse DWTP #1 SF5 samples and the high number of microplastics detected in GRZ DWTP #1 TQ. Considering GRZ DWTP #1 TQ an outlier in terms of total and leaving aside SF5 samples whose numbers depend on the volume being filtered, assumptions with other data in the literature can be made. Those numbers (from 2127 ± 96 to

12606 ± 114) are comparable to those described by WHO in 2019 (0 to 10^3 particles/L) and by Wang et al. (2021), Yusuf et al. (2022) and Xue et al. (2022) (0-6614 MPs/L). However, numbers indicated in these reviews refers to studies that 1) originate from different analytical protocols, including different analytical techniques and sampling type 2) if they refer to spectroscopic techniques, they do not present a clear indication of the accepted match percentage 3) do not take into account if subsampling followed by extrapolation has been performed or not 4) above all, investigated different particle sizes, so a direct comparison was not possible. Microplastics abundance in surface water found during the analysis can be explained considering differences in sampling sites. Although all these sampling sites are located in the final tract of the corresponding river, Adige river sampling point significantly differs from the others. The low abundance of microplastics in Adige river could be tracked down in its Alpine origin and in its flow. Adige river, with the except of Trent and Verona, do not flow through densely populated regions. Moreover, in the nearby of DWTP #2, the river intensely flows, and bights were not present, so accumulation of microplastics can be considered negligible. Actually, a similar situation can be observed for Tiber river, as DWTP #1 is located near Rome. However, in this tract, the river slowly flows, and water capture takes place in a river bight, so an accumulation of microplastics could be present and abundance can be justified. As GRZ DWTP #1 SF5 is missing and GRZ DWTP #1 TQ was difficult to process, a direct comparison with another site is not possible. Conversely, Po river, although its Alpine origins, flows through densely populated regions (e.g., Turin, Piacenza, Cremona) and receives water from many tributaries throughout the Po Valley. As water flow in the sampling point was slow and in proximity of a river bight, an accumulation of microplastics could be present and the high abundance of microplastics, compared to DWTP #2 samples, can be justified. In terms of polymer composition, heterogeneity was observed, probably due to differences between the various sampling sites. In surface water SF5 samples, PES, FC, PTFE and AC were retrieved in both samples while, in their corresponding TQ samples, only FC and PO are common to all sites. Thus, only FC is common to all surface water sample. Fluorocarbon (FC) is a trade name for a polyvinyl fluoride plastic (Polyvinylidene fluoride, PVDF), mainly employed in new fish lines. PVDF it is also employed in pipes and fittings, valves, cables, membrane materials and is being examined for applications in batteries, biomedical research, chemical engineering and wastewater management as a membrane material (Hu et al., 2022b) due to its resistance to harsh halogenated chemicals, acids, peroxides, aromatics and ozone. Fluorocarbon (FC) is rare in surface water while PTFE, found in discrete amounts within the samples, is more common (WHO, 2019; Wang et al., 2021, Yusuf et al., 2022; Xue et al., 2022). PVDF has a low density (1.78 g/cm^3) compared to other fluoropolymers (e.g., 2,20 g/cm^3 for PTFE) but it is still higher than common consumer non-fluorinated plastics (ranging in density from 0.85 to 1.41 g/cm^3). Its density may be the reason why it is not frequently found in surface water as other extraction techniques (e.g., density separation) are unable to recover high-density plastics. The oil extraction, as a non-density-based extraction technique, could have played a significant role in the detection of relevant quantities of fluorinated polymers in surface water (PVDF, PTFE, PFA and FEP). High recoveries of fluorinated

polymers were also observed applying an oil extraction method to seawater and other environmental matrices (Corami et al., 2020b; Corami et al., 2021; Rosso et al., 2022). Polyester (PES) is a generic terminology used to indicate fabrics made from polyester yarns or fibers, commonly composed by PET as a matrix. Its presence in surface water is usually related to fiber release in washing discharges (Corami et al., 2020). PES can be commonly found also in soil amending agents, plant support, and wind protection netting (Sattler and Schweizer, 2011). PES was present in significant quantities in all samples except for GRZ DWTP#2 TQ. PES was indeed the most common polymer in GRZ DWTP #3 SF5, the second most common polymer in GRZ DWTP #1 TQ, the third most common polymer in GRZ DWTP #3 TQ. PES was not in the list of the five most abundant polymers in water drafted by Koelmans et al. (2019) but is the third most common polymer detected in drinking water according to the JRC (JRC, 2022). PES is reported only in 7 studies out of 42 in Wang et al. (2021) investigation and in 1 study out of 6 in Xue et al. (2022) investigation. However, the diffusion of PES among surface water samples could again be explained by considering its density (1,23-2.3 g/cm^3). A higher percentage of PES may indeed have been recovered compared to those retrieved in other studies due to the oil-extraction method. Vinyl Ester Resin (VER) is a generic designation for resins by the esterification of an epoxy resin with acrylic or methacrylic acids. VER, together with unsaturated polyester resins are among the most commercially important thermosetting matrix materials (Kandelbauer et al., 2014). Vinyl ester resins can be included in automobiles and other vehicles parts (as boats), in fascias for buildings and as reinforcements for bridges. VER was the most common polymer in GRZ DWTP#1 sample, but it was not found in any other surface water samples. Its presence, given its relativity rarity, could be due to a spot contamination. Nylon, or Polyamide 6, is a member of the family of synthetic polymers known as polyamides. PA 6 has been widely used in fabrics for clothes and carpets, fishing nets, fish tackles, ship hulls, and disposable plastics. PA 6 is a very hydrophilic polymer, making it highly susceptible to water absorption and with a T_g (transition temperature) ranging between 37 °C and 55 °C. Nylon was found in every surface water sample, but also in procedural blanks. The adjustment on the total number due to the subtraction of Nylon values in procedural blanks resulted in his absence in DWTP#1 and DWTP #2 samples. Following the adjustment, the presence of Nylon was only considered in DWTP #3 samples. However, Nylon represented the major contributor of microplastics in procedural blanks and its presence should be carefully assessed, even in DWTP #3. Findings during the experimental part of the PhD thesis could be explained assuming a certain percentage of contamination in analytical steps performed outside of the Clean Room. Regarding other microplastics detected, several of them are commonly found in inland water (Wong et al., 2020; Wang et al., 2021, Yusuf et al., 2022; Xue et al., 2022). Polyethylene (PE), usually described as one of the most common polymers, was found in only 2 samples (GRZ DWTP #3 SF5, GRZ DWTP #1 TQ). A similar situation was observed with Polypropylene (PP); polypropylene was found only in GRZ DWTP#2 samples (GRZ DWTP #2 SF5 BIS and GRZ DWTP #2 TQ). However, considering Polyolefin (PO) found also Polypropylene, the situation appears different, with PP found in every GRZ sample except for GRZ DWTP #3 SF5. A higher homogeneity was noted by comparing the

