ISSN 1831-9424

# Analysing microplastics in drinking water

## *Practical considerations*

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2025

EUR 40430

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JRC143205

EUR 40430

PDF ISBN 978-92-68-31100-4 ISSN 1831-9424 doi:10.2760/1240697 KJ-01-25-430-EN-N

Luxembourg: Publications Office of the European Union, 2025

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How to cite this report: Mehn, D., Cella, C., Bucher, G., Fumagalli, F.S., El Hadri, H. et al, D. *Analysing microplastics in drinking water - Practical considerations,* Publications Office of the European Union, Luxembourg, 2025, https://data.europa.eu/doi/10.2760/1240697, JRC143205.

# Contents

## Abstract

The recast Drinking Water Directive (EU) 2020/2184 empowers the European Commission (EC) to adopt a methodology to measure microplastics in water intended for human consumption. When the directive entered into force in January 2021, no suitable standardised or harmonised methodologies were available. The Joint Research Centre (JRC), on request of Directorate General Environment (DG ENV), undertook a technical study to develop and test an appropriate analytical methodology. Through a comprehensive review of scientific literature, discussions with DG ENV, and consultations with experts from Member States and stakeholders, it was determined that InfraRed or Raman optical micro-spectroscopy techniques would be most effective approach for detecting microplastic at levels typical of real-world drinking water in the EU. This methodology was subsequently developed and tested by the JRC before being formally adopted by the European Commission through the Delegated Decision (EU) 2024/1441 of 11 March 2024 supplementing Directive (EU) 2020/2184 of the European Parliament and of the Council. The present report provides a summary of the published methodology with additional insights into some critical elements and limitations and – in Annexes - includes also experimental examples used at the Joint Research Centre's Nanobiotechnology Laboratory during the feasibility studies. Finally, consideration has been given to the applicability and adaptability of the methodology for other applications where legislation will require the detection or monitoring of microplastics.

## Acknowledgements

The support of the following JRC colleagues for fruitful discussion, review of this report, suggestions for improvements, and the layout of this document is much appreciated (in alphabetical order): Heidi Olsson, Gabriella Schirinzi, Birgit Sokull-Kluettgen, Veronique Vanherck, Stefania Vegro.

The authors are thankful to Bert Leemans (DG Environment) and the editor and reviewers of the JRC Editorial Review Board (JERB) for their careful reading of the manuscript and their insightful comments and suggestions.

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## Executive Summary

The recast Drinking Water Directive (EU) 2020/2184) empowered the European Commission (EC) to adopt a methodology to measure microplastics in water intended for human consumption. When the directive entered into force in January 2021, no suitable standardised or harmonised methodology was available. The Joint Research Centre (JRC), on request of Directorate-General for Environment (DG ENV) of the European Commission (EC), undertook a technical study to develop and test an appropriate analytical procedure. The methodology developed under this study was formalized through a delegated act, specifically the Commission Delegated Decision (EU) 2024/1441, issued on March 11, 2024, and complements Directive (EU) 2020/2184 by detailing the approach for measuring microplastics in drinking water.

The methodology was developed starting from a consideration of both scientific literature and feasibility tests performed at the JRC laboratories. A detailed review of the scientific literature reporting microplastics in drinking water led to the conclusion that sizes and (low) concentrations of microplastics in European drinking water would require methods with sensitivity achievable only by the use of vibrational spectroscopy techniques such as infra-red (IR) and Raman spectro-microscopy. From this evaluation, a preliminary draft of a methodology was prepared in which IR or Raman optical spectro-microscopy were proposed as the means to determine the number, size and polymer type of microplastic particles. Following extensive consultations with DG ENV and the expert group on the Drinking Water Directive, including specialists in microplastics, the initial proposal was enhanced and developed into the comprehensive methodology presented in this report. This document details the implementation of the methodology’s core components—sampling, pre-treatment, and instrumental analysis—illustrated with examples of commercially available instruments and equipment. The report summarizes the developed strategy, providing additional insights into key elements and limitations. It also includes, in the annexes, equipment-specific examples of its application at the JRC Nanobiotechnology Laboratory during feasibility studies. Moreover, the report considers the methodology's applicability to other prospective legislative needs where microplastic detection or monitoring will be necessary. Specifically, it explores potential adaptations for ground and surface waters, as well as influent, effluent, and sludge from urban wastewater treatment plants, urban runoff, and storm-water overflows. Finally, the report addresses the relevance of the methodology to the Commission Regulation (EU) 2023/2055 - better known as “the microplastics restriction” - which restricts the marketing of products containing intentionally added synthetic microplastics.

# 1 Introduction

Microplastics are small particles of synthetic polymers that can be formed by degradation of macroscopic plastic items or may be intentionally manufactured particles which have been added to consumer products and subsequently released during use.[1–4] They have become widespread in the environment[5–7] and can be found also in food[8–10] and drinking water[11–14] and thus likely to be ingested by humans.[15] The potential impacts of microplastics on human health has become a source of concern for both scientists and the general public although the weight of evidence of adverse effects of microplastics on human health is currently still low.[16,17] This is, in large part, due to the limited information about real-world exposure and eventual assimilation of ingested material. In the absence of systematically acquired analytical data on exposure through food, water and air level, generating reliable causal links with biological effects will remain difficult or impossible to achieve. Microplastics are very heterogeneous with widely variable dimensions, compositions and shapes and can be composed of one or more different polymers, can contain additives and have physical-chemical characteristics influenced by their degradation history.[1,3,18] This diversity makes the measurement and evaluation of microplastics monitoring data complex. With respect to specifically exposure, there is a need to better understand microplastics occurrence throughout the water supply chain, using appropriate methods and harmonized reporting criteria to determine the number concentrations, shapes, sizes and composition of microplastics.[19]

## 1.1 Designing the methodology

As a first step, the Joint Research Centre (JRC) undertook a detailed analysis of the scientific literature reporting the measurement of microplastics in drinking water. Results and references are already reported in a previous JRC technical report, “Analytical methods to measure microplastics in drinking water – Review and evaluation of methods”.[11] The objectives of the review were to determine (1) the methods used to separate and collect the microplastics, (2) the analytical techniques used to identify and quantify the microplastics in the collected samples, (3) the capabilities and limitations of the analytical techniques used, and (4) the quantities, size, composition and shape (particle, fragment or fibre) of microplastics recovered from the drinking water. The reported analytical techniques were from two distinct families. The first group of techniques, vibrational micro-spectroscopy methods (namely, Raman micro-spectroscopy (μ-Raman) and Infrared micro-spectroscopy (μ-FTIR or QCL-IR), can identify the type of polymer in individual particles and additionally provide information on size and shape. Identification of polymer compositions by these methods requires comparison of the acquired particle spectra with a library of spectra from known polymers. The key limitation of these methods is the lower limits of particle size which can be identified – the absolute lower limits depend on the technique (IR or Raman) and the characteristics of the instrument used but for high specification instruments around 1 μm for Raman and 5-6 μm for IR are the best which are achievable. In practice, such low values cannot be considered for routine measurements due to the very long analysis times. The second family of techniques, thermo-analytical methods (namely, pyrolysis-gas chromatography mass spectrometry, Py-GC/MS, and thermal extraction and desorption gas chromatography mass spectrometry, TED-GC/MS), can identify the types of polymers in a sample and quantify the total mass of each polymer type. Identification of polymer compositions requires comparison of their thermal decomposition products with a library of mass spectra of pyrolysis products from known polymers. Quantification of identified polymers requires a calibration for each polymer. Thermo-analytical methods alone are unable to provide information on particle numbers, size or shape. Thermo-analytical methods have no intrinsic lower limit for particle size but are limited by the minimum mass detection levels which are typically in the range 0.1 to 2 μg depending on the polymer. In the case where all the polymers of interest in a sample can be quantified simultaneously, the measurements time (1-2 hours) offers advantages over the optical methods (0.5-1 day).[11]

The Commission consulted with Member State nominated experts in the field to supplement the information from published studies and to guide the development of a methodology, which would be most appropriate for the range of microplastics concentrations which can be expected in European drinking water. Globally, reported levels of microplastics in drinking water ranged from 0.0001 to 440 particles per litre but specific consideration of data from European studies finds concentrations primarily in the lower part of this range. Results of inter-laboratory studies related to quantification of microplastics in water and ongoing standardisation efforts were also considered when evaluating which of the two families of analytical methods would be most suitable for the case of drinking water. Overall, very few reports provided information on the mass-based concentration of microplastics in drinking water, presumably because typical concentrations and sampling volumes did not permit the recovery of enough material to be detectable by the less sensitive thermo-analytical methods.

When all the information was considered, in particular the required detection limits, it was concluded that the only realistic analytical strategy for monitoring would be to undertake particle counting and identification based on vibrational spectroscopy - more specifically IR or Raman optical micro-spectroscopy. This analytical approach, combined with sample collection by in-line cascade filtration of water volumes of at-least 1 $m^3$, was judged to be the most appropriate basis on which to develop a detailed methodology which could best harmonize the collection, analysis and reporting. Much of the uncertainty in comparing microplastics data reported in the literature is due to the lack of harmonization of key aspects of sampling and presentation of data. For example, without clearly defined limits on the size of particles to be collected (filter cut-off) and reported (size binning) data becomes difficult or impossible to compare. Other key aspects of the methodology were determined by choices which were made to overcome technical limits of instrumentation, spectral databases or analytical practices. For example, identification of polymers by the spectroscopic techniques listed above requires comparison with spectral libraries of known polymers. Microplastics may be composed of a very wide range of polymers, copolymers and additives and spectral libraries cannot be guaranteed to contain all possible variants. Consequently, a pragmatic approach to monitoring should firstly obligatorily analyse and record the presence of a smaller group of specific polymers (**Table 1**), which are known to be commonly present in the environment and drinking water. In addition, where the analysis method positively identifies particulates of other synthetic polymer materials they shall be recorded (see the polymer classification workflow in **Figure 2**).

In view of the complex multifaceted nature of the information obtained from the analysis of microplastics in water - microplastics concentration, composition, size and shape - a pragmatic approach had to be taken to reduce the level of complexity of the data collected for monitoring purposes. Data recorded for eventual reporting thus has to be done by classifying microplastics on the basis of predefined size bins (see the size classification list in **Table 2**), shape categories (particle/fragment or fibre) and composition categories (priority polymers). Details on this are described in Chapter 3.5 Data reporting.

## 1.2 Delegated act

Following the literature review, laboratory tests and stakeholder consultations, the methodology to measure microplastics in water intended for human consumption (referred as “the Methodology” in this report) was published in the form of a Delegated act supplementing the EU Directive 2020/2184 of the European Parliament and of the Council[20]. The first part of the document published in the Official Journal of the European Union outlines the background and rationale for the act, including the potential impacts of microplastics on human health and the environment. The document then discusses the different analytical techniques that can be used and the requirements for sampling equipment, data analysis, and reporting. The act describes the methodology itself in the second section of its Annex, while the first part provides definitions of the terminology used in the act. It should be noted that the methodology is intended for use under the Drinking Water Directive and therefore concerns water “intended for human consumption”. Specifically this is defined as “*all water, either in its original state or after treatment, intended for drinking, cooking, food preparation or other domestic purposes in both public and private premises, regardless of its origin and whether it is supplied from a distribution network, supplied from a tanker or put into bottles or containers, including spring waters; and all water used in any food business for the manufacture, processing, preservation or marketing of products or substances intended for human consumption*”. Natural mineral water and bottled water are subject to requirements from other EU legislation.

In chapter 2 of the present report, text boxes containing the wording of Section 2 (the “Methodology”) of the annex to the delegated act[20] are included and accompanied by comments, illustrative figures and suggestions on the interpretation of the legal text. Detailed considerations on the various steps of the Methodology are described in Section 3.

This document illustrates the practical application of the Methodology, including instrument-specific procedures and figures. However, the figures and procedure details presented herein are for illustrative purposes only and should not be considered as exclusive solutions. Furthermore, under no circumstances should they be interpreted as implying the superiority of any commercial instrument, software, chemical, or other products.

The present report provides a summary of the published methodology with additional insights into some critical elements and limitations and – in Annexes - includes also experimental examples used at the Joint Research Centre’s Nanobiotechnology Laboratory during the feasibility studies. The chemicals, tools and instruments described in the Annexes serve only as examples, and readers may choose alternative products or vendors that suit their needs, as the aim is to illustrate the experimental approach rather than to endorse specific products or vendors.

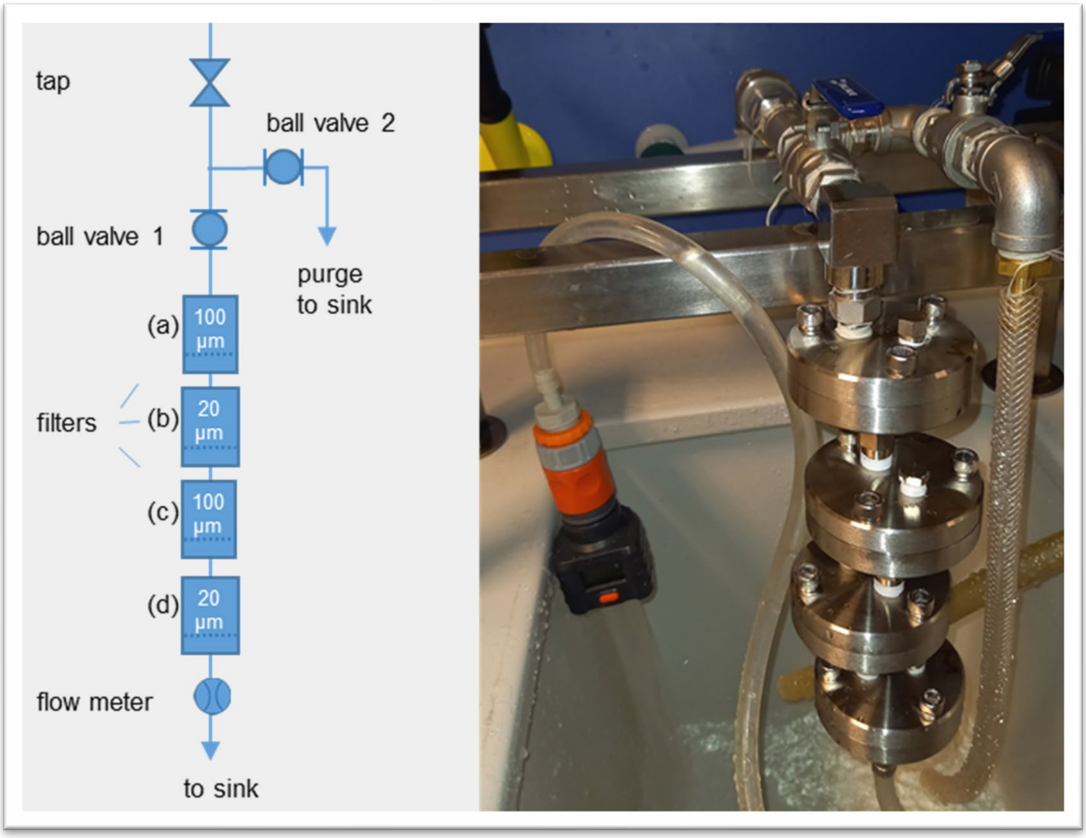
## 1.3 Extending the applicability of the methodology

The methodology described above was specifically developed for use in monitoring microplastics in drinking water but this is only one of several area of legislation where such analytical methods will be required in the future. Consequently, consideration was given to the extent to which it could be adapted to other areas such as surface and groundwaters (water framework directive, WFD / groundwater directive, GWD), urban runoff and storm-water overflows, treated and untreated urban wastewater as well as the related sewage sludge as required under the revised Urban Wastewater Treatment Directive (UWWTD). In addition to these environmentally orientated applications the implementation of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) restriction on the placing on the market of synthetic polymer microparticles[21] in substances and mixtures will also need methods to detect and quantify microplastics. These applications all pose additional and diverse challenges compared to drinking water, in some case technical due the increased complexity of the samples but, as in the case of REACH, the formulation of the legislation may introduce a factor (weight-based metric and particle size range) which are not compatible with the output of the drinking water directive (DWD) methodology.

## 2 The methodology

The first step of the methodology[20] is sample collection (**Box 1**) which is done by cascade filtration in order to avoid clogging of smaller cutoff filter(s). It foresees the collection of two different size fractions which could be analysed separately, possibly using two different techniques if needed. **Figure 1** shows the schematic of the filter system and a photographic example of a filter device assembled from commercially available components. The device is built of four filtration units: a, b, c, d. Each of which contains a 47 mm stainless steel filter in a stainless-steel filter housing. The valves are rotating ball valves (metallic) and apart from polytetrafluoroethylene (PTFE) sealings the system contains plastic elements (connection tube and flow meter) only downstream of the filtration cascade.

**Figure 1.** Schematic drawing (left) and photograph (right) of sampling device



*Source: own production*

The methodology specifies only the cut-off for the filters (20 µm and 100 µm) without any obligation related to any specific type of filter construction (*i.e.* mesh filter, pore filter or others). This serves to allow for future development/availability of better performing commercial filters (*e.g.* reusable metal foil pore filters would likely improve recovery).