composition of plastics in SF5 samples with the corresponding TQ samples of the same site. However, only 5 polymers out of 9 (approx. 56%) were common to GRZ DWTP #2 SF5 BIS and GRZ DWTP #2 TQ and only 5 polymers out of 12 (approx. 42%) were common to GRZ DWTP #3 SF5 and GRZ DWTP #3 TQ. These differences underline the necessity of also performing studies on sampling methods when developing protocols for microplastics analysis, as results may be very different by changing the sampling input. Discrete sampling returns a "picture" of the microplastic content at that particular time but may not give a reliable assessment of the contamination. Collecting several samples within a short time frame during the sampling day (e.g., 3 hours) could allow to understand the fluctuation in microplastic content in the area of interest. Sampling systems are based on this concept and allow to avoid repetitive sampling. However, as this experience has shown, the development and validation of a sampling system requires a great deal of effort, also considering that additional analytical steps (e.g., resuspension) are required. In terms of size, a higher homogeneity was observed. Only approximately 5% of particles found was bigger in size than 100 µm (max 515,9 µm) and approximately 95% of particle size varied between 20 and 100 µm. By stratifying for sampling type, notable differences from these values were only observed in surface water TQ samples with approximately 24% of particles > 100 µm and 67% particles with a size from 20 to 100 µm. This could indicate the efficiency of the first filtration unit in retaining particles > 300 µm. Summing up, with both sampling methods, SMPs constituted the majority of particles retrieved. Since studies on microplastics in surface water are usually different in terms of size range investigated due to different sampling and analytical techniques, a direct comparison of proportion MPs/SMPs with literature data was not possible. However, SMPs are commonly found in surface water (WHO, 2019; JRC, 2022) and size data are similar to those observed in other studies performed in a similar manner (Pivokonsky et al., 2020; Wang et al., 2020a). Observing Wang et al. findings (2020a) approximately 97% of particles retrieved were < 100 µm. A similar situation was observed by Pivokonsky et al. (2020) as, for fragments, in two different sites, MPs < 100 µm were approx. 99%. Fibres showed instead a different behaviour (approx. 50% of particles < 100% for site #1 and 80% for site #2). Both studies employed Raman microscopy. In any case, particles < 20 µm were not retrieved in any samples analyzed during PhD project. This could simply be a consequence of the effective absence of microplastics in samples. FTIR microscopy (resolution ≃ 5-10 µm; 7 µm for the one employed at CNR-ISP) FTIR microscopy proved indeed to be able to identify particles < 20 µm (Strand et al., 2018; Corami et al., 2021). Data retrieved by Raman microscopy and literature review (Pivokonsky et al., 2020; Wang et al., 2020a) showed that microplastics can be found also below the resolution limit of FTIR microscopy (< 5 µm). Two suspected microplastics with a size ≃ 2µm were indeed identified in two surface water samples. Particle size definition is extremely important in microplastics analysis as it could be implicated in particle toxicity and health implication. Thus, a detailed dimensional analysis of particles 20 – 100 µm fraction was performed for every sample. Data showed that 40-50 µm size cluster was the most populated for every sample. The 40-50 µm size cluster remained the most populated even if stratifying by sampling techniques and assuming a 1000 L volume

for filtration system samples. A general trend in which particles are more condensed in the region to the left of the 60-70 µm cluster can also be observed. Similar results were obtained by Pivokonsky et al. (2020) and Wang et al. (2020a). This could mean, although further evidence is needed, that the majority microplastics in surface water are closer to the 20 µm border than the 100 µm border. Considering that the uptake and transport of particles <10 µm into intestinal cells appears possible, a meticulous dimensional analysis should be carried out to check if particles of this size actually enter DWTPs. In terms of shape, a high degree of homogeneity was observed among samples. Non-elongated particles were by far the most common particles (AR< 2) in all samples. The proportion of non-elongated particles over the total remained essentially the same stratifying data for sampling type (approx. 65% for SF5 samples assuming 1000 L as a reference volume and approx. and 65% for TQ samples). A direct comparison with WHO (WHO, 2019) and other data (e.g., Pivokonsky et al., 2020, Wang et al., 2020a) was not possible because, in these reviews, shape data are shown as 3D shapes (fibres, fragments, spherical particles, film, foam…), suitable only for particles > 100 µm. In addition, several studies found in literature employed strong pre-treatment methods (e.g., digestion temperature and/or reagent assisted) possibly modifying microplastics shape during the analysis and rendering comparisons even more difficult. However, fragments (assignable to non-elongated particles group) are reported as the most common shape identified in WHO review and prevailed in site #1 (approx. 80%) and site #2 (87–92%) in Pivokonsky et al. (2020) research.

4.2 CONCLUSIONS AND FUTURE RESEARCH NEEDS

This PhD project, developed to fulfil the institutional role of ISS in accordance with the European Directive and following the holistic approach as the rationale of the entire research, allowed collecting data on several critical issues concerning microplastics. First of all, following the JRC guideline, an assessment of the national preparedness about microplastics analysis has been carried out. The national survey showed that, despite microplastics is a generally new topic, it was already being approached also by Universities/CNR and SNPA members. However, in the analytical field, several knowledge gaps emerged, not only for water suppliers but also for Universities/CNR and SNPA members. Activities related to the National Working Group could help to fill this gap in the near future, also through publications about the topic to be submitted (e.g., a technical report on the analytical methods for microplastics in drinking water from contributions of every National Working Group member) and already submitted. In this sense, a first technical report on sampling methods (Martellone et al., 2021), produced as part of the PhD project with the collaboration of Venice ISP-CNR and Padua University, has already been published serving as guideline for those approaching microplastics for the first time. The upcoming meeting of the National Working Group will be held in accordance with the activities organised by the JRC. In this framework are included results of the experimental part. The developed method allowed an identification of microplastics in surface water, even with water with a high organic

content. In this regard, *Particle measurement* approach proved to be suitable for microplastics analysis and a mild pre-treatment step (oil extraction), allowed a clear identification on filters and recovering of fluorinated microplastics. However, it might be useful, in the near future, to repeat the analysis in the same sites (both with filtration system and discrete sampling) to confirm the method suitability for surface waters, possibly employing certified standard. As reported in the bibliographical research part, there is a lack of certified reference standards as they are currently difficult to produce due the heterogeneity of microplastics. However, results of international ring tests showed that situation is evolving, and more reliable non-certified standards are now available. In any case, *Particle measurement* proved to be more rapid, intuitive and practical (especially in view of a routine analysis) than *Imaging*. Experimental also showed that microplastics are present in surface water in significant amounts and have the opportunity to reach DWTPs. Each site showed different polymers composition but small microplastics (SMPs) (especially those < 70 µm) proved to be the most abundant group. However, no microplastics between 7 and 20 µm, potentially the most harmful ones, were identified. Repeated analyses should be carried out in order to confirm these findings. Although in the beginning research on microplastics was in a tangle in which it was not easy to get out, now the problem is beginning to thin out, especially in the analytical field. All this has been only possible through the regulatory intervention, implying the recognition of the key word, namely that of "standardization". Only standardization allows the comparison of analytical methods and data. The ultimate goal, which is the risk assessment, can only be achieved with these prerogatives and through collaboration between regulatory authorities, researchers and, in case of drinking water, water suppliers.

Acknowledgements

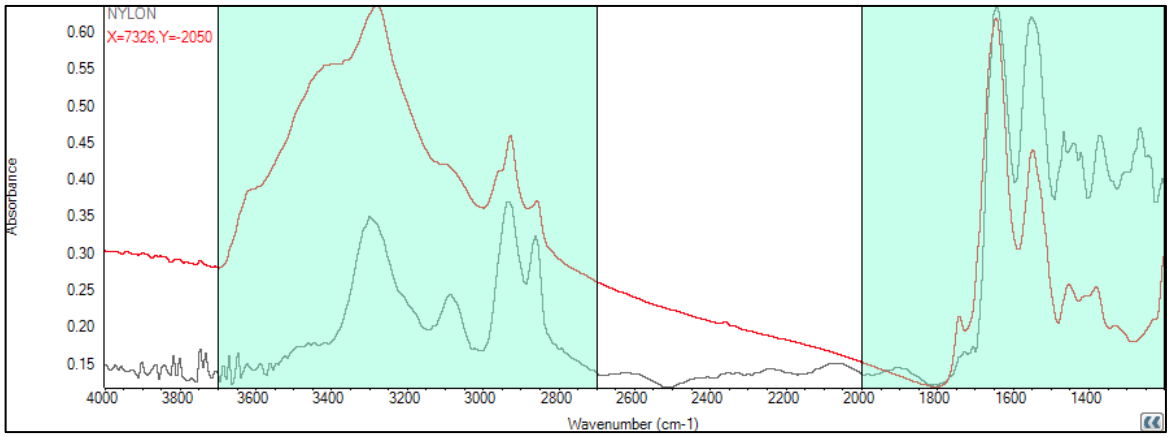
The conception, development and management of this tangled institutional and experimental project became only possible through the active collaboration between the participating entities. First of all, special thanks go to drinking water suppliers who allowed water sampling from pressurized systems within DWTPs and provided data on water quality. Their voluntary participation was essential for the project's success. Special thanks go also to John Fawell and Pablo Campo Moreno from Cranfield University, for their outstanding support during the design of the filtration system. Regarding the other analytical phase, the expertise of CNR-ISP and Padua University was prominent for providing quality data in such a challenging field as microplastics analysis is. Special thanks therefore go to dott.ssa Fabiana Corami, dott.ssa Beatrice Rosso and dott.ssa Giulia Vitale from ISP-CNR. Their expertise on microplastics analysis allowed a critical interpretation of the results, also considering that analytical methods are constantly evolving. In this sense, a systematic literature review, such as the one performed jointly with our colleagues in Padua when drafting the ISTISAN technical report (Martellone et al., 2021), is in fact always necessary in order to remain updated on this mutable topic. Special thanks therefore go also to prof.ssa Sara Bogialli and dott. Lucio Litti, for their support in Raman analysis, including the data management. Their help was indispensable for the comparison of Raman data with FTIR data acquired at Venice ISP-CNR. Last but certainly not least, gratitude goes to the central coordination of the PhD project at Italian National Institute of Health, Water Quality Unit, namely in dott. Luca Lucentini and dott.ssa Daniela Mattei, and prof. Gabriele Favero from Sapienza University. Their scientific expertise was decisive for the accomplishment of the project, despite the issues encountered due to the pandemic.

Appendix I: Acronyms for plastic polymers found or cited

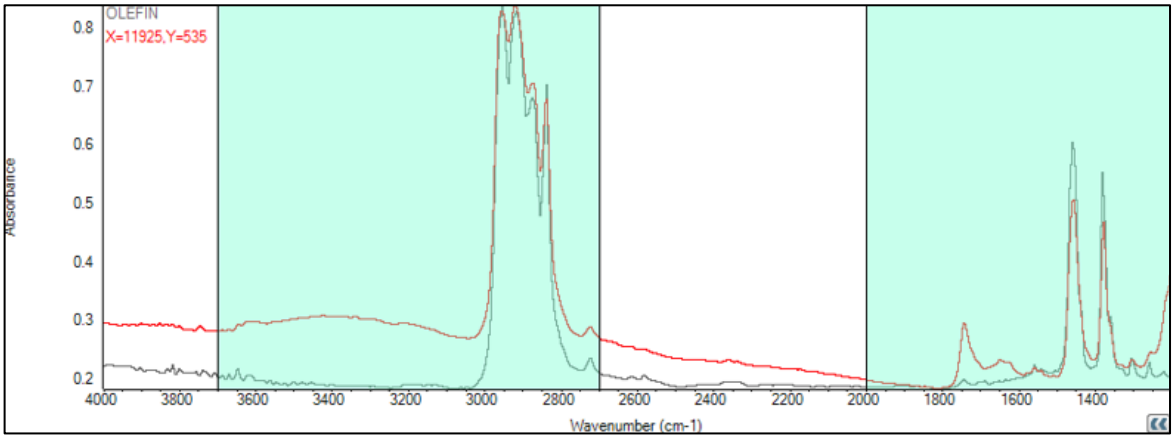
ACRONYM	POLYMER
ABS	Acrylonitrile Styrene Butadiene
AC	Acrylic
EMA	Methyl Acrylate Copolymer
EVA	Ethylene vinyl acetate
EVOH	Poly (Ethylene Vinyl Alcohol)
FC or PVDF	Fluorocarbon/ Polyvinylidene fluoride
FEP	Fluorinated ethylene propylene
PA	Nylon
PARA or PAA	Polyarylamide
PC	Polycarbonate
PE	Polyethylene
PES or PEST	Polyester
PET	Polyethylene terephthalate
PFA	Pefluoroalcoxy Fluorocarbon
PAM	Polyacrilamide
PBT	Polybutylene Terephthalate
PI	Polyimide
PO	Polyolefin
POM	Polyacetal/Polyoxymethylene
PP	Polypropylene
PPA	Polyphtalamide
PS	Polystyrene
PTFE	Polytetrafluoroethylene
PTX	Polymethylpentene
PU	Polyurethane
PVC	Polyvinyl chloride
VER	Vinyl Ester Resins