Analytical laboratories are foreseen to work as much as possible under plastic free conditions but avoiding plastic components might not be always possible (for example, the presence of PTFE sealings in the setup depicted above). In this case, deviation from the methodology as foreseen by point 17(g) of the Annex to the delegated act should apply.

Any solvents or liquid reagents needed during the sample processing (*i.e.* pre-treatment and resuspension of particles) should be free of particles which may interfere with later particle counting or identification when using optical instrumentation. To achieve this is normally sufficient to pass the reagent through ≤0.45 µm cut-off filters which will remove any suspended particles in the size range relevant to the Methodology.

**Box 1.** Methodology to measure microplastics in water intended for human consumption. Annex, section 2 Point 1-2

Methodology to measure microplastics in water intended for human consumption

A filter cascade shall be used to collect particles and fibres from water intended for human consumption. Images from optical microscopy or chemical mapping are then used to determine individual particle size and shape, while vibrational micro-spectroscopy is used to identify particle compositions. The methodology shall be limited to particles with a dimension between 20 μm and 5 mm, and to fibres with length comprised between 20 μm and 15 mm. The methodology shall be used to determine the microplastics concentration expressed as the number of microplastics per cubic metre of water and concentrations of microplastics classified according to pre-determined size ranges, shape and composition categories.

(1) Samples shall be collected using filtration by passing water intended for human consumption through a cascade of four filters. The filters should be mounted in filter holders suitable for operating under positive pressure. The first filter, denominated (a), shall have a cut-off of 100 μm and the second filter, denominated (b), shall have a cut-off of 20 μm. The third filter, denominated (c), shall have a cut-off of 100 μm and the fourth filter denominated (d) shall have a cut-off of 20 μm. Filters (a) and (b) shall serve to collect the suspended matter from the water intended for human consumption. Filters (c) and (d) shall be used, where required, to produce procedural blanks to assess levels of microplastic contamination, in particular from laboratory equipment, reagents and surrounding atmosphere, occurring during the steps of sampling, treatment and analysis. To minimise atmospheric contamination of samples, the required volume of water should be piped directly from the sampling point through the filter cascade without the use of an intermediate collection or storage vessel. Intermediate collection/storage vessels may only be used when immediate, direct cascade filtration at the sampling point is impossible or impracticable, notably for technical or safety reasons.

(2) During all steps of collecting, treating, storing and analysing samples, all reasonable precautions shall be taken to avoid contamination of the samples with extraneous plastic particles from the surrounding environment, personal protective or laboratory equipment. All liquids used in sample processing shall be filtered (0.45 μm or less) prior to use.

Literature information on the concentrations of microplastics in European drinking water is limited, but the concentration is expected to be at a level which is lower or much lower than 1-10 particle/litre. Similarly, based on literature analysis, it is expected that the combined steps of sample collection, processing, manipulating and instrumental analysis have a high probability of introducing microplastics contamination with levels ranging from a minimum of a few tens of particles to several hundred particles per sample. To ensure that measurement data can reflect the actual microplastics concentrations in drinking water without being dominated by background contamination it is necessary to increase the ratio of true analyte to background contamination. In the case of drinking water this requires that sample volumes in excess of 500 L be used. The chosen value of 1000 L (**Box 2**) is in line with the value of 1500 L which is has been recommended by the ASTM standard D8332 (ASTM D8332-20 "Standard Practice for Collection of Water Samples with High, Medium, or Low Suspended Solids for Identification and Quantification of Microplastic Particles and Fibers")[22]. Such volumes may appear high compared to that of conventional chemical and biological monitoring of drinking water and consequently consideration was given to this cost when developing the methodology. The price of tap water in various cities in the EU depends strongly on country - in 2021, it was the highest in Norway and lowest in Italy - but in general was below 10 €/m.[3,23] Thus, the cost of collecting particles from 1 $m^3$ of sample (and the simultaneous collection of blanks) can be considered to be negligible compared to the costs associated with the staff, materials and instrumentation needed for each sample treatment and analysis.

The typical pressure of 5 bar in a domestic water supply system allows the sampling of such a volume in less than 2 hours - see **Figure 3** which shows flow rates and collection times achievable at a sampling point that mimics the conditions of a tap in a household. Throughput might be even higher at sampling points at the water source (point of compliance) if higher pressures are applied. It should be noted that the methodology was developed with the view that bottled water is not directly covered by the drinking water directive. For water, which is destined for distribution in bottled form, the methodology could be used at the point of compliance where the water supply enters the bottling plant but is not designed for the detection microplastics in water after the bottling process.

By employing a sampling device comprising four filters arranged in a cascade series, filter clogging is reduced, and the generation of sample blanks can be done without the need for additional water from the main supply or any another source of particle-free water. If necessary, the compact design of the filtration device and use of closed filter-housings allows the complete unit to be transferred directly to the analytical laboratory without any need to disassemble the closed filter-housings. Once in the laboratory, the filter housings may be opened under controlled conditions, *e.g.* under laminar flow hood, thus minimising the possible contamination of the sample or blank filters from the surrounding environment.

It is essential that the filters used as procedural blanks are the same type as the corresponding sample collection filters and should be treated and analysed in the same way, i.e. filters (a) and (c) must be treated identically and filters (b) and (d) must be treated identically. It should be noted that particles collected from filter (a) could be treated and analysed using different methods to filter (b). For example, transmission μFTIR microscopy may be effective for identifying particles in the 20-100 μm range while particles in the 100-5000 μm range can be too strongly absorbing and require analysis by Raman microscopy.

By collecting blanks with each sample, the analyst has the possibility to detect any unforeseen increase of background contamination by comparing sample blanks with the normal background level This level should be determined in advance by performing a statistically relevant number (at least 10) of procedural blank measurements – each done in the same laboratory, using the same sample preparation and measurement methods. It should be noted that the Methodology advises the analysis of filters (c) and (d) "where required", meaning that their collection should always be done, but it is not mandatory to undertake a complete analysis of each blank during a monitoring campaign.

**Box 2.** Methodology to measure microplastics in water intended for human consumption. Annex, section 2 Point 3-8

(3) A minimum volume of 1 000 (thousand) litres of water shall be sampled. The total volume of water passed through the filter cascade shall be measured and recorded.

(4) A sample analysis by vibrational micro-spectroscopy may be done directly on the original collection filters, if they are compatible with the analytical method used. Incompatibility of the original collection filter may be due to insufficient smoothness of the filter surface, interference from scattered signals from the filter, fluorescence or absorption of optical signals when used in transmission.

(5) If a sample analysis cannot be done directly on the collection filter, the particulate materials may be re-suspended in liquid and transferred to an alternative support for subsequent analyses. If necessary, density separation and/or chemical/enzymatic treatment measures may be applied to reduce the presence of non-plastic materials such as minerals, metal oxides and natural organic matter.

(6) Experimental verifications shall be performed to assess the recovery of material on each of filters (a) and (b) when applying the methodology as implemented by the user. This may be done by spiking the water flow into the filter cascade sample with a known quantity of clearly identifiable microplastics and verifying the quantity recovered following the analysis procedure. The spikes shall include particulates with sizes, densities and numbers appropriate for assessing recovery on filters (a) and (b). It is recommended to use spike particles in the size range from 120 to 200 μm to assess the recovery on filter (a). To assess recovery on filter (b) it is recommended to use particles in the size range from 30 μm to 70 μm. Recovery shall be assessed using particles of at least two of the priority polymers. The polymers used shall include at least one with higher density than water (e.g., PET) and at least one with lower density than water (e.g., PE). In each case, the number of spike particles shall be within the range of 50 to 150. The analysis procedure shall be considered acceptable if the recovery rate is within the range of 100 % to +/- 40 %.

(7) When material is transferred from collection filters (a) or (b) to an alternative analytical support (secondary filter or other appropriate surface) this shall preferably be done without sub-sampling. If the analytical procedure includes sub-sampling steps, then the final analysed sample shall represent at least 10 % of the material recovered from the original volume of water sampled. Analysis shall be done separately on materials collected on each of the filters (a) and (b).

(8) Filters (c) and (d) shall be used to produce procedural blanks. The procedural blank produced with filter (c) shall consist of a 100 μm filter and shall be subjected to the same processing and analysis steps as collection filter (a). The procedural blank produced with filter (d) shall consist of a 20 μm filter and shall be subjected to the same processing and analysis steps as collection filter (b). To quantify the typical levels of background contamination occurring during the performance of the analytical procedures, it is recommended to collect, process and analyse a minimum of ten procedural blanks of each filter type. These values shall be used to calculate the mean (μ) and standard deviation (σ) of the background microplastic contamination. Subsequently, further procedural blanks shall be collected periodically and analysed to monitor variations in the level of background contamination. If any periodic blank exceeds the mean background contamination (μ) by more than three times the standard deviation (σ) then the laboratory shall investigate the source of the increased contamination and take measures to reduce it.

It is often not possible to analyse particles directly on the filter used for collection, as the filter's properties can interfere optically with particle observation, and the high concentration of other particles captured from the sample can also hinder analysis. Nevertheless, the methodology does not rule out this possibility and thus this option remains open to potential future advancements in the field.

The recovery of microplastics can be impacted by various factors, including sample treatment, particle transfer to a different analysis filter, and sub-sampling. Since the treatment and analysis procedures may differ for the two size fractions collected, it is essential to evaluate recovery for both size ranges (20-100 µm and above 100 µm). Additionally, density of the polymers can significantly influence recovery, so preliminary validation studies should be conducted using plastic particles with densities both higher and lower than that of water.

When analysing particles collected on filters it would be preferable to avoid introducing possible sub-sampling bias by analysing particles over the entire surface of the filter, but this may result in very long measurement times. To counter this a number of valid strategies have been described in the literature to scan partial filter areas in order to reduce data collection time and increase throughput[14] without compromising the data. These approaches are particularly useful for techniques that involve measurements at single particle positions, such as Raman spectroscopy-based analysis. The Methodology allows for this type of 'sub-sampling' strategy, provided that it is performed to ensure sample representativeness. To generate final concentration values valid for the original sample, the fraction of the total filter area considered must be taken into account, as specified in point (16) of the delegated act Annex. As already expressed in this report, vibrational micro-spectroscopy techniques were found to be the more realistic analytical solutions because of the low detection levels that can be reached. These techniques include Fourier transformation IR spectroscopy and Raman spectroscopy since both are reported to perform in a reliable way above 20 µm of particle size.[24,25] Vibrational micro-spectroscopy instruments and their software are part of a rapidly developing field and the Methodology does not specify further instrument details so as to leave open the possibility for incremental improvements of existing technology as well as the adoption of new, innovative alternatives.

**Table 1.** Priority polymers to be reported, their application field(s) and yearly production

| Polymer | Acronym | Common applications | European demand* Mt/year |
|---|---|---|---|
| Polyethylene | PE | Packaging, agriculture, building and construction | 14.8 |
| Polypropylene | PP | Packaging, automotive, construction, household | 10 |
| Polyethylene terephthalate | PET | Packaging, household | 4 |
| Polystyrene | PS | Packaging, building and construction | 3.1 |
| Polyvinylchloride | PVC | Building and construction, packaging, agriculture | 5.2 |
| Polyamide | PA | Automotive, electrical and electronics, others | 0.9 |
| Polyurethane | PU | Building and construction, automotive, others | 4.1 |
| Polymethylmethacrylate | PMMA | Glass substitute, architectural, medical and dental uses | 0.2 |
| Polytetrafluoroethylene | PTFE | Construction, aircraft, household | <0.1 |
| Polycarbonate | PC | Building and construction, Automotive, electrical, electronics | 0.8 |

* Plastic converters demand in Europe in 2021

*Source: Plastics Europe, Plastics, the facts 2022, 22 October 2022*

**Box 3.** Methodology to measure microplastics in water intended for human consumption. Annex, section 2 Point 9-11

(9) Prior to undertaking analyses by vibrational spectroscopy, optical microscopy or chemical mapping shall be used to measure or estimate the number of generic particles (≥ 20 μm) on the full filter or sample support. Where the total number of generic particles on the filter is too high to measure in a practical time, the operator may limit the analysis to one or more smaller sub-areas of the filter: the selection of the area shall follow appropriate subsampling strategies which maintain a representative sample. The sub-sampling shall cover at least 20 % of the area of the sample support or filter. Where sub-areas of the filter are used, the operator shall analyse all particles and fibres in the size range ≥ 20 μm.

(10) The compositional analysis of microplastic particles and fibres shall be carried out using vibrational spectroscopy methods such as μ-FTIR, μ-Raman or equivalent variations such as QCL-IR. The instruments shall be capable of acquiring IR/Raman spectra from particles within the size range of 20 μm or less. Optical images or chemical maps shall be used to determine the size of microplastic particles and fibres. Optical images shall be acquired using an objective of at least 4x magnification. The particle size classification shall be based on area-equivalent diameter whenever this option is available to the instrument operator. Alternative measures of diameter shall be used only if this option is not available. The type of alternative diameter shall be reported.

(11) The identification of particles and fibres from acquired spectra shall be carried out by comparison with spectra of known materials contained in a spectral library. The spectral library used for identification shall contain examples of all the priority polymers and shall in addition contain examples of proteins and minerals and natural polymers such as cellulose that might commonly be present in water intended for human consumption.

Vibrational micro-spectroscopy techniques all rely on the use of spectral databases for material identification and the methodology is intentionally flexible regarding the origin of the database. However, it does oblige that the database contains, as a minimum, the spectra of the indicated priority polymers. These were selected based on their usage in the EU and the frequency that the scientific literature reports their presence in the environment (**Table 1**). Given the similarity of some of these polymers to natural organic compounds, and to minimize the likelihood of false positives, the Methodology also requires the inclusion in the databases of spectra that facilitate the identification of non-polymeric materials, such as natural organic compounds, proteins, and minerals. The methodology does not cover tyre wear (rubber) particles as these particles cannot be identified by micro-spectroscopy methods.

Information about procedural blanks is recorded as part of the report, but sample data should not be corrected with blanks, *i.e.* number concentrations determined for blanks should not be subtracted from sample measurement results. At this time, both scientific and standardisation communities have yet to agree on what is the most reliable or relevant way to utilize data from background blanks. Consequently, it is best to future-proof the data by ensuring that blank data and uncorrected sample data both remain available in the report.

Reporting of identified microplastics in predetermined size classes and as a concentration on a particle/m$^3$ basis allows easier intercomparison of monitoring between laboratories. The size categories according to the point (16) of Definitions are collected in the table below.

**Table 2.** Size categories for reporting of particle size

| Size category | Area equivalent diameter (D, µm) |
|---|---|
| 1 | 20 µm ≤ D <50 µm |
| 2 | 50 µm ≤ D <100 µm |
| 3 | 100 µm ≤ D <300 µm |
| 4 | 300 µm ≤ D <1000 µm |
| 5 | 1000 µm ≤ D <5000 µm. |

*Source: own production*

It should be noted that Area Equivalent diameter (D) has been chosen as the mandatory descriptor for reporting particle size. A number of other alternative size descriptors exist (e.g. Feret min) but Area Equivalent diameter (D) was chosen on a pragmatic basis as it is most commonly available descriptor in commercial instrument software solutions that support automatised analysis as described in section 3.5.3 and shown in **Table 5**.

Microplastics are very heterogeneous, not only regarding widely variable dimensions, and compositions but also shape which can be near spherical particles, irregular fragments, films and fibres. In the case of fibres, measuring their length is not straightforward and very difficult to automatise, and consequently fibre concentrations and compositions are reported but without the complication of also reporting length/width.
The classification workflow for data reporting is shown in **Figure 2**.

Finally, a report should contain also metadata as requested in point (17) of the delegated act Annex for better interpretation of future monitoring data regarding geological locations and sampling dates.

**Box 4.** Methodology to measure microplastics in water intended for human consumption. Annex, section 2 Point 12-18

(12) Where automated identification procedures are used, an experimental verification shall be performed to assess the appropriate positive acceptance criteria for spectrum matching. The verification shall consider the specific features of the applied instrumentation, spectral library and identification strategy. This may be done by using pure polymer microparticles, but the evaluation has to cover the relevant size ranges to be retained by the sampling filters, notably, (a) > 100 µm and (b) 20-100 µm. Once the minimum quality level applied for positive spectral identification has been established, that level shall remain fixed for the protocol applied by the analytical laboratory.

(13) Data shall be recorded separately from the materials collected on each of the two collection filters (100 µm and 20 µm cut-off). Where procedural blank samples are collected data shall be recorded separately from the materials collected on each of the blank filters (20 µm or 100 µm cut-off).

(14) Measurement requirements: the filter or sub-area of the filter shall be analysed so as to examine all microplastic particles and fibres as defined in the size ranges detailed in section 1, points (3) and (4).

(15) Data acquired on microplastic particles and fibres shall be elaborated to categorise each object on the basis of its size, number, shape and composition as follows: (a) shape: particle or fibre according to the definitions in section 1, points (3) and (4) (b) size (if particle): the size category listed in section 1, point (16); (c) composition (if particle): identified as a priority polymer as defined under Section 1, point (14) or identified as a non-priority polymer under section 1, point (15)(ii) or identified as other material under section 1, point (15)(iii); (d) polymer type (if fibre): where fibre dimensions and instrument capabilities allow for a positive identification of polymer type, this shall be identified in accordance with the categories defined in section 1, points (14) and (15) otherwise it shall be indicated as unidentified fibre.

(16) If the analysis of the materials on the filters or sample support does not address all collected particulates (e.g., due to sub-sampling) in the relevant size range, the data shall be appropriately scaled so as to correctly represent the concentration of microplastics in the original sample of water intended for human consumption. The content of microplastics in water intended for human consumption shall be expressed as the number of microplastic particles or fibres per cubic metre.

(17) Users of this methodology shall ensure that all of the following additional information is recorded in relation to each sample collected and measured:

(a) total volume of water sampled;

(b) location and time of sampling and sample analysis;

(c) sample treatment details;

(d) spectroscopic method and instrument applied;

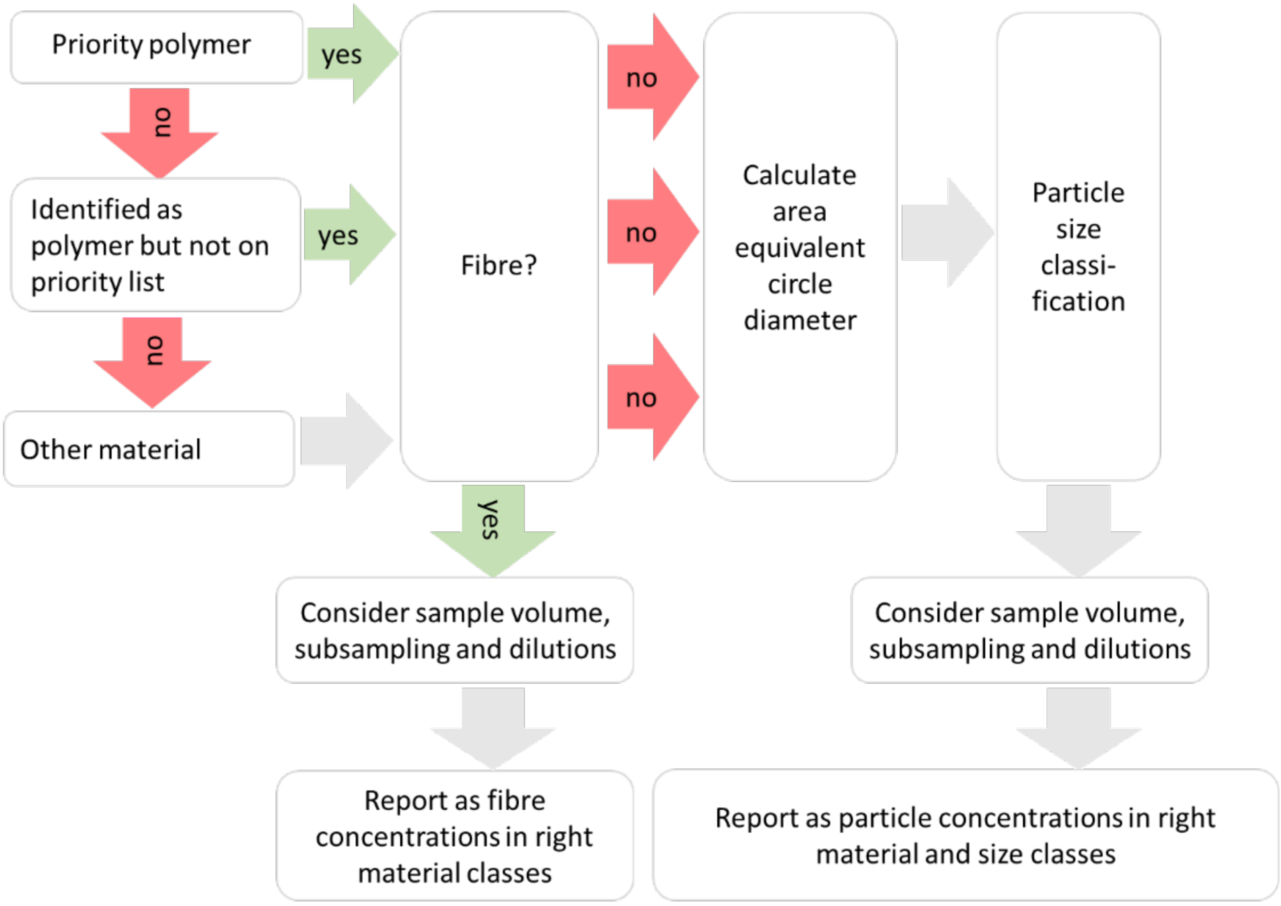
(e) details of any sub-sampling during analysis or sample preparation;

(f) chemical nature of any plastic component(s) in sampling device or in equipment used during sample preparation;

(g) any deviation from the methodology including justification.

(18) When using this methodology, standard laboratory and environmental safety rules shall apply.

**Figure 2.** Classification workflow for data reporting



*Source: own production*

# 3 The Methodology in practice

## 3.1 Sampling

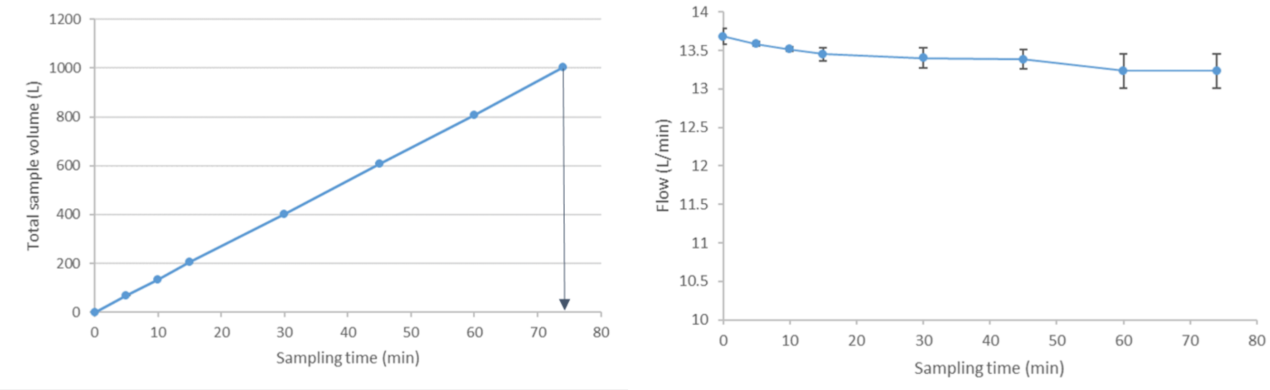
The specifications for the sampling of the drinking water are a key part of the Methodology because sampling (volume, size fractions, generation of blanks) and sample pre-treatment steps have a strong effect both on the analytical procedure that can be applied and on data reporting. The typical source of drinking water in the EU is tap water, *i.e.* pressurised water, thus the methodology uses in-line filtration to collect particles. In specific cases, where the drinking water cannot be sampled from a pressurised system, a pump could be applied to pass the water through the filter cascade.

The sampling procedure foresees sequential filtration by passing pressurised water through a cascade of four filters.

Size cut-off values were selected considering the current capabilities of analytical instrumentation and to limit the amount of captured particles that are smaller than the size range to be analysed. Between 20 and 100 µm particle size, the suggested vibrational micro-spectroscopy methods show reliable performance. Above 100 µm size, transmission Fourier-Transform Infrared (FTIR) measurements become less appropriate, as the low transmission through thick particles may result in truncated spectra. The collection of different size fractionations (collection filters (a) and (b)) permits the operator to pre-treat and analyse the two different fractions using different protocols or even different analytical solutions (*e.g.* FTIR microscopy in transmission and Attenuated Total Reflectance (ATR) mode or µ-Raman). The additional filters (c) and (d) serve as blank filters which can be subjected to the same processing steps as the respective collection filters permitting the background contamination levels to be assessed.

Annex I of the present report describes the sampling procedure tested in the JRC Nanobiotechnology Laboratory. It uses commercially available, stainless steel filter holders that are compatible with the positive pressure (~5 bar) typical in domestic water supply systems. The utilised stainless steel mesh filters were proven to be compatible with the filter holders and were re-usable after cleaning, but – as suggested by expert comments - special care should be dedicated to their quality control as bending or harsh chemical treatment might affect their pore-size.

**Figure 3.** Total filtered volume (left) and typical flow rates (right) obtained by using the suggested cascade filtration with 100 µm – 20 µm - 100 µm - 20 µm filters in 47 mm filter housings. Average of three measurements.



*Source: own production*

Our sampling procedure foresees the removal of the collected particles from the filters and the subsequent treatment of the concentrated suspension before analysis.

Tests to evaluate sampling times were performed in the autumn period of 2023, attaching the filtration apparatus to the water supply system of JRC. **Figure 3** shows that particles from 1000 L of water can be collected by using the suggested filter cascade without clogging the filters and in a reasonable time (about 75 min). The absence of significant clogging was demonstrated by the flow rate which remained quite stable (average of 13.5 L/min) during the entire sampling period. In this case the filter units used were designed to hold 47 mm diameter filters which were suitable for the quality of water available in JRC. In the unlikely case of filter clogging due to higher levels of suspended solids, commercial filter holders of 90 mm diameter could be used.

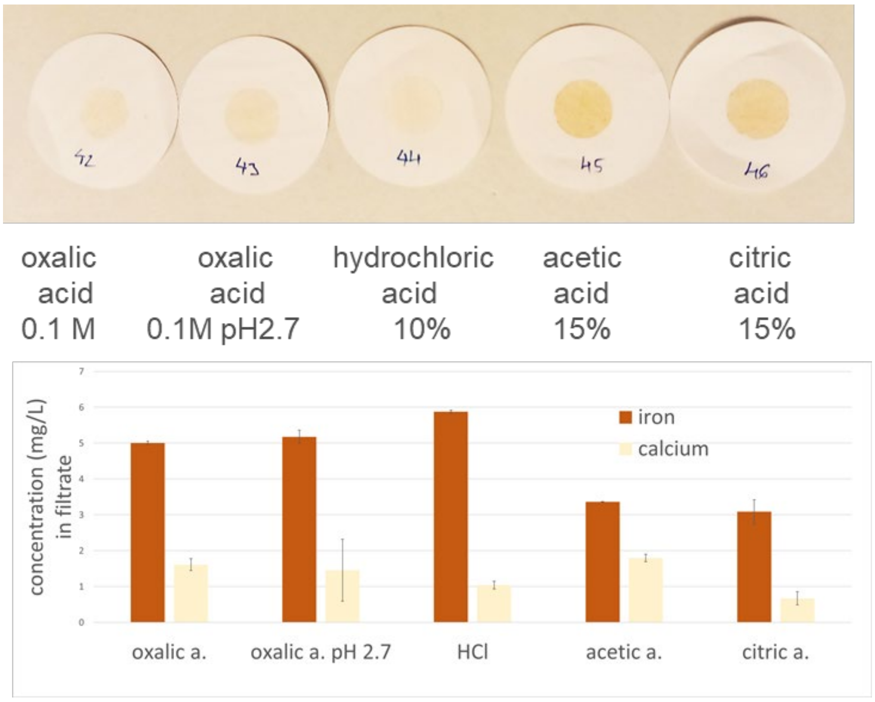
## 3.2 Sample treatment

Micro-spectroscopic analysis of microplastics can be compromised if the sample supports (filters) are overloaded with other, non-plastic particles. Too high number of objects to be analysed might result in unrealistically long measurement times, particles overlaid or coated by other materials might not be spectroscopically identifiable while the formation of aggregates might bias size measurements. In such cases it may be necessary to adopt physical or chemical measures to reduce these problems.

Water intended for human consumption usually presents low concentration of organic contaminants but may contain a high concentration of minerals. This was the case for the drinking water tested at the JRC sampling point. The water distributed at the JRC is produced from lake water (Lago Maggiore, 40 m depth) after sand-bed filtration and subsequent oxidative treatment to neutralise microbiological contamination. Our samples contained large amounts of inorganic particles including iron-oxides and hydroxides, calcium and magnesium salts, and – in our specific case - diatom algae. The relatively high concentration of suspended particles and the filter morphology (woven stainless steel mesh filters) did not allow for the direct analysis of particles on the collection filters. However, it cannot be excluded that drinking waters from underground sources containing lower amounts of suspended solids could allow the use of pore filters instead of woven meshes and these would allow direct analysis on the collection filters (for example by Raman micro-spectroscopy). Direct analysis on the collection filters would also decrease the risk of particle loss during transfer onto a secondary analysis filter. Consequently, the suggested methodology described in this document does not exclude direct analysis on collection filters.

Various sample pre-treatment steps can be applied to decrease the amount of contaminant particles in the concentrated samples. Annex II of the present report describes the sample preparation steps optimised in the JRC Nanobiotechnology Laboratory. In our case the first step was the removal of minerals (by acid treatment), followed by a density separation procedure. Finally, the resulting particles are collected on a secondary filter that is compatible with the analysis method. Efficiency of various acid treatments tested in our laboratory is illustrated in **Figure 4.**

**Figure 4.** Filtration residues on 0.1 µm cut-off filters (top) and iron and calcium concentration of filtrates (bottom) after sample treatment with various acids (aliquots from the same sample, corresponding to 200 L of sampled water). Concentrations in filtrate refer to concentrated samples, measurements were done using a Bruker S4 T-star Total Reflection X-ray Fluorescence (TXRF) instrument).



*Source: own production*

Typical solutions applied in density separation are saturated salt solutions in the 1.2-1.8 kg/L range such as sodium chloride (1.15-1.3 kg/L), zinc chloride (1.5-1.8 kg/L), sodium iodide (1.55-1.8 kg/L) and sodium or lithium metatungstate (1.4 and 1.6 g/mL, respectively).[26] Among the solutions of inorganic salts, sodium chloride is the cheapest, and most environmentally friendly, but has also the lowest density, thus excluding the separation of high density polymers (such as PTFE, PVC or PET). Our choice (*i.e.* $ZnCl_2$) has higher cost and needs more careful handling. However, the solutions can be recycled by filtration after use, and their density can be set back to the starting value (*i.e.* 1.7 kg/L) by adding small amounts of additional salt.

## 3.3 Recovery and spiking

To the best of our knowledge, there is no single, simple filtration device available on the market that would be specific for sampling drinking water as required for the DWD. It is therefore likely that laboratories will adopt in-house constructed devices, and these will be have to be tested regarding their performance in capturing particles in the requested size range. Moreover, microplastic particles are prone to be adsorbed on vial/pipette surfaces, or sediment or float in water depending on their density, thus they are rarely homogenously distributed in a water-based suspension. Sub-sampling of the concentrated sample might result in increased uncertainty of the results. Additionally, sample pre-treatment steps might lead to particle loss during the process. Therefore, the recovery of particles in the applied sampling and possible sub-sampling procedure has to be carefully evaluated.

Particles that have higher density than water behave differently during the sampling and sample pre-treatment process from particles that float because of their low density. Thus, it is recommended to perform the recovery evaluation with both types of materials (*i.e.* at least one material with lower density and one material with higher density than water).

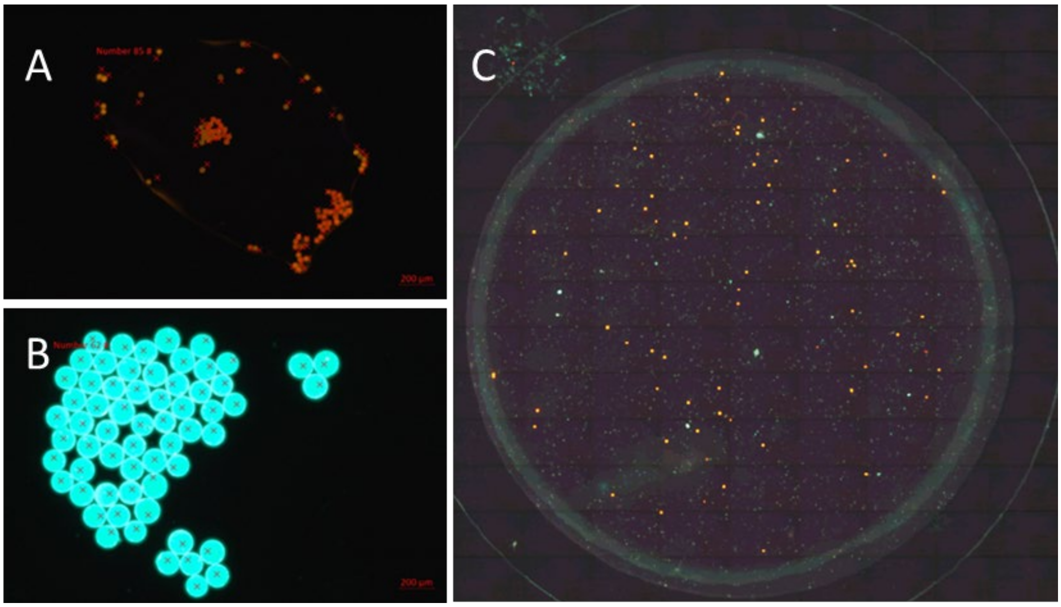
**Table 3.** Bulk density of the polymers included in the priority list

| Polymer | Acronym | Bulk density (g/cm$^3$) |
|---|---|---|
| Polyethylene (low density) | PE | 0.84-0.94[27–29] |
| Polyethylene (high density) | PE | 0.94-0.98[30–32] |
| Polypropylene | PP | 0.83-0.92[28–30] |
| Polyethylene Terephthalate | PET | 1.29-1.45[27–29] |
| Polystyrene | PS | 1.04-1.11[29,31] |
| Polyvinylchloride | PVC | 1.16-1.58[27,28,31] |
| Polyamide | PA | 1.02-1.16[28,32] |
| Polyurethane | PU | 1.17-1.28[27] |
| Polymethylmethacrylate | PMMA | 1.09-1.20[27,29,32] |
| Polytetrafluoroethylene | PTFE | 2.10-2.30[29,32] |
| Polycarbonate | PC | 1.20-1.22[29] |

*Source: own production*

Particle numbers in the spike sample have to be high enough to make statistical evaluation reliable, but not unrealistically high – considering reported microplastic number concentrations in drinking water in Europe. According to our knowledge, reference material development is currently ongoing, but none with the needed number and size is commercially available at the moment.