Appendix II: List of selected microplastics FTIR spectra based on the highest matches

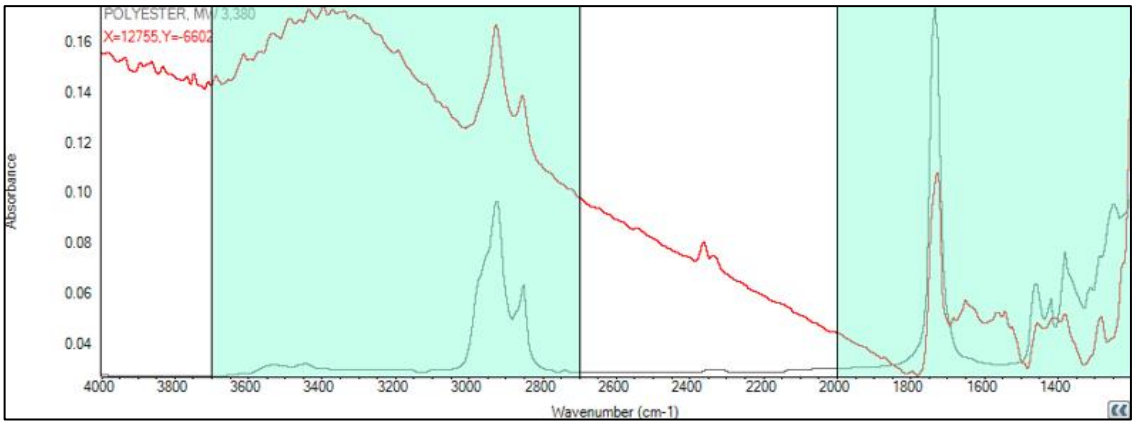
GRZ DWTP #2 SF5 BIS (Match 76,1%)



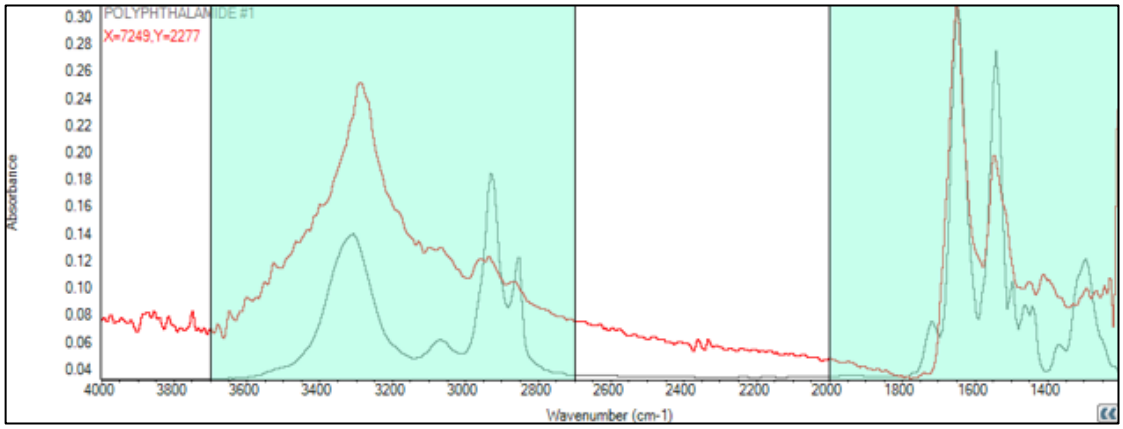
GRZ DWTP #2 SF5 BIS (Match 78,7%)



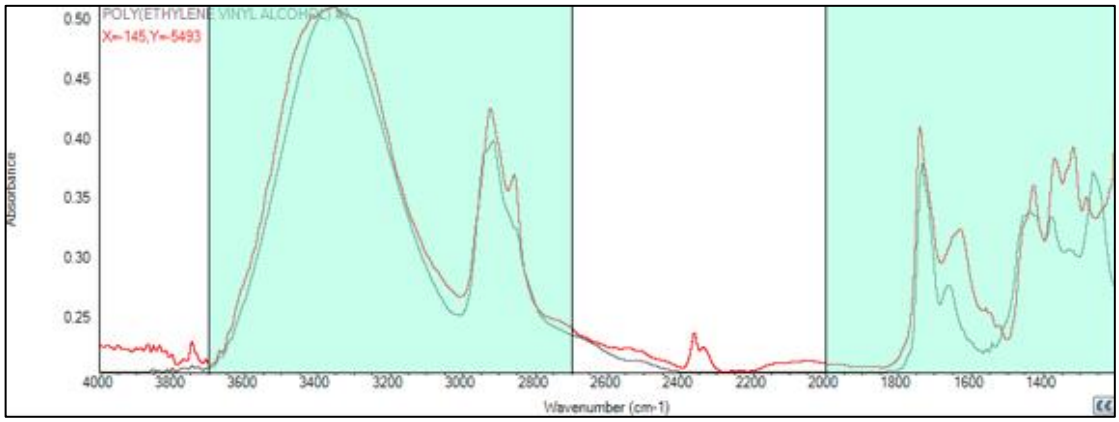
GRZ DWTP #2 TQ (Match 95,4 %)



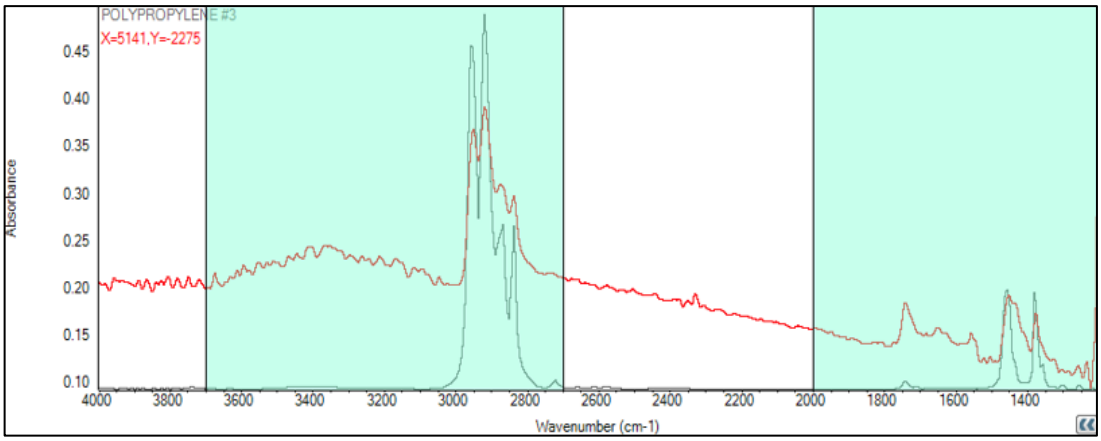
GRZ DWTP #2 TQ (Match 78,6%)