**Figure 4.** A) in house labelled, Nile Red tagged 30 μm PMMA, B) commercial 120 μm PS fluorescent particles on a potassium bromide (KBr) tablet (red crosses show event count), C) spike particles collected on a 25 mm aluminium oxide filter together with other particles from drinking water.



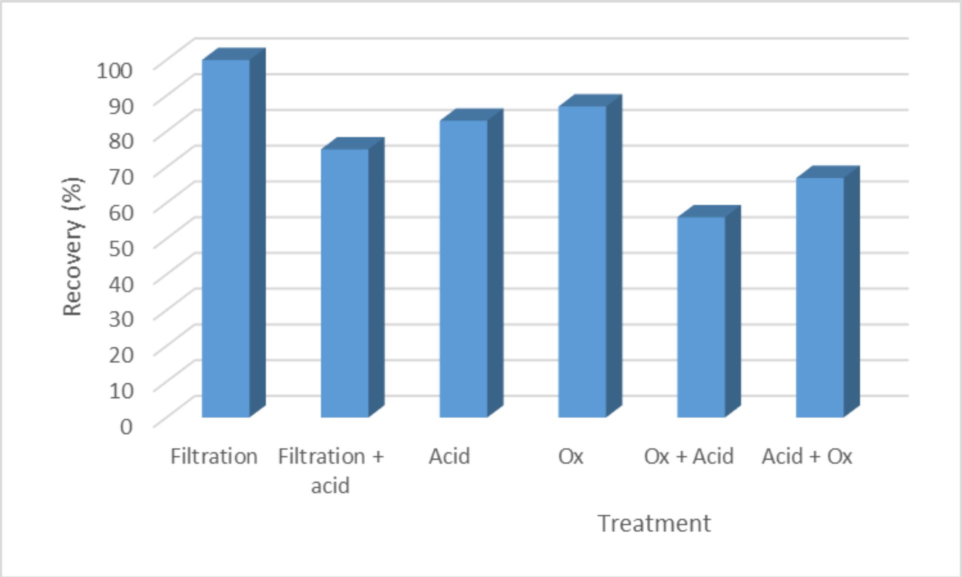
*Source: own production*

Annex III of the present report describes the spiking procedure that we applied at the evaluation of our sampling procedure. In an ideal situation the recovery evaluation would be done with irregular shaped particles mimicking the case of secondary microplastics and modelling better their adsorption behaviour on surfaces. On the other hand, it is extremely difficult to generate/find a reference material with irregular shaped particles and narrow particle size distribution. Moreover, counting of irregular shaped particles becomes difficult or often compromised by agglomerates formed on filter surfaces. Spherical particles (or particles with regular shape) are much easier to quantify as particle boundaries are well defined. Fluorescent labelling might also make the recovery evaluation easier by facilitating spike particle recognition in the haystack of other particles. Using fluorescent labelled spheres also speeds up the recovery studies as it decreases the readout time in the experiment from hours to minutes.

We characterised the recovery of the various (potential) steps of sample preparation by spiking samples with Nile Red tagged 30 µm PMMA particles. During the evaluation of the recovery at the filtration step, the fluorescent labelled particles were deposited on a water soluble KBr support, counted using a fluorescent microscope and placed on the first 100 µm cut-off filter (filter (a)) of the filtration device. After filtration, the spiking PMMA particles (together with all other particles obtained from the drinking water) were redispersed from filter (b) following the steps described in Annex I of the present report. When evaluating recovery of the individual sample treatment steps, the water-soluble support with the spike particles was directly placed in the beaker containing the concentrated suspension of particles obtained from drinking water on filter (b) in a solution of 10% hydrochloric acid or in a solution applied for mild oxidative digestion of cellulose. Following the given treatment step(s), particles were collected on a 25 mm, 0.1 micron cut-off aluminium oxide filter and counted by using a fluorescent microscope.

**Figure 5** illustrates that the recovery is close to 100% in the filtration process. When filtration was combined with subsequent acid treatment, the recovery was found to be lower (75%). Individual treatment steps (without filtration) provided 83-87% recovery while their combination (two subsequent treatment steps in various order) resulted in recovery values of 56-67%. These data suggest that additional sample preparation steps might lead to reduced recovery. Particles might be "lost" because of adsorption on various surfaces: on filters, on the walls of glass vials or removed from filter surface(s) by air flow or electrostatic forces. Imperfect closure of the filtration device might also lead to sample (particle) loss between the filtration funnel and the filter. As a conclusion, it is recommended to minimise the number of sample preparation steps and to find the appropriate (sample specific) balance between sample clean-up supporting the analysis step and acceptable recovery.

**Figure 5.** Recovery values determined for the various steps of sample preparation using Nile Red tagged 30 µm PMMA particles for spiking. (Acid: treatment with 10% HCl solution, Ox: mild oxidative treatment (NaOCl and TEMPO catalyst))



*Source: own production*

## 3.4 Analysis procedures

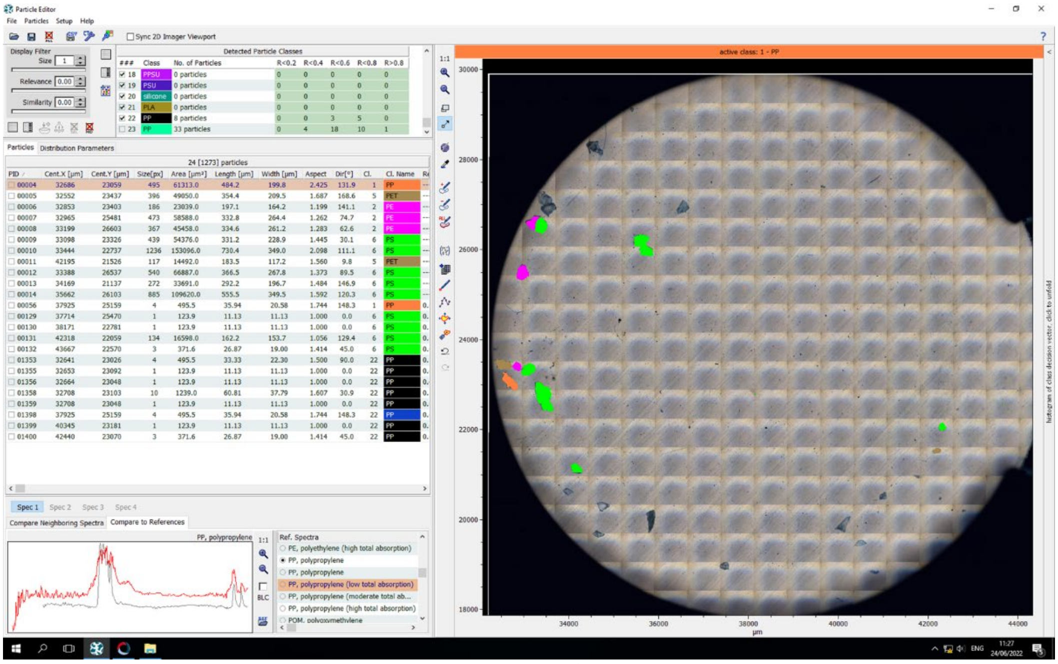
We tested two out of the three commercially available micro-spectroscopy based techniques (μ-FTIR and μ-Raman) in the JRC Nanobiotechnology Laboratory and collected information about QCL-IR through literature review and discussion with instrument manufacturer. We have verified the applicability of the tested methods regarding the particle size range and generated in-house procedures for spectrum acquisition and data analysis. These procedures are instrument and software specific. Accordingly, any method transfer to another laboratory should consider the differences in instrumentation. However, lessons learned can still be shared. Because of the high number of generic particles in the sample, the use of automatised image and spectrum collection is necessary if a sufficiently high throughput is to be reached. The instrument used for the analysis has to have a motorised stage and a software that is able to perform the comparison of large number of spectra with a spectral database.

As mentioned above, our procedure foresees the transfer of the particles to an optically more adequate substrate, *i.e.* a secondary filter. We applied aluminium oxide filters that are partially transparent for IR light and additionally do not disturb Raman analysis. These filters are also available in small diameter (13 and 25 mm) thus allowing the optical imaging and scanning of a full filter area by image stitching. These filters (and most available filters in general) are round shaped. Regardless of this, all tested instrument software collects rectangular shaped images. For full filter analysis it means acquisition and storage of a large amount of data (21% of total) in the four corners which has no analytical value (see for example the black corner areas in **Figure 6**). Ideally, it would be desirable to have the option to collect data only from selected area of the filters but unfortunately the current generation of chemical mapping software has no option to specify user defined areas and consequently a strategy such as the circle-sector shape strategy[14] is not a viable solution. Instead, multiple rectangular shaped areas following a spiral pattern can be collected - keeping in mind that the automatized acquisition and analysis for separate rectangles is not part of any of the evaluated software solutions. Thus, the more efficient and unbiased way according to our experience is still the analysis of full filters.

### 3.4.1 FTIR microscopy-based analysis and chemical mapping example procedures

Real world microplastic materials often contain colorants and other additives that might be fluorescent under specific conditions. μ-FTIR has the clear advantage that it is not disturbed by fluorescence. It can work in ATR, reflection and transmission mode. Micro-ATR is suitable for the analysis of few, larger objects, but less convenient for small particle analysis: the physical contact between the instrument and the small objects can be difficult and might result in contamination of the ATR crystal and vice-versa. Using the instrument in reflection mode is suboptimal for particles in the tens to hundreds of micron size range because of the behaviour of light resulting in multiple reflection and interference in transparent materials with curved surfaces.

**Figure 6.** Screenshot from µ-FTIR chemical mapping analysis software. Right: optical image of a 13 mm filter collected by image stitching overlaid by chemical mapping results in the central area. Left: Data reporting including comparison with reference spectrum (bottom left)



Detected Particle Classes

| ### | Class | No. of Particles | R<0.2 | R<0.4 | R<0.6 | R<0.8 | R>0.8 |
|---|---|---|---|---|---|---|---|
| ☑ 18 | PPSU | 0 particles | 0 | 0 | 0 | 0 | 0 |
| ☑ 19 | PSU | 0 particles | 0 | 0 | 0 | 0 | 0 |
| ☑ 20 | silicone | 0 particles | 0 | 0 | 0 | 0 | 0 |
| ☑ 21 | PLA | 0 particles | 0 | 0 | 0 | 0 | 0 |
| ☑ 22 | PP | 8 particles | 0 | 0 | 3 | 5 | 0 |
| ☐ 23 | PP | 33 particles | 0 | 4 | 18 | 10 | 1 |

24 [1273] particles

| PID | Cent.X [µm] | Cent.Y [µm] | Size[px] | Area [µm²] | Length [µm] | Width [µm] | Aspect | Dir[°] | Cl. | Cl. Name | Re |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ☐ 00004 | 32686 | 23059 | 495 | 61313.0 | 484.2 | 199.8 | 2.425 | 131.9 | 1 | PP | --- |
| ☐ 00005 | 32552 | 23437 | 396 | 49050.0 | 354.4 | 209.5 | 1.687 | 168.6 | 5 | PET | --- |
| ☐ 00006 | 32853 | 23403 | 186 | 23039.0 | 197.1 | 164.2 | 1.199 | 141.1 | 2 | PE | --- |
| ☐ 00007 | 32965 | 25481 | 473 | 58588.0 | 332.8 | 264.4 | 1.262 | 74.7 | 2 | PE | --- |
| ☐ 00008 | 33199 | 26603 | 367 | 45458.0 | 334.6 | 261.2 | 1.283 | 62.6 | 2 | PE | --- |
| ☐ 00009 | 33098 | 23326 | 439 | 54376.0 | 331.2 | 228.9 | 1.445 | 30.1 | 6 | PS | --- |
| ☐ 00010 | 33444 | 22737 | 1236 | 153096.0 | 730.4 | 349.0 | 2.098 | 111.1 | 6 | PS | --- |
| ☐ 00011 | 42195 | 21526 | 117 | 14492.0 | 183.5 | 117.2 | 1.560 | 9.8 | 5 | PET | --- |
| ☐ 00012 | 33388 | 26537 | 540 | 66887.0 | 366.5 | 267.8 | 1.373 | 89.5 | 6 | PS | --- |
| ☐ 00013 | 34169 | 21137 | 272 | 33691.0 | 292.2 | 196.7 | 1.484 | 146.9 | 6 | PS | --- |
| ☐ 00014 | 35662 | 26103 | 885 | 109620.0 | 555.5 | 349.5 | 1.592 | 120.3 | 6 | PS | --- |
| ☐ 00056 | 37925 | 25159 | 4 | 495.5 | 35.94 | 20.58 | 1.744 | 148.3 | 1 | PP | 0. |
| ☐ 00129 | 37714 | 25470 | 1 | 123.9 | 11.13 | 11.13 | 1.000 | 0.0 | 6 | PS | 0. |
| ☐ 00130 | 38171 | 22781 | 1 | 123.9 | 11.13 | 11.13 | 1.000 | 0.0 | 6 | PS | 0. |
| ☐ 00131 | 42318 | 22059 | 134 | 16598.0 | 162.2 | 153.7 | 1.056 | 129.4 | 6 | PS | 0. |
| ☐ 00132 | 43667 | 22570 | 3 | 371.6 | 26.87 | 19.00 | 1.414 | 45.0 | 6 | PS | 0. |
| ☐ 01353 | 32641 | 23026 | 4 | 495.5 | 33.33 | 22.30 | 1.500 | 90.0 | 22 | PP | 0. |
| ☐ 01355 | 32653 | 23092 | 1 | 123.9 | 11.13 | 11.13 | 1.000 | 0.0 | 22 | PP | 0. |
| ☐ 01356 | 32664 | 23048 | 1 | 123.9 | 11.13 | 11.13 | 1.000 | 0.0 | 22 | PP | 0. |
| ☐ 01358 | 32708 | 23103 | 10 | 1239.0 | 60.81 | 37.79 | 1.607 | 30.9 | 22 | PP | 0. |
| ☐ 01359 | 32708 | 23048 | 1 | 123.9 | 11.13 | 11.13 | 1.000 | 0.0 | 22 | PP | 0. |
| ☐ 01398 | 37925 | 25159 | 4 | 495.5 | 35.94 | 20.58 | 1.744 | 148.3 | 22 | PP | 0. |
| ☐ 01399 | 40345 | 23181 | 1 | 123.9 | 11.13 | 11.13 | 1.000 | 0.0 | 22 | PP | 0. |
| ☐ 01400 | 42440 | 23070 | 3 | 371.6 | 26.87 | 19.00 | 1.414 | 45.0 | 22 | PP | 0. |

Ref. Spectra
○ PE, polyethylene (high total absorption)
● PP, polypropylene
○ PP, polypropylene
○ PP, polypropylene (low total absorption)
○ PP, polypropylene (moderate total ab...
○ PP, polypropylene (high total absorption)
○ POM, polyoxymethylene

*Source: own production*

On the other hand, in the 20-100 micron range FTIR performs very well in transmission mode. Combined with a focal plane array detector it can produce chemical map of a full (13 mm) filter or partial filter area in a reasonable time (1-3 sample/day as a function of the focal plane array dimension).

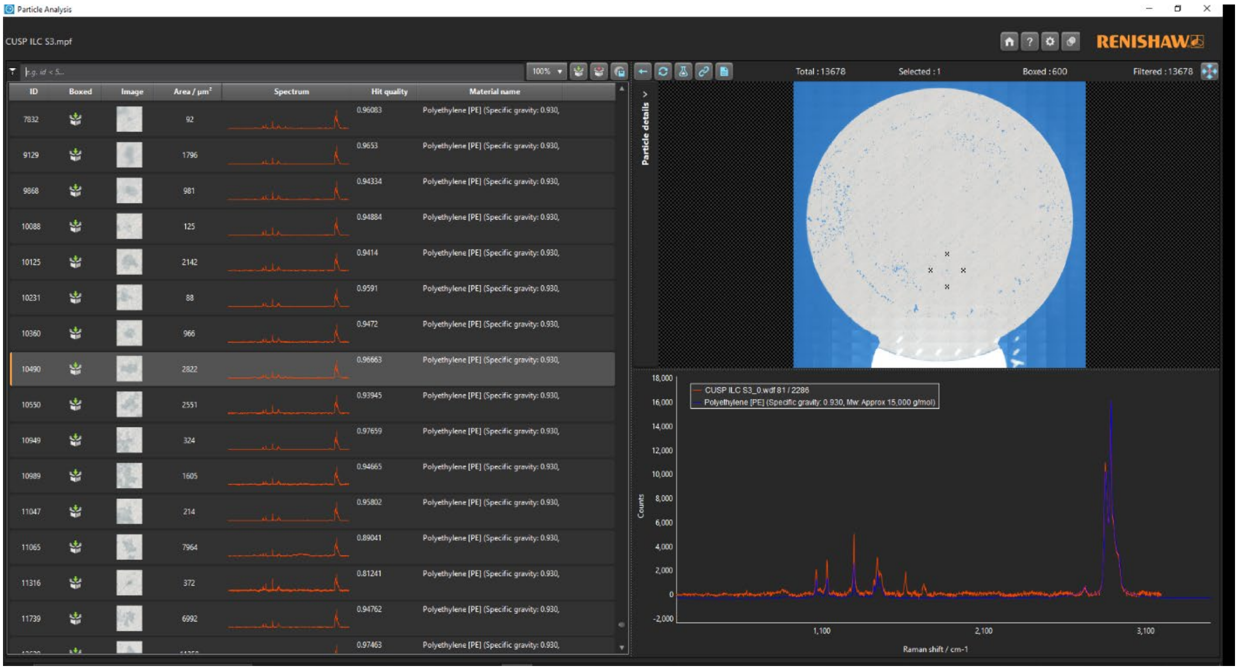
Annex IV or the present report contains our transmission FTIR based procedure for the analysis of the particles in the 20-100 micron size range using an FTIR microscope with focal plane array (FPA) detector, the manufacturer's software for data collection and a commercial software for data analysis (see also **Figure 6**).

Chemical mapping has additional benefits. Firstly, the spectrum collection time does not depend on the number of particles on the substrate, only on the analysed filter area. In addition, more spectra are collected for each particle which means that material identification is not based on a single spectrum, but on a series of spectra belonging to the same particle. Moreover, spectra are collected from various positions within an object that can help to find better quality spectra for identification of large particles, where transmission spectra are often truncated because of too high thickness at some areas but can have much better quality close to the edges where particles are less thick. Finally, binning of pixels (*i.e.* averaging spectra collected by adjacent pixels of the FPA) allows the number of scans to be decreased while still keeping the total number of averaged spectra to improve signal to noise ratio.  On the other hand, chemical mapping needs serious computational power and more data analysis time than the analysis of single spectra from point-by-point measurements.

### 3.4.2 Raman microscopy-based analysis and point by point measurement

Raman microscopy-based identification of particles analyses scattered light and has the clear advantage that, under optimal conditions, it can cover the full-size range (20-5000 µm) defined by the methodology. No separate instrumentation or setup is needed to analyse the particles collected on the 20 µm and the 100 µm filters. On the other hand, µ-Raman have much lower throughput in imaging than an µ-FTIR with FPA detector, as chemical images are collected through point-by-point measurements. As a result, the practical approach to analyse many particles on a filter surface is particle-by-particle measurements after optical image analysis using automatized procedures. This approach has the major drawback that only one spectrum per particle is obtained and a proper optimisation is not always possible, since measurement position is automatically determined on the basis of the acquired optical image and the method setup (**Figure 7**).

**Figure 7.** Screenshot from Raman particle analysis software. Right: Image of a 13 mm filter collected by image stitching and overlaid by binarised image generated for particle recognition. Left: Individual particle images with corresponding size data, processed spectra and results of identification



CUSP ILC S3.mpf

Total : 13678
Selected : 1
Boxed : 600
Filtered : 13678

| ID | Boxed | Image | Area / µm$^2$ | Spectrum | Hit quality | Material name |
|---|---|---|---|---|---|---|
| 7832 | | | 92 | | 0.96083 | Polyethylene [PE] (Specific gravity: 0.930, |
| 9129 | | | 1796 | | 0.9653 | Polyethylene [PE] (Specific gravity: 0.930, |
| 9868 | | | 981 | | 0.94334 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10088 | | | 125 | | 0.94884 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10125 | | | 2142 | | 0.9414 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10231 | | | 88 | | 0.9591 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10360 | | | 966 | | 0.9472 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10490 | | | 2822 | | 0.96663 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10550 | | | 2551 | | 0.93945 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10949 | | | 324 | | 0.97659 | Polyethylene [PE] (Specific gravity: 0.930, |
| 10989 | | | 1605 | | 0.94665 | Polyethylene [PE] (Specific gravity: 0.930, |
| 11047 | | | 214 | | 0.95802 | Polyethylene [PE] (Specific gravity: 0.930, |
| 11065 | | | 7964 | | 0.89041 | Polyethylene [PE] (Specific gravity: 0.930, |
| 11316 | | | 372 | | 0.81241 | Polyethylene [PE] (Specific gravity: 0.930, |
| 11739 | | | 6992 | | 0.94762 | Polyethylene [PE] (Specific gravity: 0.930, |
| | | | | | 0.97463 | Polyethylene [PE] (Specific gravity: 0.930, |

*Source: own production*

Annex V of the current report contains our Raman microscopy-based procedure for the analysis of microparticles. Our procedure relies on the use of automatic optical image analysis, spectrum collection and software evaluation provided by the instrument manufacturer and specifically developed for particle analysis.

Our procedure profits from the specific feature of the instrument (LiveTrack) that allows to automatically optimise the zeta (height) position of the motorized stage (and thus the focal plane) for each single particle. This setting becomes vital for automatic analysis of a large number of particles with wide size distribution such as analysis of particles in the 100-5000 µm range.

Generating a good quality, high contrast image that helps to recognise particles is another key point. In bright field (BF) illumination for example, some particles appear darker and some brighter than the background depending on their optical properties, making it impossible to process all particles in a single analytical chain. We applied transmission type illumination that results in dark particles on a brighter background and allows to work with a single image for the optical contrast-based localisation of particles. Our experience with the automatic analysis highlighted also the extreme importance of sample preparation when the point-by-point measurement strategy is used. Overlapping particles are difficult to separate by automatic image analysis and the operator’s choice of threshold might introduce bias in the selection of measurement positions and in the size evaluation. Additionally, very closely located, touching or overlapping particles with different chemical composition might result in spectra that contain the spectral features of multiple materials, making identification less reliable. Therefore, the deposition of particles on the filter surface in homogenous way, without generating many aggregates is the prerequisite of collecting good quality data.

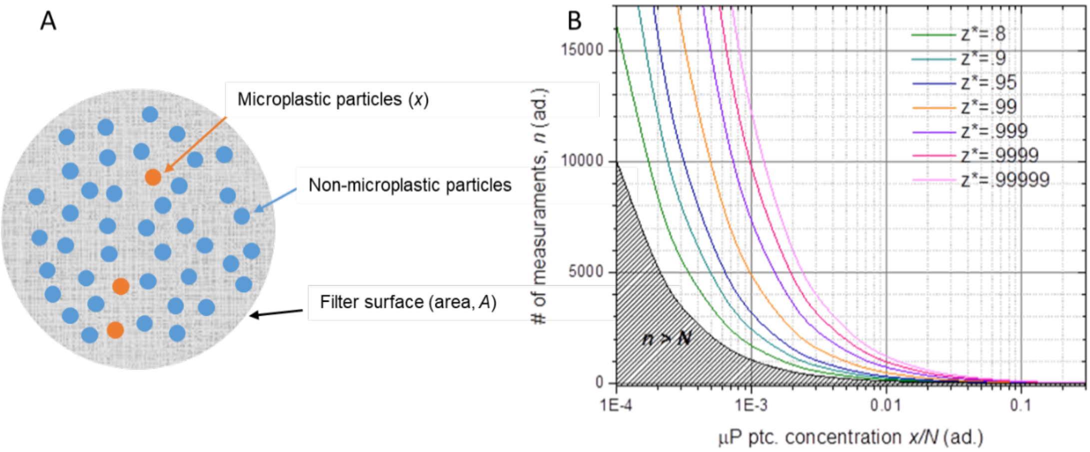
### 3.4.3 Minimum analysed filter fraction vs minimum number of analysed particles

The number of microplastic particles in a sample of 1000 L is supposed to be compatible with the micro-spectroscopy analytical procedures described above. However, if the samples contain many other (non-polymeric) particles, the data collection and processing time might become unacceptably long. The solution for this is sample treatment and – even if not optimal from recovery point of view - subsampling of the concentrated suspension or reducing the analysed filter area.

For large MPs, all particles on the filter can be detected and analysed at a given magnification. In this case it is typically possible to analyse the whole filter area or quite significant fraction of it, sample size can be quantified and the level of confidence of random sampling can be easily calculated along the lines presented below.

In this case fraction of the total number of particles present on the filter is analysed (Figure 9).

**Figure 8.** A) Schematic representation of the filter surface loaded with particles, among them x microplastic particles (in orange), B) number of measurements needed to detect at least one microplastic particle depending on microplastics concentration and confidence levels



*Source: own production*

If N is the total number of independent objects (e.g. particles) optically identified on the filter surface (A) and x is the number of microplastics present on the filter, the number of independent measurements (n) needed to detect at least one plastic particle, with confidence level z*, in a sample with micro(nano)plastic concentration x/N can be calculated using formula (1):

$$n = \frac{log\left[1 - \frac{(1-P(\bar{E}))z^*}{P(E)}\right]}{\log\left(P(E)\right)} \tag{1}$$

where P(E) is the probability that the analysed particle actually is a microplastic and $P(\bar{E}) = 1 - P(E)$ is the probability that the analysed particle is not microplastic. Under our assumptions P(E) can be written simply as P(E) = x/N and therefore equation (1) can be simplified to (2):

$$n = \frac{\log(1-z^*)}{\log x - \log N} \tag{2}$$

This calculation assumes that the chosen analytical technique can assign chemical identity to an unknown particle without ambiguity and that particles are interrogated serially, chosen in a random order. Results are shown in Figure 9(B) for some selected concentrations and confidence levels. If the original sample is deposited on a filter without sample treatment these calculations could be used to predict how many objects, visually identified on a filter, must be verified for their chemical identity to exclude the presence of microplastics above a certain threshold concentration in the original sample.

When the filters size, analytical technique or time constrains limitations do not allow full filter scanning, subsampling according to a windows-based analysis scheme is often the solution of choice. In this case, error quantification and confidence interval calculations, as well as windows selection schemes are described by Schwaferts et al.[33] and references therein.

However, the sample arriving to the spectroscopic investigation step is often treated before analysis to allow better imaging and measurement conditions. Thus, deposited particle numbers on a filter are not reflecting the original total particle concentrations. As a result, no conclusion about the presence of microplastics above a certain threshold in the original sample can be drawn. Moreover, the concept might be incompatible with protocols that apply chemical imaging for recognition of particles. On the other hand, selecting a well-defined, representative fraction of the filter area that has to be fully analysed (and considering potential subsampling) allows in a simple way to estimate original microplastic concentrations. For example, if $V_{tot}$ [$m^3$] of water was filtered, it was resuspended in a volume of $V_{conc}$ [mL] but only $V_{proc}$ [mL] was processed and transferred to a filter, where x% of the filter area was analysed, and n microplastic particles were found on the filter, the original concentration of particles is calculated as (3):

$$c\ [1/m^3] = n*(100/x)*(V_{conc}/V_{proc})/V_{tot} \qquad (3)$$

Considering that according to the current methodology laboratories should investigate at least 20% of the filter area, that subsampling allows to use a minimum of 10% of the sample and recovery can be as low as 60%, the limit of detection (by size fraction) is supposed to be at least 84 microplastic particles / $m^3$ or better.

### 3.4.4 Hit quality considerations

Hit quality index (HQI) is a numerical value applied to describe the similarity between the acquired spectrum and the reference spectrum or set of spectra. Ultimately, this is the number that will help the analyst to decide about a spectrum if it is a spectrum of a synthetic polymer material. It is calculated and expressed in various ways typically on a scale between 0-1, 0-100 or 0-1000. We have tested 4 different software solutions for spectrum identification and some features of these are collected in the **Table 4** below.

**Table 4.** Software solutions tested in spectrum identification: comparison methods and HQI index ranges

| Source/Software | Technique | Strategy | Comparison method | HQI(1) range |
|---|---|---|---|---|
| Bruker/OPUS | µ-FTIR | Single spectra | Search for bands position or correlation based on vector normalisation or min-max normalisation raw spectrum, 1st and 2nd derivative | 0-1000 |
| Freeware/siMPle | µ-FTIR | Chemical map | Pearson correlation based<br>"match score"<br>raw spectrum, 1st and 2nd derivative | 0-1 |
| Purency/ImageLab | µ-FTIR | Chemical map | Machine learning | 0-1 |
| Renishaw/ ParticleAnalysis | µ-Raman | Single spectra | Correlation(2) or first derivative correlation | 0-1 |

(1) Hit Quality Index.

(2) No information available in the software on calculation method

*Source: own production*

Many software use the simple way of point-by-point comparison of the acquired spectrum (and/or its derivatives) with spectra from the database for example by generation of a Pearson correlation coefficient. An example for this mathematical method is represented by the freeware siMPle,[34,35] where the analyst can select the weight of parameters and fine tune the analysis process. The use of derivatives instead of raw spectra helps to decrease the effect of baseline fluctuations and gives more trustable results. The analyst can usually also set up a lower cut-off value to simplify the evaluation of analysis results by excluding hits below a certain similarity level. However, because of the many interaction possibilities between evaluation software and user, the results are clearly exposed to human bias. Commercial products are often less transparent, might allow much less interaction and do not share direct information about the mathematical method applied at HQI generation.

A very diverse approach that we tested was developed by Hufnagl et al.[36] and applied by the software from Purency (ImageLab). It uses classifiers, i.e. complex algorithms combined with machine learning to sort data into classes. A training dataset of microplastic particles, silicon and matrix particles was used to generate 22 information categories. The process allows to predict the affiliations of unknown spectra from training data categories. Processing of the spectra acquired by the user allows limited interaction, thus the resulting hit quality descriptors are less prone to human bias. On the other hand, post-processing is possible and potentially allows strong operator interference with the data, i.e. in the case of the addition of pixels to particles to correct their size based on confrontation of optical image and spectra of close pixels. Accordingly, the automatically generated hit quality results disappear from the report table.

Based on our hands-on experience with various software solutions and discussion with experts, we can conclude that there is no standardised or mutually agreed *best way* to express the similarity in spectrum analysis. The situation is further complicated by the inherent nature of chemical mapping where the chemical identity of the particle is not evaluated based on the HQI of a single spectrum but relies on more values corresponding to neighbouring pixels in a chemical image. Even within the same technique many strategies exist and the actual fast development in the field of machine learning and artificial intelligence in data processing imply that upcoming high throughput spectrum analysis methods will more and more rely on these types of solutions. As a consequence, establishing a single accepted protocol for calculation of HQI and setting an acceptable lowest hit quality value in the identification of microplastics could have more negative than positive outcomes. It could exclude commercially available techniques and software solutions from microplastics analysis and hinder further development of future data analysis methods. Moreover, the additional information from visual observation added to the spectral information can also improve the identification of particles. For example, a smooth, uniform 30 µm diameter fibre with fluorescent green colour and sharp edges might be still judged to be a microplastic particle based on additional information on its appearance even if HQI is under the previously set automatic acceptance level. On the other hand, for comparability of the results at least within the same analytical laboratory, a lowest HQI index cut-off value for automatic identification should be fixed, i.e. the lowest HQI that can be accepted without additional control by the analyst should be set and kept unchanged between samples. This HQI might be different depending on the various polymers which may pose different challenges in identification, especially in the presence of particles of natural origin as described in more detail in 3.5.4.

## 3.5 Data reporting

Data generated by applying the suggested methodology have to be comparable between measurement locations and timepoints. Thus, it is crucial to settle the content and format of data reporting, including i) the polymer types to be reported, ii) how laboratories should record the shape and dimensions of particles, iii) whether the detected blank values should be or should not be subtracted from measured quantities. Apart of these, some key features of sampling, sample treatment and the analysis process also have to be described in a structured form that – if needed - will allow future metadata analysis aiming to reveal possible correlations between procedure details and reported values.

### 3.5.1 Polymer types

The throughput of the analysis by micro-spectroscopy methods does not depend so heavily on the number of polymers to be reported as in the case of thermo-analytical methods. On the other hand, the immense – and increasing - number of various synthetic polymer materials makes it impossible to include all existing plastics in databases and to in-house validate sample preparation and measurement protocols considering all of them.

Both for optimising the performance of sample treatment and identification steps and for the generation of statistics on reported data is advantageous to limit the material classes to be recorded to some *priority polymers*, *i.e.* plastic types that were shown to be present as microparticles in the environment or detected in drinking water. **Table 1** summarises the suggested selection of priority polymers, together with some info on their use (possible source when appearing as contaminants) and yearly word production.