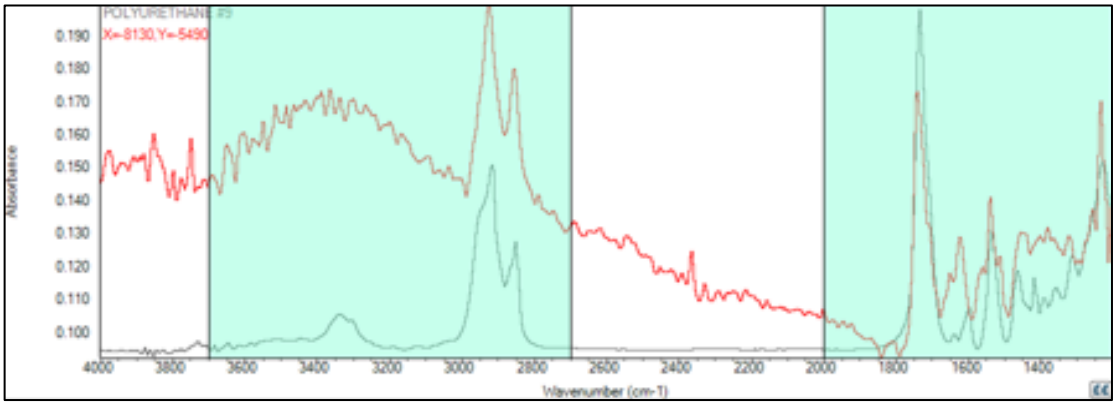
GRZ DWTP #2 TQ (Match 72,5%)



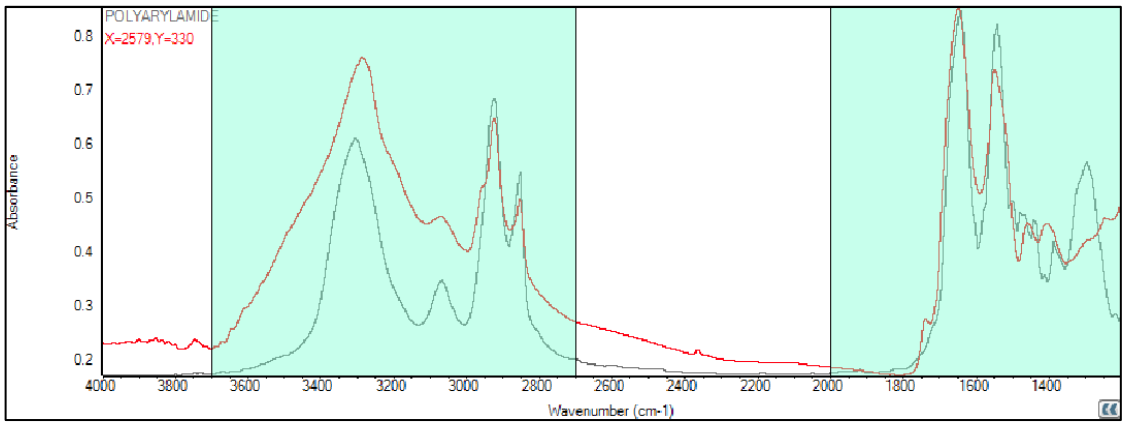
GRZ DWTP #2 TQ (Match 77,8%)



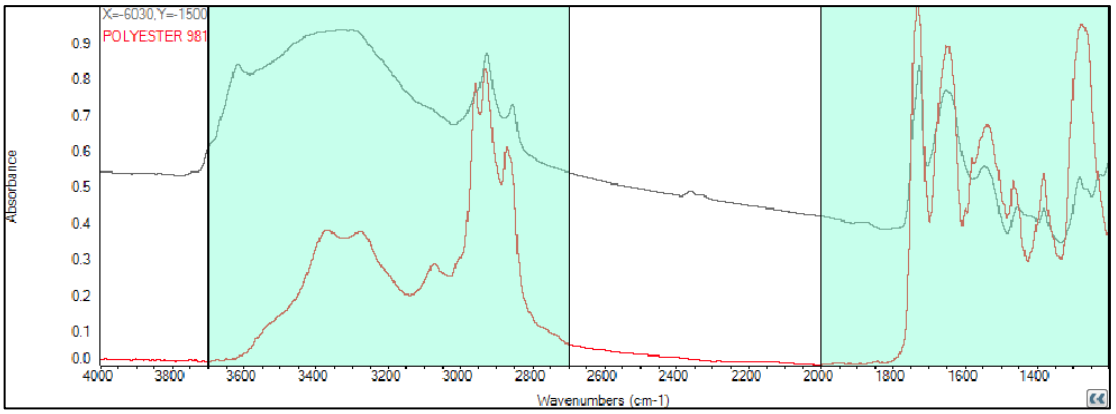
GRZ DWTP #2 TQ (Match 76,2%)



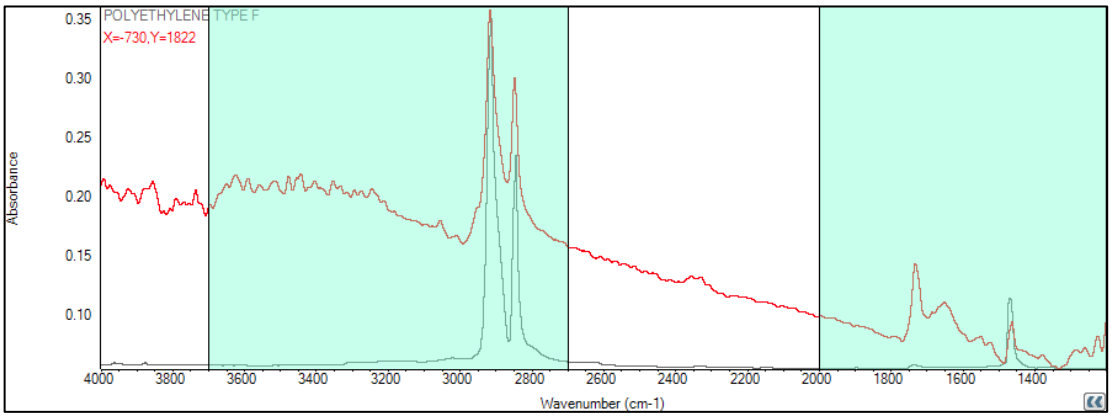
GRZ DWTP #2 TQ (Match 75,7%)