We tested the following spectral databases as components of the analysis software solutions: Hummel Polymer FTIR library (software independent commercial database), Purency Imagelab material classes (inbuilt), Renishaw's polymer database (inbuilt, but possibly augmented by user spectra), siMPle automated analysis library (free download, containing more spectra for each material)[37] (**Table 4**). All these spectral databases contained the selected material classes. All tested software that works with spectrum to database comparison allows the user to expand the database with in-house collected (or imported) data in order to include more spectra of the same polymer and spectra of possible matrix compounds as suggested by many experts to reach better reliability in identification. On the contrary, the machine learning based solution is built with the same concept but does not allow further modification of the reference data pool.

### 3.5.2 Blanks

As the expected concentration of the target analyte is low and at the same time plastic microparticles are known to be omnipresent in our environment, it is very important to collect information on what is the detected concentration of microplastics using the same sample treatment and analysis procedure but without exposure to the sample. The procedural blank generation suggested by the Methodology uses the pragmatic approach of placing blank filters after the filters used for sample collection. Supposing that the first two filters collect all the particles larger than 20 µm, the two blank filters are not supposed to be exposed to microplastic particles >20 µm from the sample. The two different sizes, 100 and 20 µm were introduced after evaluating technical feedback received during the expert consultation. It allows the operator to apply the same sample treatment and analytical procedure for the blanks as for the samples collected at the corresponding cut-off. This becomes especially relevant if different micro-spectroscopy techniques are used for the analysis of the two size fractions (*e.g.* ATR FTIR for >100 µm and transmission FTIR for 100-20 µm). The suggested strategy ensures that blanks follow exactly the same path and are exposed to exactly the same environmental conditions as samples. Moreover, collection of blanks does not require additional sampling time and (discarded) drinking water volume.

During expert consultations this strategy has received the criticism that some fibres with smaller diameter but higher length than the filter cut-off might pass the sample collection filter and still be captured on the corresponding blank filter. This peculiar behaviour cannot be fully excluded but can be considered at the processing of monitoring data.

To allow flexible and intelligent evaluation of the data, the form (particle or fibre), composition and size of microplastics in blanks have to be fully reported in the same way as in samples and the numbers detected in blanks and should not be subtracted from the numbers observed in samples. This approach allows also to monitor possible fluctuations in blank levels during time that can indicate a contamination problem. Reporting the microplastics in blank(s) together with their descriptors in the same way as for microplastics observed in samples contributes also to the critical evaluation of method performance for certain material types and to consider more carefully or selectively discard data on specific materials - if needed. This might become interesting especially for polymer types which are spectroscopically very similar to some natural materials, as described more in details in 3.5.4.

### 3.5.3 Sizing

Shape is often discussed as a possible parameter that might affect interaction of microplastics with biological systems. Moreover, our former literature review[11] indicated that about 50% of the microplastics detected in drinking water are fibres. Polymer based fibres are often flexible and might behave in a different way in filtration compared to irregular shaped particles. As mentioned also above, they might pass filters that have smaller cut-off as their larger dimension. They might not "lay down" on the analysis filter and appear with their full length in one focal plane at optical investigation. Size descriptors that are typically used for "fragment" shaped particles are difficult to generate automatically or are just less meaningful in case of fibres. Thus, fibres are complicated to size, but their morphology is important to be reported. Therefore the methodology asks only reporting the number of fibres without size information in the size fraction where they were captured.

The cascade filtration step generates two size fractions from the collected particles. However, the nominal size of the filter cut-off might be affected by clogging, *i.e.* filters might capture also particles that are smaller than the nominal pore size. Measuring the particle size by microscopy helps not only to confirm the size bin but allows also to sort the identified microplastics in more size categories allowing a later comparison with other studies that might have used more size fractions. Therefore the methodology requires reporting particle size data in 5 size categories: 20≤ D <50 µm, 50≤ D <100 µm, 100≤ D <300 µm; 300≤ D <1000 µm and 1000≤ D<5000 µm (**Table 2**), where D is area-equivalent diameter: diameter of a circle that has the same area as the area of the 2D projection of the particle.

**Table 5.** Available size descriptors at various instrument manufacturers' software

| Instrument / Software<br>Size descriptor | Horiba Raman | Renishaw Raman | WiTEC Raman | Agilent QCL | Bruker FT-IR | Purency FT-IR | freeware FT-IR |
|---|---|---|---|---|---|---|---|
| | | WiRE Particle Analysis | Particle Scout | | OPUS | Image Lab | siMPle |
| Area | + | + | + | + | + | + | + |
| Feret min and Feret max | | | + | | | | |
| Equivalent circle diameter | | + | + | | | | |
| Volume estimation | + | | | | | | + |
| Rectangle | | | + | + | + | | |
| Shorter and Longer axis<br>Length and width<br>Major and minor dimension | + | + | | + | | + | + |
| Circularity | | | | + | | | |
| Perimeter | + | + | + | + | | | |
| Eccentricity | | + | + | + | | | |
| Orientation | | + | | | + | + | |
| Solidity | | + | | + | | | |
| Ellipse ratio or aspect ratio | | + | + | + | + | + | |
| Elongation | | + | | | | | |
| Brightness | | | | | + | | |

*Source: own production*

Size of a particle can be measured in many different ways and the resulting value might refer to various dimensions. The "area-equivalent diameter" as size descriptor was chosen based on a combination of our experience with in-house tested software and information obtained for other instrumentation following and electronic search or by verbal inquiry to instrument manufactures. **Table 5** summarises our findings and shows that all instrument providers offer the possibility to measure the area of particles in some way that can be easily transformed to area equivalent diameter. In contrast, we found only one manufacturer whose analysis software actually included minimum and maximum Feret diameters as a built-in feature.

Particle area is the most obvious size descriptor choice by software developers as both in single position measurements and in chemical mapping-based analysis a group of adjacent pixels with known size correspond to each particle. Automatised single position measurements start with the collection of an optical image that is then analysed to determine which pixels correspond to particles and which pixels do not - according to the optical contrast between particles and the surrounding area. This "binarised" image is used then for size measurements with a resolution arising from the pixel size of the original optical image. Similarly, chemical mapping results in hyperspectral images made of pixels. In this case, pixel size depends on the combination of microscope's optics and the detector array. After spectrum identification, the pixels corresponding to the same particle (same spectral identity) will be used to determine the size of the particle.

The use of area as descriptor has also the advantage of being probably the size descriptor which is most closely linked to the corresponding volume of the particles. As suggested by some experts, it can be used for the estimation of particle volume and - by considering material specific densities - also for the estimation of particle mass. This feature might keep open the possibility to compare concentration data generated by number-based micro-spectroscopy and mass-based thermo-analytical methods.

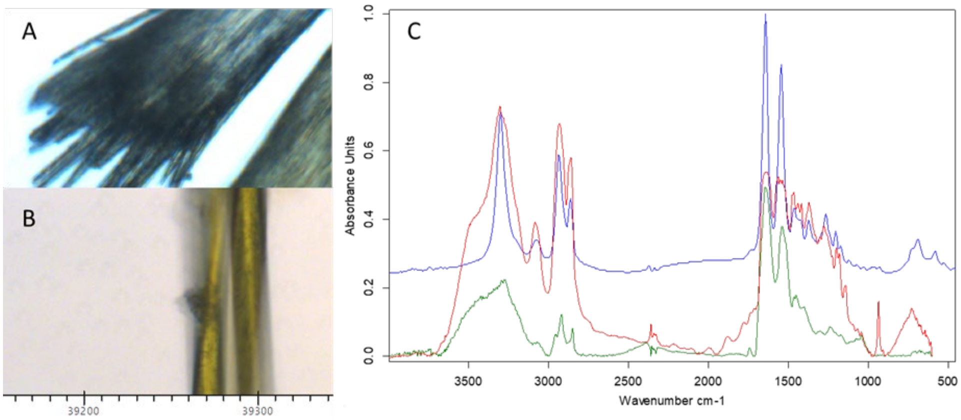
We have to note, that according to our experience both methods might underestimate particle size. When particles are defined based on optical contrast, some parts of the particle might be lighter and do not appear as selected pixels in the binarised image. A simple example is the case of polymeric microspheres where the centre of the sphere appears less "compact" in a transmission image and often remains unrecognised. On the other hand, in chemical imaging the too thin or too thick parts of irregular shaped particles might fail to be recognised as a certain material type because of the low quality (bad signal to noise ratio or saturated) spectra. The consequence in both cases is that less pixels are considered to correspond to the particle than the human observer would select based on the optical image. One of the tested software solutions (Purency, ImageLab) allowed to perform corrections by comparing the results to the optical image which is a powerful feature – provided that the quantity of detected particles is low enough to consider manual post-processing as a viable option.

Finally, it means that in case of automatic analysis the information on size will have an uncertainty that makes it reasonable to permit reporting other size descriptors, in case the equivalent area circle diameter is not available. This allows also to keep the methodology compatible with possible upcoming standards.

### 3.5.4 Limitations

We have also confirmed some other known limitations of micro-spectroscopic methods. One drawback which is often underestimated is the spectral similarity between some natural polymeric materials and plastics. Typical example is natural vs. synthetic polyamides. Figure 9 illustrates the μ-FTIR analysis of a natural, protein-based fibre (human hair, A) and that of a fluorescent green polyamide flock particle (B). Both materials were found to be the most similar to polyamide when using a library containing only polymeric materials.

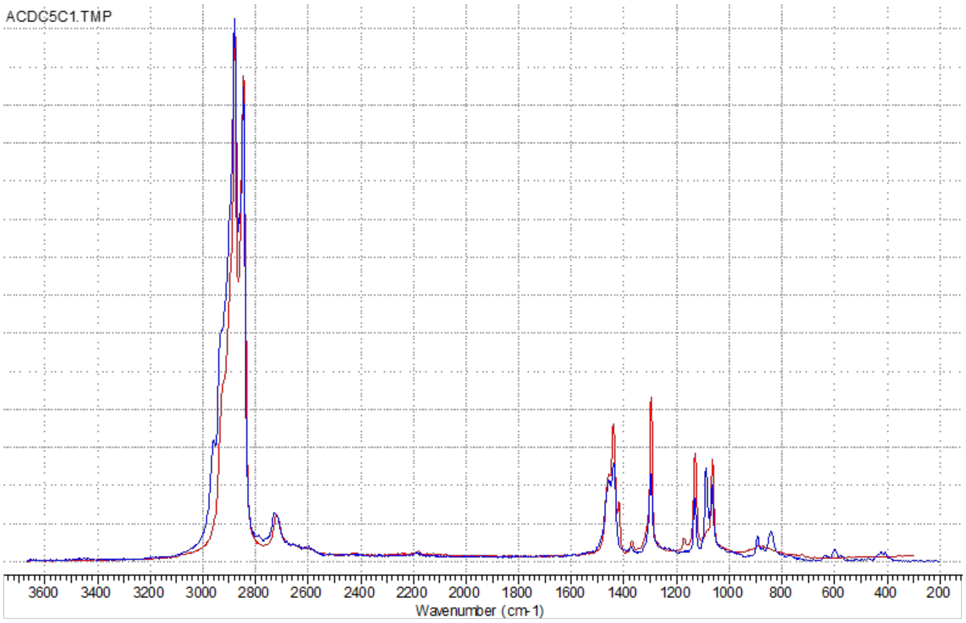
**Figure 9.** Microscopic image of A) human hair and B) polyamide fibres and C) FTIR spectral match between their spectra and polyamide. Best hit result of library search (blue, polyamide-6+polyamide-6,6 mixture) for spectrum of human hair (green) and polyamide fibre (red) with HQI of 386 and 314 for hair and polyamide, respectively



*Source: own production*

Certain lines, like C-H stretching vibrations in Raman spectroscopy have a higher weight in automatic spectrum identification, for example using Pearson coefficient-based analysis. As a result, materials with high quantity of -$CH_2$- groups might appear to be very similar to polyethylene. Natural waxes (e.g. bees wax), fatty acids and even some detergents regularly used in laboratory belong to these classes of materials. The example presented in Figure 10 shows the similarity between the Raman spectra of sodium dodecyl sulphate (SDS, an often used surfactant) and polyethylene, resulting in 84% HQI (on a 0%-100% scale) in one of the tested spectrum analysis software solutions. These examples emphasise the importance of amending the database with spectra of natural matrix compound materials and with spectra of materials that might appear in the sample due to the sampling and sample treatment process.

**Figure 10.** Similarity between Raman spectral fingerprint of SDS (blue) and polyethylene microparticle (red)



*Source: own production*

# 4 Adaptability of DWD methodology for alternative applications

The Delegated Decision (EU) 2024/1441 specifically defines a methodology for monitoring microplastics in drinking water as required by the recast-DWD. However, there are other policy areas where future new or revised legislation will require methodologies for detecting or monitoring microplastics. It is thus prudent to consider the extent to which the outcomes of this work can be directly or indirectly applied in the development of other legislative applications. Currently the areas relating to microplastics as a pollutant include surface and ground- waters (WFD/GWD), urban runoff and storm-water overflows, treated and untreated urban wastewater as well as the related sewage sludge (per the revised UWWTD). In view of preventative actions against release of microplastics, implementation of the legislation to restrict the placing on the market of manufactured microplastics under REACH would also require analytical methods for compliance checking.

## 4.1 Adaptability of DWD methodology to surface waters

Literature search and consideration of information gaps regarding the adaptation of drinking water methodology in relation to the other legislative applications under the WFD and UWWTD shows that the general approach based around vibrational micro-spectroscopy could probably be adapted to other applications involving water-based samples with low or moderate suspended solids – groundwater, surface water and possibly WWTP effluent. Detailed consideration must be given to the aspects of sampling and in particular whether there is a need to adjust the methodology to allow greater flexibility in establishing the minimum sampling volumes to be used for each application.

In the case of water from urban runoff and storm-water overflows, the quality and composition of the water may be too variable to provide a reasonable judgement on these points without more detailed information from real-world testing. Furthermore, urban runoff water may contain more tyre wear particles and consideration must be given to inclusion of this particular polymer pollutant in the monitoring. As discussed previously the vibrational spectroscopy methods used in the DWD methodology are not well suited to detecting tyre wear particles and consequently inclusion of thermo-analytical methods would have to be considered. Introducing the additional use of thermo-analytical methods to the methodology would permit a more complete picture of the presence of all types of microplastics to be obtained but would significantly increase the technical and economic monitoring burden.

The monitoring of microplastics in wastewater treatment plant (WWTP) sludge - a solid matrix most likely containing a high concentration of microplastics - will require a major revision of the methodology in terms of sampling and sample treatment. It is also possible that the higher levels would permit a re-evaluation of the option of using thermo-analytical methods which, in the case of Py-GC/MS, could offer cost and sample throughput benefits but only if used as an alternative to vibrational spectroscopy. For this, it is conceivable that a combination of solvent extraction of the solids followed by analysis using thermo-analytical methods would be a technically elegant and economically interesting solution.[38] It should be noted that this would produce mass-based data which is not directly comparable with the particle number data from the drinking water samples or any other water-based samples if those are analysed by micro-spectroscopy.

Finally, in the case of conformity testing of products for compliance with the REACH restriction, the adoption of a mass-based metric in this legislation makes the DWD methodology unsuitable as the latter uses a particle number metric which cannot be reliably converted into the required mass-based data. Furthermore, the particle size range adopted in the REACH restriction extends below what is resolvable by the current generation of vibrational spectroscopy instruments. Consequently, the most realistic and generally applicable solution would be to apply thermo-analytical methods. It should be noted that the ability of this solution to work will depend on the operator having prior knowledge of the types of polymers which may be in the product or alternatively being equipped with a very extensive library from which to identify materials.

# 5 Conclusions

Globally, microplastics were shown to be present in drinking water with sizes ranging from a few µm to several mm. Reported concentrations typically ranged from an upper level of several hundred particles per litre to values which are often much less than the equivalent of 0.1 particle per litre. When considering only the studies of EU water supplies, the concentrations of microplastics were all found to be below 1 particle per litre and the majority were significantly below 0.1 particle per litre. These low levels meant that instrument sensitivity had to be considered as a key factor in developing a methodology. In these same European studies, the analysis of particle composition indicated that about 90% of the particles were distributed amongst 7 polymer types with no single polymer type constituting more than 25% of the particle number.

A detailed examination of the literature showed that four main instrumental methods are currently used in microplastics analysis – two optical methods, Infra-Red (IR) and Raman micro-spectroscopy and two thermo-analytical methods, pyrolysis-gas chromatography mass spectroscopy (Py-GC/MS) and Thermal Extraction and Desorption (TED-GC/MS). Each of these methods have specific strengths and limitations in terms of the measurement metric (polymer weight or particle number), sensitivity, minimum particle size and ability to identify polymer. Overall, it was concluded that no single analytical technique was fully suited to characterise the nature and extent of microplastic contaminants in drinking water. Consequently, defining a methodology would inevitably require some degree of compromise and pragmatic choices regarding the critical parameters to monitor. Key factors in determining the most suitable method or combination of methods were the measurement metric, size range, sensitivity and reliability (specificity, accuracy, precision), but additionally the throughput, cost per sample and instrument availability also had to be considered. Overall, the conclusion of the review was that the expected sizes and (low) concentrations of microplastics in European drinking water would require analytical methods with a level of sensitivity which, with current technology, could only be achieved by the use of vibrational spectroscopy based on Infra-Red or Raman micro-spectroscopy.