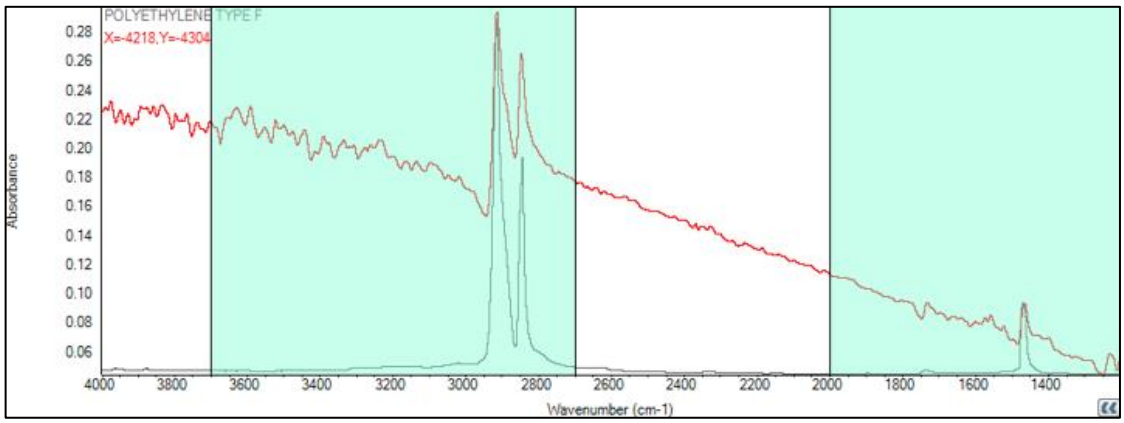
GRZ DWTP #2 TQ (Match 80,4%)



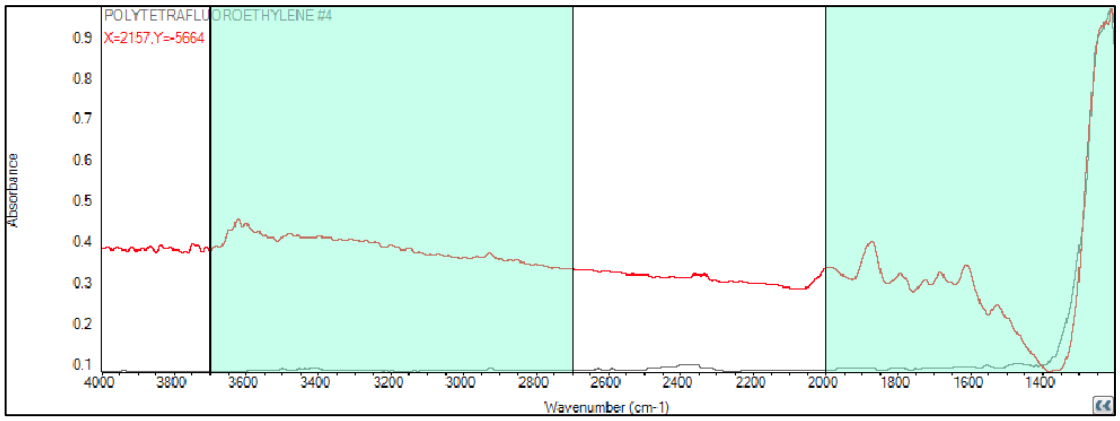
GRZ DWTP #3 SF5 (Match 74,7%)



GRZ DWTP #3 SF5 (Match 88,9%)



GRZ DWTP #3 SF5 (Match 85,3%)



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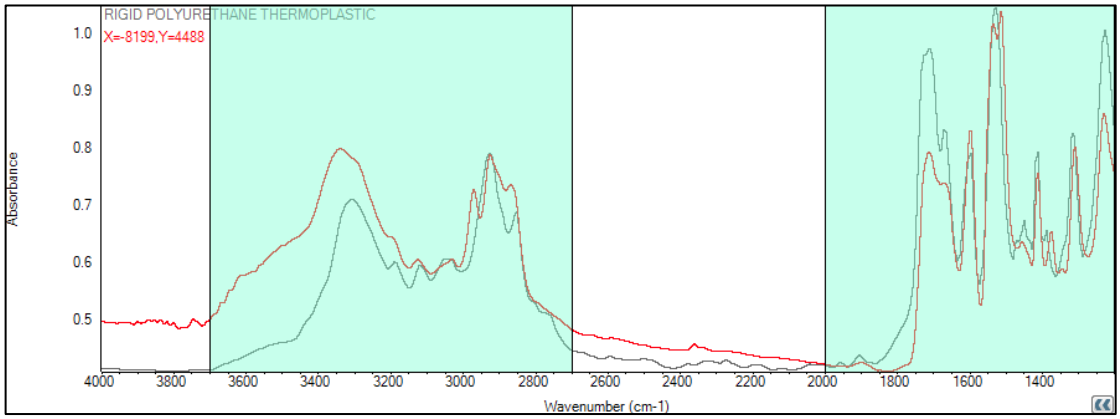
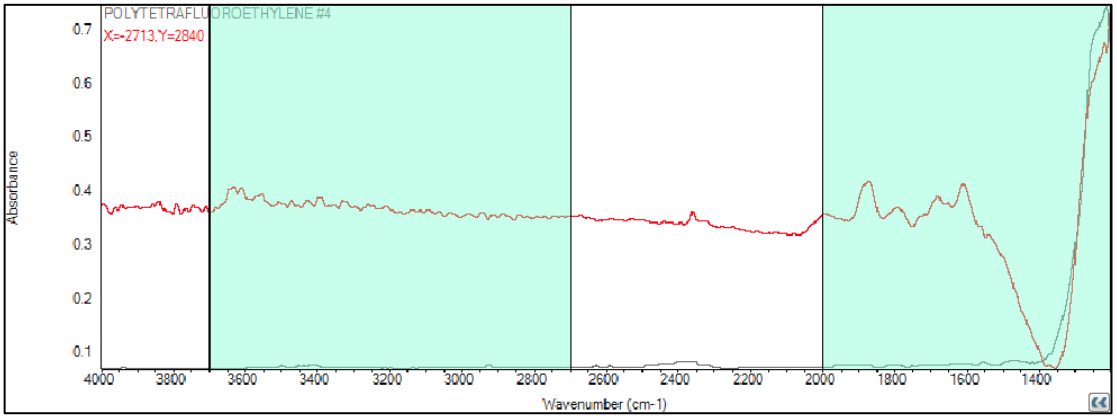
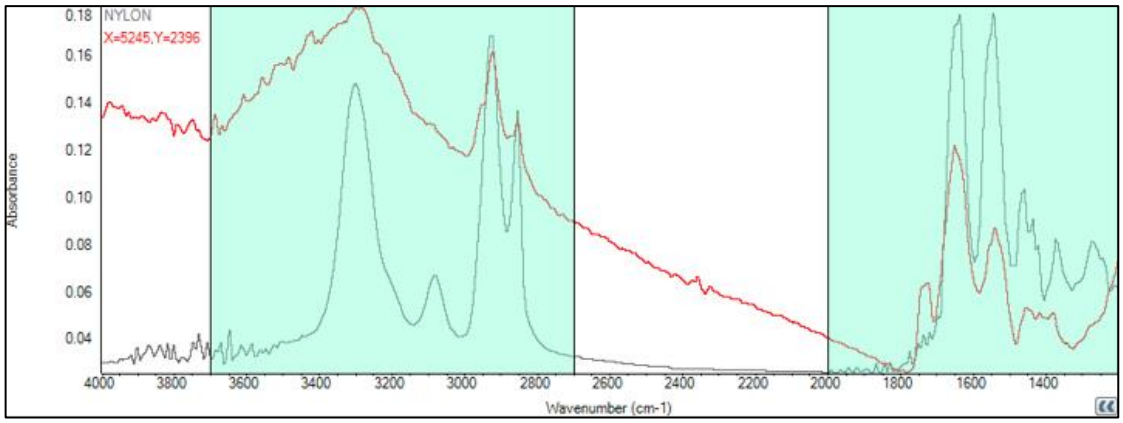


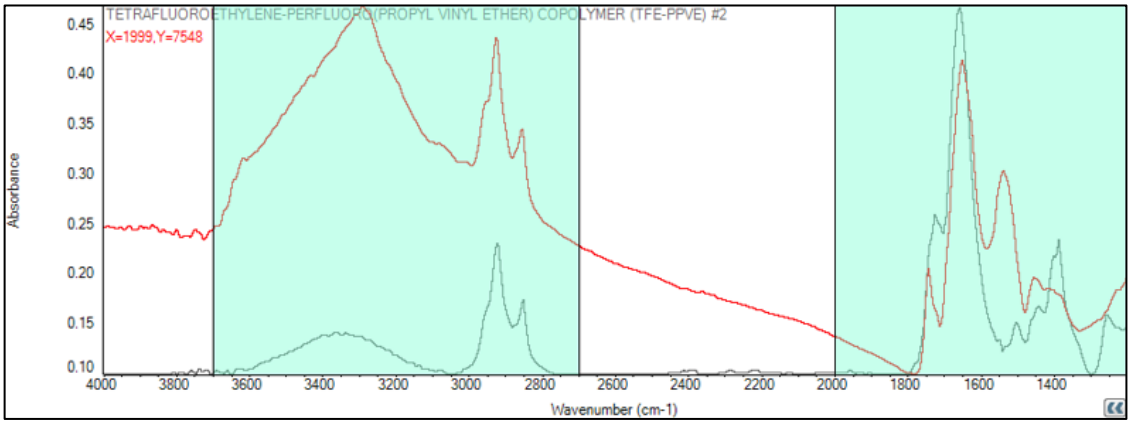
GRZ DWTP #3 SF5 (Match 78 %)



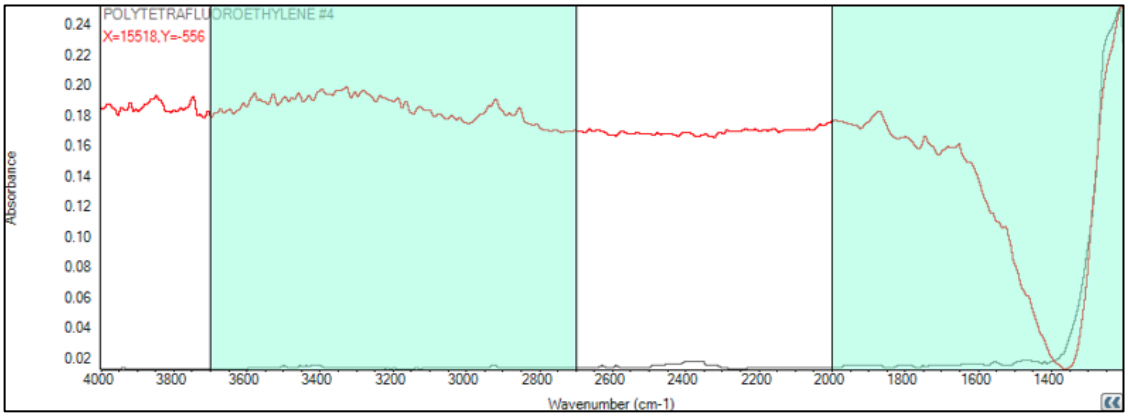
GRZ DWTP #3 TQ (Match 76,8 %)



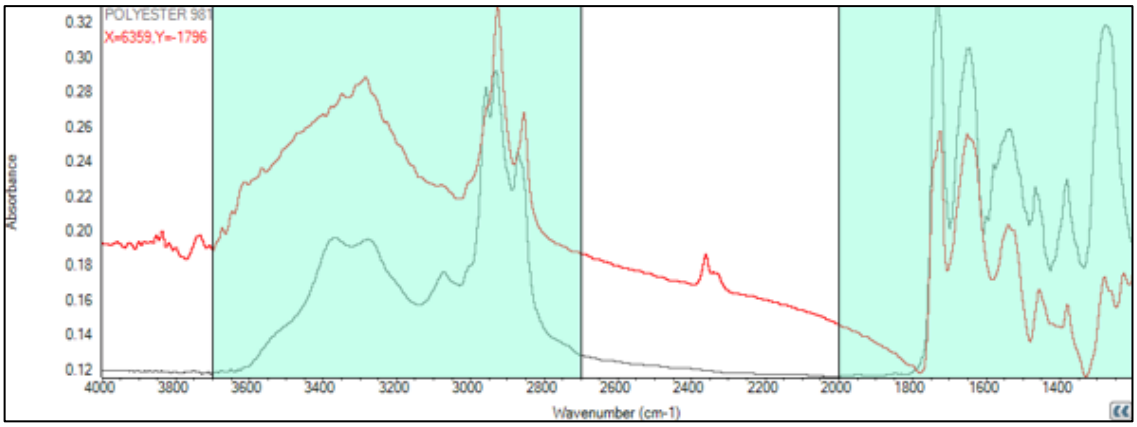
GRZ DWTP #3 TQ (Match 71,5 %)