Based on these evaluations and expert consultations, the final methodology was published as Annex of a Delegated act definitions. In the present report, it was transcribed in section 2 and accompanied with additional comments and explanation on the rationale behind while in section 3 there is a detailed description of how its main components - sampling, pre-treatment and instrumental analysis - can be put into practice using examples of commercially available instruments and equipment.

The methodology was designed in order to obtain the maximum scientific reliability with the minimum analytical effort. Measuring microplastics remains challenging because they vary vastly in size, shape, composition and chemical identity. It requires complex and expensive instrumentation which is often slow to use and needs skilled operators. The development of new and improved instrumentation to increase analysis speed and automate to reduce operator intervention is desirable. The methodology for European drinking water sets a number of specific technical or reporting requirements but has flexibility to allow future instrumental or methodological developments in the field. While the identification method and instrument family (micro-spectroscopy) and the data reporting parameters are well defined in order to gain comparable data, the methodology provides flexibility concerning the actual instrument and protocol used by laboratories. This flexibility allows also freshly developed or tailor made, more user-friendly instruments or filter devices or materials to be picked from the improving market.

Finally, consideration has been given to the adaptability of the methodology to other legislative applications such as the recast-Urban Waste Water Directive, the ground and surface water directives and the REACH restriction on the use of microplastics in products. Notably, the DWD methodology is fundamentally unsuitable for the REACH restriction because it relies on particle number concentrations, which do not align with the mass concentration metrics defining product limits under this legislation. For the other directives, selected components of the methodology could, to varying degrees, potentially be used with appropriate adjustments. For those other applications the analytical instrumentation, Raman or IR micro-spectroscopy, is generally applicable to the detection of microplastics provided that a statistically relevant number of particles can be extracted, and that the extraction can be done so as to eliminate or greatly reduce the presence of other non-plastics particulates. This, however, excludes applications which may require detection of tyre wear particles as the optical spectroscopy methods are not well suited to polymers containing high levels of carbon black. Where the detection of tyre rubber may be important, for example in urban runoff, alternative methodologies using the thermo-analytical methods would need to be developed.

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# List of abbreviations and definitions

| Abbreviations | Definitions |
|---|---|
| ASTM | American Society for Testing and Materials |
| ATR | Attenuated Total Reflectance |
| BF | Bright field (Annexes) |
| D | Area-equivalent diameter |
| DG ENV | Directorate General Environment |
| DW | Drinking Water (Annexes) |
| DWD | Drinking Water Directive |
| EC | European Commission |
| EU | European Union |
| FPA | Focal Plane Array |
| FTIR | Fourier-Transform Infrared (spectroscopy) |
| GC | Gas chromatography |
| GC/MS | Gas-Chromatography Mass-Spectrometry (analytical techniques combining gas-chromatography and mass spectrometry) |
| GWD | Groundwater directive |
| HCl | Hydrochloric acid |
| HIS | hyperspectral image (Annexes) |
| $H_2O_2$ | Hydrogen peroxide (Annexes) |
| $H_2SO_4$ | Sulphuric acid (Annexes) |
| HQI | High quality index |
| IR | Infra-Red |

| **Abbreviations** | **Definitions** |
|---|---|
| JRC | Joint Research Centre |
| KBr | Potassium bromide |
| MP | Microplastics (Annexes) |
| MS | Mass spectrometry |
| NaOCl | Sodium hypochlorite |
| Ox. | Mild oxidative treatment |
| PA | Polyamide |
| PC | Polycarbonate |
| PE | Polyethylene |
| PET | Polyethylene terephthalate |
| PMMA | Poly(methyl methacrylate) |
| PP | Polypropylene |
| PPE | Personal protective equipment (Annexes) |
| PS | Polystyrene |
| PTFE | Polytetrafluoroethylene |
| PU | Polyurethane |
| PVC | Polyvinyl chloride |
| Py-GC/MS | Pyrolysis-Gas Chromatography Mass Spectrometry |
| QCL-IR | Quantum cascade laser-Infrared (IR) spectroscopy |
| SDS | Sodium dodecyl sulphate |
| RCF | Relative centrifugal force (Annexes) |

| Abbreviations | Definitions |
|---|---|
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemicals |
| TED-GC/MS | Thermal extraction and desorption gas chromatography/mass spectrometry |
| TXRF | Total Reflection X-ray Fluorescence |
| UWWTD | Urban WasteWater Treatment Directive |
| µ-FTIR | Fourier-Transform Infrared micro-spectroscopy (FTIR spectroscopy coupled to a microscope) |
| µ-Raman | Raman micro-spectroscopy (Raman spectroscopy coupled to a microscope) |
| µm | micrometre (micron) |
| WFD | Water framework directive |
| WWTP | Wastewater treatment plant |
| $ZnCl_2$ | Zinc Chloride |

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# Annexes

## Annex I Sampling of drinking water for analysis of microplastics in water for human consumption

| Version | Authored by | Date |
|---|---|---|
| v1.1<br>Added blank 100 micron filter compared to v1.0 of 08.09.2023 | Dora Mehn | 18.10.2023 |
| | Reviewed by | Date |
| | Douglas Gilliland | 15.11.2023 |

### Scope and limitations

The scope of this document is to provide a detailed description of how to sample large volume of drinking water for microplastic analysis by micro-spectroscopic methods. Because of the large number of other microparticles in drinking water (different from microplastics), it is expected that this sampling step be followed by sample pre-treatment and the transfer of treated sample to a second filter that is compatible with the analysis procedure. This sampling procedure is designed for use with pressurised water lines and does not cover sampling from non-pressurised sources, for example wells, surface water or bottled water.

### Principle

This procedure applies in-line sequential filtration by passing water intended for human consumption through a cascade of four filters, with cut-off at (a) 100 µm (b) 20 µm (c) 100 µm (d) 20 µm (**Figure 1**). Filters (a) and (b) serve to collect the suspended matter from the water intended for human consumption, while filters (c) and (d) are used as a procedural blanks to assess levels of microplastic contamination from e.g. laboratory equipment, reagents and atmospheric occurring during the steps of sampling, treatment and analysis. To minimise atmospheric contamination of samples the water sample is piped directly from the sampling point through the filter cascade without the use of an intermediate collection or storage vessel. Analysis of materials collected on the filters (a), (b), (c) and (d) is done separately in a later stage. Filters (c) and (d) serve as a procedural blanks which are subjected to the same processing and analysis steps as filters (a) 100 µm and (b) 20 µm, respectively. Because of the high background concentration of microplastics (MP) in laboratory and household environment, special care has to be taken to avoid contamination of equipment surfaces by MP fall-out from air. All equipment parts and tools have to be washed with water-based detergent, ethanol and rinsed with MilliQ water possibly immediately before the sampling. If this is not possible, clean equipment parts has to be stored in glass containers covered with glass or Aluminium foil. In case the sampling point is outside the laboratory, the transfer of mounted (closed) sampling device is recommended. During sampling, the operator should wear a cotton laboratory coat and, if safety requirements permit, should avoid the use of plastic gloves. The operator should avoid using cosmetic products that might interfere with the sampling (make-up, nail polish, hair wax). The use of plastic tools and ingredients during the full sampling process should be circumvented if possible. In case it becomes impossible, plastic components of the sampling tool or procedures should be reported as possible sources of contamination.

### Equipment and reagents

Drinking water (DW) source (tap) with typical pressure of household DW supply systems (≤5 bar)

Sink, deep enough to allow the positioning of the 4 consecutive filters of the sampling device in a vertical arrangement

Leakage free connection to tap (in our case in-house made metallic connection) with T geometry equipped with ball valves on both flow lines

Stainless steel 47 mm filter holders (x4, Millipore XX4404700) equipped with rubber sealing ring and connected in line by stainless steel hexagonal fittings (HOKE-316, male at both ends).

Stainless steel hose connector (Millipore, Z566647)

Stainless steel 47 mm mesh filters with 20 µm cut-off (x2, fteu, Germany)

Stainless steel 47 mm mesh filter with 100 µm cut-off (x2, fteu, Germany)

Hexagonal key fitting the screws of the filter holders

Stainless steel tweezers (pointy and round tip)

Flow meter and counter downstream connected to last filter holder (Gardena, 8188-20) that is able not only to measure actual flow, but also to measure the total passed volume

Ultrasonic bath

Nitrogen line with spray gun

Glass beakers 200 mL (x4) with enough diameter to host a 47 mm filter in horizontal position

Glass petri dish 60 mm (x4)

Erlenmeyers 100 mL with ground glass stopper (x2)

Laboratory detergent solution (freshly prepared)

MilliQ water

Ethanol (99.9%, 0.22 µm filtered, stored at room temperature in Erlenmeyer with glass stopper)

Triton X 100 solution (0.1 w/w% in MilliQ water, X100, Sigma-Aldrich. Stored at room temperature in Erlenmeyer with glass stopper)

Piranha solution (freshly prepared, cc$H_2SO_4$: 30%$H_2O_2$ - 3:1)

Aluminium foil

## Sampling device

The sampling device is a filter cascade built of 4 filter holders and a flow meter, connected to the water source by a T tube element with 2 ball valves. The second line is used for purging the system and allows to depressurise at the end of the filtration process. The flow meter is connected by a silicone tube downstream to the filtration device (no contamination from back-flow is expected) to allow a positioning for easy readout and safe from water. The filter cascade is dismounted after each sampling by opening each of the filter holders using the hexagonal key (3 screws) to allow removal of the filters from each device. Opening the filter housings (without dismounting the connector tubes) provides access to the inner surfaces for washing with an aqueous detergent and then rinsing with ethanol and MilliQ water. **Figure 1** of the main document shows a schematic drawing and a photograph of our sampling device.

Filters

Clean, 47 mm stainless steel mesh filters with appropriate nominal cut-off - (a) 100 µm, (b) 20 µm, (c) 100 µm, and (d) 20 µm are used for each sampling. Stainless steel filters can placed in the filter housings by using round tip tweezers, but they are easier to be removed using pointed tweezers. Care should be taken to avoid scratching the filter housing when using pointed tweezers.

Recycling of stainless steel filters is possible after cleaning. The recommended steps for cleaning are as follows.

1 Cleaning filters with detergent solution, sonication in water bath and rinsing with MilliQ water.

2 Apply proper personal protective equipment. Perform operation under fume hood. Prepare the acid piranha solution in a glass beaker (diameter large enough to host a 47 mm filter in horizontal position) by adding 5 mL 30% $H_2O_2$ to 15 mL $ccH_2SO_4$ slowly. Wait till the solution cools down.

3 Place the filter in the solution using stainless steel tweezers and leave it there for 5 min. Pour the major part of the liquid in the acid waste container. Add carefully MilliQ water to the rest, discard the liquid in acid waste container. Wash the filter with MilliQ water and store for later use under the conditions described above.

Avoid dropping filters that are wet with ethanol or traces of HCl in the Piranha solution. Both might cause violent reaction, the second might lead to the damage of the filter.

Bending the mesh filters might change their pore size. Take care of not to bend or mechanically damage the filters during all handling procedures.

**Sampling procedure**

The total volume to be sampled might depend on experiment, typical volume is 1000 L. The flow depending on sampling point is usually 12-16 L/min, typical sampling time for 1000 L is less than 2 hours. The system has to be purged before sampling in order to remove any accumulation of particles from the supply line that may deposit there with time (mainly inorganic, iron-oxide, calcium carbonate, etc.).

1 Attach the upper part of the sampling device with the two ball valves and the top part of filter-housing (a) to the tap.

2 Close ball valve 1, open ball valve 2, open the tap and purge the line for 10 min at maximum possible flow (>100 L). Check if exit of the sink is clean from obstructions and that the water can easily flow to the drain.

3 Close ball valve 2 and open ball valve 1 to purge the top part of the sampling line for an additional 5 min. Close the tap, close all valves.

4 Mount the sampling device starting from connecting the flow meter to the exit of filter housing (d) 20 µm filter. Place the large support grid on the bottom part of the filter housing (Annex I Figure 1/2). Place the fine support grid on it (Annex I Figure 1/3). Place the filter on the support grid (Annex I Figure 1/4). Place the red rubber sealing ring to fit to the perimeter of the groove (Annex I Figure 1/5). Place the upper part of the filter housing (that carries already also the bottom part of second filter housing) on top, carefully aligning the holes for the 3 screws. Insert the 3 screws and tighten gently by hand, then pass with the hexagonal key to close the filter holder tight.

5 Mount the (c), 100 µm filter on top in a similar way. Insert the 3 screws and tighten all of them gently by hand before using a hexagonal key to close the filter holder tight.

6 Mount the (b), 20 µm filter on top in a similar way. Insert the 3 screws and tight all of them gently by hand, then pass with the hexagonal key to close the filter holder tight.

7 Mount the (a), 100 µm filter on top in a similar way, close the device with 3 screws tight.

8 Place the flow meter in a position protected from water splashes and with well readable screen. Set the total volume to zero by pressing the button longer (3 seconds). Place the exit tube in the sink.

9 Open ball valve 1. Open the tap slowly till reaching highest possible flow.

10 Inspect the sampling device for leakage. In case of leakage stop flow, adjust, and restart procedure from beginning with clean filters. Inspect flow rate and total volume values if realistic (10-16 L/min). Check that water leaves free and does not accumulate in sink

11 Regularly check the filtered total volume.

12 Place 4 clean beakers close to the sampling point and label them. Place 4 clean glass Petri dishes close to the sampling point and label them. Place both tweezers close to the sampling point.

13 When total required volume is reached, close ball valve 1. Close the tap. To avoid back flow of water Move the flow meter to a position such that all the tubing and flow meter is below the level of filter-housing (d). Open ball valve 1, open ball valve 2. Air will enter from the purge line and water will exit through sampling line allowing collected particles to settle on the filters.

14 Dismount filter housing (a) by releasing the 3 screws using the hexagonal key. A little bit more water might still flow out through the flowmeter. Keep the filter device in vertical (filters in horizontal) position during the next operations. Remove the rubber ring using the round tip tweezers and place it on a clean Petri dish. If it is not visible, it is still attached to the upper part of the filter housing. Take out the 100 μm filter keeping it carefully in a horizontal position (pointy tweezers) and place it in a clean beaker.

15 Dismount filter housing (b) by releasing the 3 screws using the hexagonal key and repeat the procedure above to collect the sealing ring and the 20 μm filter.

16 Dismount filter housing (c) by releasing the 3 screws using the hexagonal key and repeat the procedure above to collect the sealing ring and the 100 μm blank filter.

17 Dismount filter housing (d) by releasing the 3 screws using the hexagonal key and repeat the procedure above to collect the sealing ring and the blank 20 μm blank filter.

18 Pick up the sealing ring taken form filter (a) with a round tip tweezer and rinse the surface in the corresponding beaker containing filter (a) with about 10 mL 0.1% Triton X 100 solution. The liquid should be enough to cover the filter and it should not be allowed to dry.

19 Repeat the same procedure for the other three rings and filters.

20 Sonicate the beakers for 30 s in a water bath.

21 Remove the filters from the liquid, rinse them with further 20 mL Triton X solution, 20 mL ethanol and dry them with a nitrogen gun while keeping inside the beaker with tweezers. Avoid using high nitrogen flow that would cause liquid spilled from the beaker.

22 The four suspensions are ready for sample pre-treatment preferably without storage period.

23 Equipment should be cleaned with detergent containing water, rinsed with MilliQ water, ethanol and MilliQ water and stored as described above. Filters should be cleaned as described above.

**Reporting**

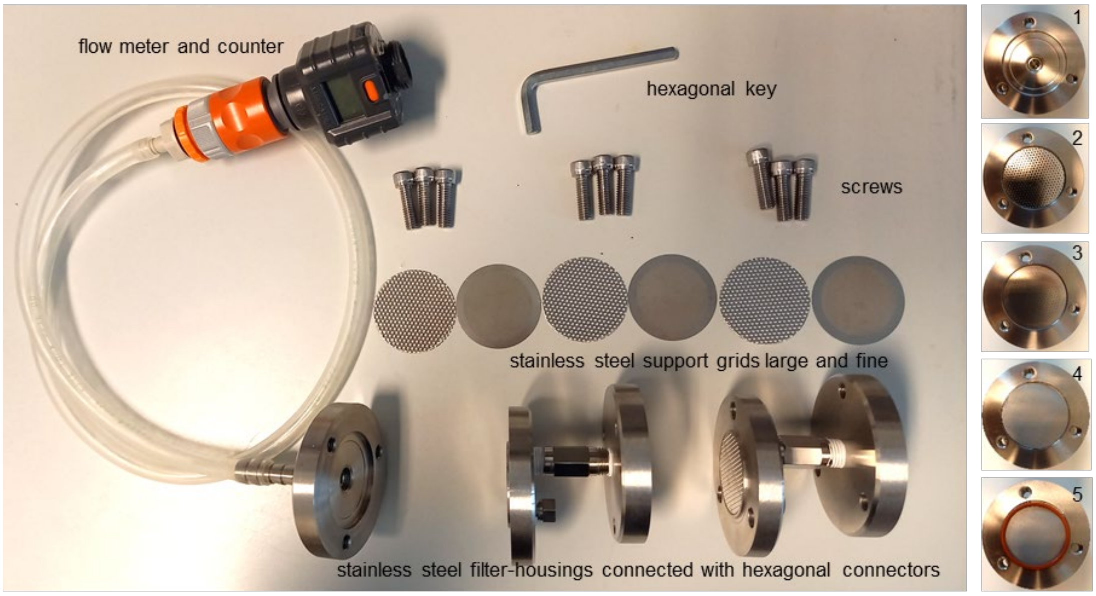
Data registered at sampling should include the

- date of sampling
- place of sampling
- total volume
- any deviation from procedure
- sources of possible plastic contamination with plastic type

- other observations

Samples should be labelled with identifier that allows also the identification of the filter (a), (b), (c) and (d).