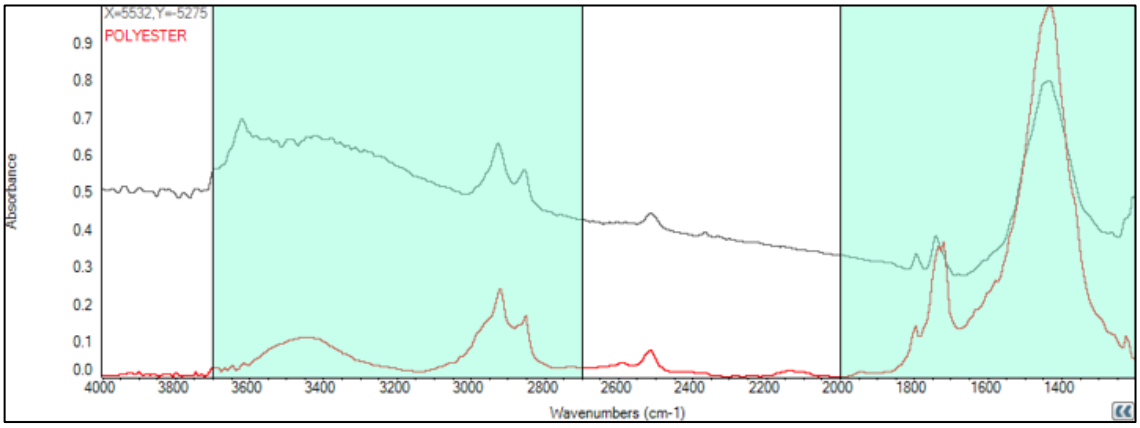
GRZ DWTP #3 TQ (Match 72,8 %)



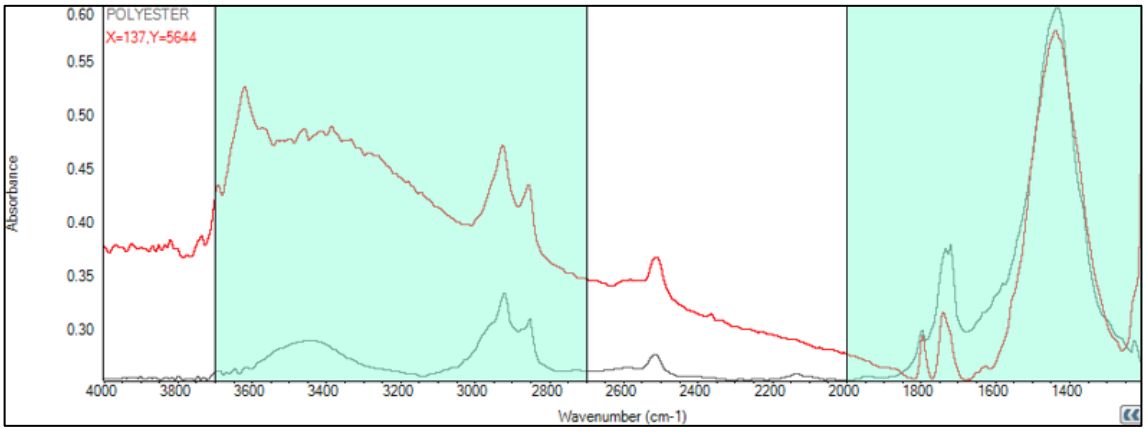
GRZ DWTP #3 TQ (Match 80 %)



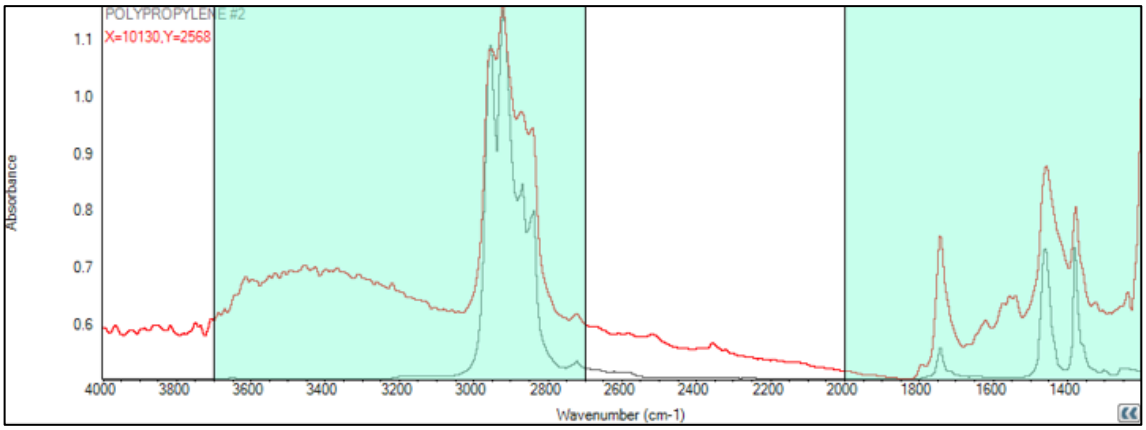
GRZ DWTP #3 TQ (Match 76,2 %)



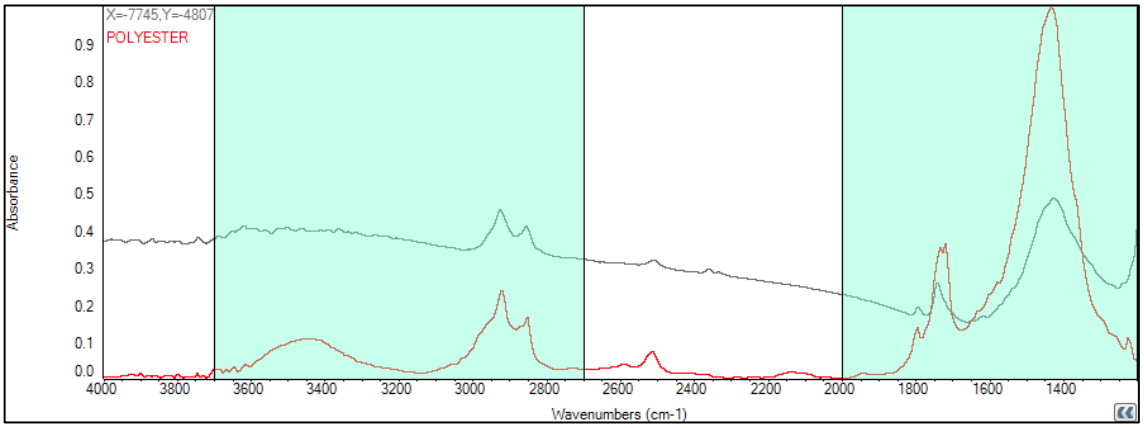
GRZ DWTP #1 TQ (Match 81,5 %)



GRZ DWTP #1 TQ (Match 78,8 %)



GRZ DWTP #1 TQ (Match 82,3 %)



GRZ DWTP #1 TQ (Match 77 %)

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