Annex I Figure 1. Left: stainless steel elements of dismounted sampling device showing 3 units with flow-meter attached to exit, Right: mounting steps of the sampling device



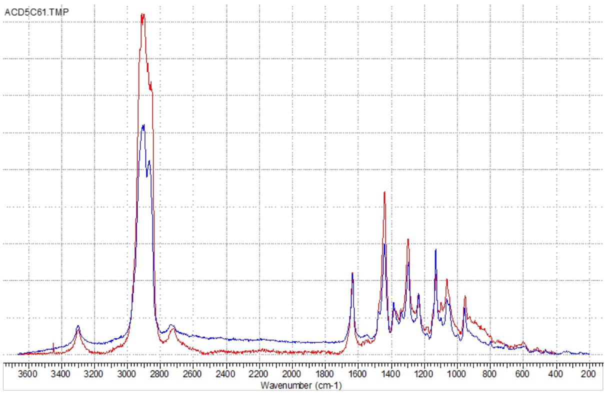
*Note: only 3 elements of the cascade filter series are shown in the figure out of four in total. Ball valves and the purge line are also not shown in this image.

*Source: own production*

Plastic elements in this procedure

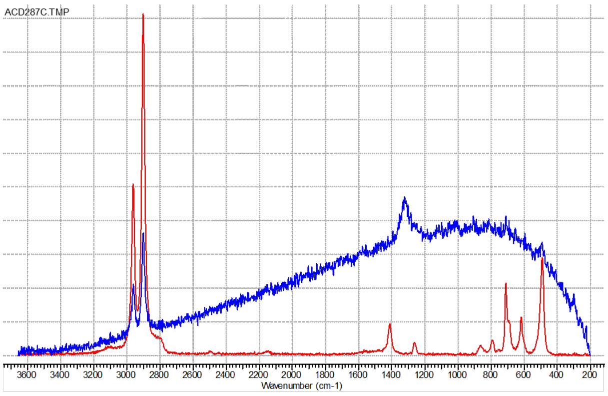
This protocol was developed knowing that a number of component in the system contain plastics. Specifically, polyamide sealing is used in the tubing joints of the water distribution lines, PTFE sealing tape is used in mounting the filtration device and the filter housings were fitted with red rubber sealing rings. The Raman spectra of these materials is shown below (Annex I Figure 2, Annex I Figure 3, Annex I Figure 4)

Annex I Figure 2 Blue: fiber like sealing material used at JRC, Red: polyamide-6,6



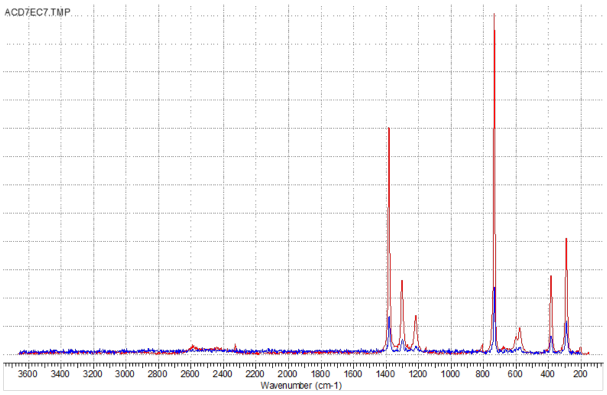
*Source: own production*

Annex I Figure 3 Blue: rubber sealing ring, Red: silicone (polysiloxane)



*Source: own production*

Annex I Figure 4. Blue: PTFE tape, Red: reference PTFE



*Source: own production*

## Health and safety considerations, waste treatment

The sampling device is built from stainless steel elements that are heavy. The operator should wear proper, closed shoes to avoid any damage in case of heavy pieces falling.

If the sink is blocked and/or does not allow a proper exit flow, water might flood the place of sampling. Proper exit flow has to be verified and attention has to be dedicated that no electric devices can get in contact with water during the sampling process.

During the cleaning of stainless-steel filters in Piranha solution the operator should wear the appropriate personal protective equipment (PPE, *i.e.* gloves, laboratory coat, closed shoes, goggles). The operation should be performed under a fume hood. Piranha solution and acid residuals should be collected in liquid waste containers together with other acid waste that is stored under fume hood. The container should not be immediately closed after addition of Piranha waste to allow the free exit of formed gases.

## Annex II Sample treatment

| Version | Authored by | Date |
|---|---|---|
| v1.0 | Dora Mehn | 23.03.2024 |
|  | Reviewed by | Date |
|  | Douglas Gilliland | 15.04.2024 |

### Scope and limitations

The scope of this document is to provide detailed description on how the concentrated samples containing particles collected from the various cut-off filters (including blank filters) can be treated before transferring the particles to the analysis filter. These preparation steps serve to decrease the amount of non-plastic microparticles in the sample. This helps to avoid overloading the analysis filter with particles which would result in too large a number of particles to be analysed (extended analysis time) or in overlapping particles (atypical spectra). Sample treatment steps can include density separation, treatments with acids, bases, enzymes, oxidative agents, and solvents. This document does not discuss treatment with bases and enzymes as the organic material content of the matrix is expected to be relatively low. The various treatment steps are optional and might depend on the quality of the sample. In some cases, the order of the steps is important, as explained below for oxidative treatment. Each purification step is intended to improve the quality of the sample for analysis, but carries the risk of losing particles, which can compromise recovery.

### Principle

Acid treatment is applied to dissolve iron-oxides, calcium and magnesium salts in order to decrease the amount of inorganic particles. Hydrogen peroxide treatment is used to reduce the concentration of organic components in the sample. Density separation can be applied to eliminate non-acid soluble and non-oxidisable particles, such as silicon-oxide (sand, diatom) particles. Rinsing with ethanol helps to dissolve organic contaminants and to produce a more homogeneous distribution of particles on the surface of the analysis filter.

All equipment parts and tools have to be thoroughly washed with water-based detergent, filtered ethanol and rinsed with MilliQ water possibly immediately before use. If the latter is not possible, cleaned equipment parts have to be stored in glass containers covered with a glass lid or aluminium foil prior to use. During sample treatment, the operator should use gloves when working with acids and oxidising agents and should wear cotton laboratory coat. The operator should avoid using cosmetic products that might interfere with the sample (make-up, nail polish, hair wax). The use of plastic tools and ingredients should be avoided during the full sample treatment process if possible. In case it becomes impossible, plastic components of the tools should be carefully reported as possible sources of contamination.

The volume and surface area of the vials used during the procedure should be kept to a minimum. The larger the surface area of containers and vials, the greater the possibility of losing particles by adsorption on surfaces. The surfaces of beakers, funnels and containers should be as smooth as possible, without scratches or physical features that favour the attachment of microscopic particles.

Particles collected from different cut-off filters can be treated in different ways, but their respective blanks must go through the same treatment steps as the corresponding filter for sample collection, i.e. particles collected from filter (c) have to be treated as those from filter (a) and particles from filter (d) have to be treated as those from filter (b). For description of filters, refer to Figure 1 in the main text and to Annex I.

### Equipment and reagents

Particle suspensions (in 1:1 Triton X-100 0.1% w/w solution: ethanol mixture) in 200 mL glass beaker

Vacuum pump for filtration device (Laboport Knf)

Glass filtration funnel 47 mm with PTFE base (Millipore, XX1014704)

Stainless steel 13 mm or glass 25 mm filtration funnel (Sigma Aldrich, XF3001200)

Stainless steel 47 mm mesh filters with 20 µm cut-off (x2, fteu, Germany) + silicone O ring

Anodisc 13 mm or 25 mm filters with 0.1 µm cutoff

Stainless steel tweezers (round tip)

Ultrasonic bath

Nitrogen line with spray gun

Glass beakers 200 mL (x4) with appropriate diameter to host a 47 mm filter in horizontal position

Glass petri dish 30-60 mm (x4)

Erlenmeyers 100 mL with ground glass stopper (x2)

Microwave digester vessel, CEM 35 mL, 908035

Laboratory detergent solution (Aquet F17094, freshly prepared)

MilliQ water

Ethanol (99.9%, 0.22 µm filtered, stored at room temperature in Erlenmeyer with glass stopper)

Triton X-100 solution (0.1% w/w in MilliQ water, X100, Sigma-Aldrich. Stored at room temperature in Erlenmeyer with glass stopper)

Concentrated hydrochloric (37%) acid (Sigma Aldrich, 30721)

Concentrated phosphoric acid (85%) (Sigma Aldrich, 438081)

Hydrogen peroxide 30% (Sigma Aldrich, H1009)

Zinc chloride (Sigma Aldrich, 208086)

Aluminium foil

Laboratory Centrifuge for 50 mL tubes, Eppendorf 5804R equipped with swing rotor

### Acid treatment procedure

Taking into account the final volume of the sample, the appropriate amount of concentrated acid should be added slowly to the beaker under continuous stirring to reach a final concentration of 10% for HCl or 25% for phosphoric acid.

*For example: In case of 40 mL starting volume, 15 ml 37% HCl is needed to reach a final concentration of 10%.*

*The volume of concentrated acid to be added would be estimated as follow:*

$$V_{acid} = \frac{\%_{final} \times V_{sample} \times d_{sample}}{d_{acid} \times (\%_{acid} - \%_{final})}$$

Where:

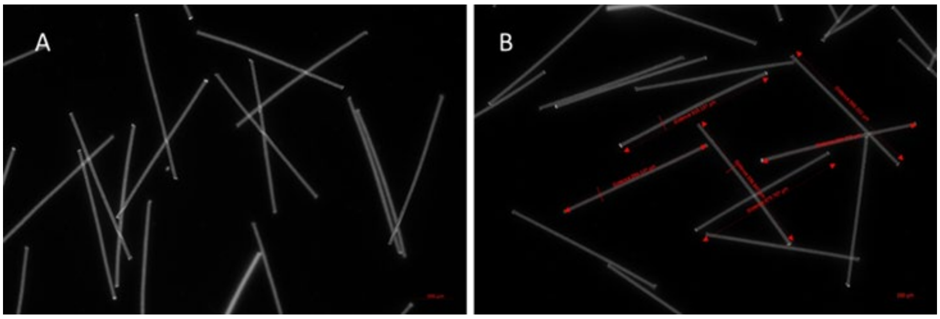
$V_{acid}$ is the volume of concentrated acid to add (mL)
$V_{sample}$ is the starting volume of sample (mL)
$d_{sample}$ is the density of the starting sample diluent (≡ 1 g/mL if water)
$\%_{final}$ is the weight percentage of acid aimed at the end (e.g. 10% for HCl, 25% for $H_3PO_4$)
$\%_{acid}$ is the weight percentage of concentrated acid (e.g. 37% for HCl, 85% for $H_3PO_4$)
$d_{acid}$ is the density of the concentrated acid (e.g. 1.2 g/mL for 37% HCl, 1.7 g/mL for 85% $H_3PO_4$)

Stirring can be achieved by agitating the beaker manually or using a glass rod. Application of PTFE coated magnetic stirrer is not recommended, because microplastic particles tend to adhere to the hydrophobic PTFE surface. After at least 3 h of incubation at room temperature the acid digested sample can be further processed. It is not recommended to use treatment times longer than overnight digestion (at room temperature).

If further oxidative treatment with hydrogen peroxide is planned, the dissolved iron ions should be removed by filtration prior to oxidative treatment. This filtration step can be performed using 20 µm cut-off stainless steel filters, after diluting the acidic solution to about 100 mL and rinsing the beaker three times with a similar volume of 0.1% Triton X-100 solution.

The filter can then be transferred into the original beaker that fits the size of the 47 mm filter. Particles have to be resuspended from the filter in a similar way as described at the final step of sampling. If the sample is to be subjected to a hydrogen peroxide treatment, the rinsing step with ethanol is not recommended.

Annex II Figure 1 Fluorescence image of polyamide fibres before (A) and after (B) acid treatment with 10% hydrochloric acid. Red arrows show size measurements confirming that dimensions of the fibres did not change during the treatment.



*Source: own production*

### Density separation

Density separation is designed to separate microplastics particles from inorganic particulate contaminants based on their different densities. Most microplastic particles are expected to float in a zinc chloride solution with a density of 1.5 g/mL while sand and other silica-based particles should sediment. It is recommended that a more concentrated zinc chloride solution is prepared, filtered through a 0.2 µm filter and added to the suspension to reach a density of 1.5 g/mL while taking into account the volume and mass of the initial sample.

*For example: about 40 mL of 1.63 g/mL* $ZnCl_2$ *solution has to be added to 10 mL sample suspension in water (with 0.1% Triton X-100) to produce a suspension with a density of about 1.5 g/mL.*

*The volume of concentrated* $ZnCl_2$ *to be added would be estimated as follows:*

$$V_{concentrate} = \frac{V_{sample} \times (d_{final} - d_{sample})}{d_{concentrate} - d_{final}}$$

Where:
$V_{concentrate}$ is the volume of concentrated $ZnCl_2$ to add (mL)
$V_{sample}$ is the starting volume of sample (mL)
$d_{sample}$ is the density of the starting sample diluent (≡ 1 g/mL if water)
$d_{final}$ is the density aimed at the end (e.g. 1.5 g/mL)
$d_{concentrate}$ is the density of the concentrated $ZnCl_2$ solution (e.g. 1.63 g/mL)

The required time for the separation can be estimated based on the Stokes equation, considering that floatation speed (V) will depend on the gravitational acceleration (g), diameter of the particles (D), density of the particles ($d_P$), density of the medium ($d_m$) and viscosity of the medium (v).

$$V = \frac{g \times D^2 \times (d_p - d_m)}{18 \times v}$$

The slowest floating microplastic particles are those that are the closest in density to the density of the medium. Applying only normal gravitational acceleration (*i.e.* 9.81 $m/s^2$) the floatation time of a 20 µm PVC particle (density of about 1.38 g/mL) is expected to be more than 10 hours on a 15 cm length path. Floatation times can be markedly shortened by applying centrifugal acceleration force. At about 260 RCF in a typical laboratory centrifuge the separation time in 35 mL glass vessels becomes less than 10 min for 20 µm PVC particles.

It is important to note, that certain polymers have a higher density than 1.5 g/mL. For instance, in case of PTFE it is 2.2 g/mL, very close to the density of silica particles. As a consequence, the above-described procedure is not applicable to separate PTFE from inorganic matrix components.

**Oxidative treatment procedure**

If hydrogen peroxide treatment is required, it is recommended that it be carried out after acid treatment and subsequent removal of iron ions, as previously described.

Considering the final volume of the sample, the appropriate amount of concentrated hydrogen peroxide should be added slowly to the beaker under continuous stirring to reach the requested final concentration for $H_2O_2$.

*For example: In case of 20 mL starting volume, about 20 ml 30%* $H_2O_2$ *is needed to reach a final concentration of 15%.*

*The volume of concentrated* $H_2O_2$ *to be added would be estimated as follow:*

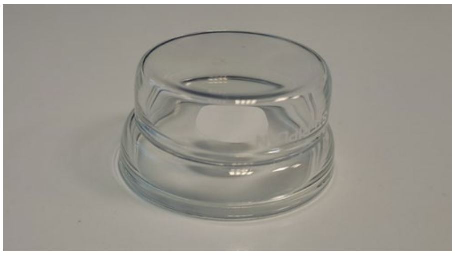
$$V_{H_2O_2\,Conc.} = \frac{\%_{H_2O_2\,final} \times V_{sample} \times d_{sample}}{d_{H_2O_2\,Conc.} \times (\%_{H_2O_2\,Conc.} - \%_{H_2O_2\,final})}$$

Where:
$V_{H2O2\ Conc.}$ is the volume of concentrated $H_2O_2$ to add (mL)
$V_{sample}$ is the starting volume of sample (mL)
$d_{sample}$ is the density of the starting sample diluent (≡ 1 g/mL if water)
$\%_{H2O2\ final}$ is the weight percentage of $H_2O_2$ aimed at the end (e.g. 15%)
$\%_{H2O2\ Conc.}$ is the weight percentage of concentrated $H_2O_2$ (e.g. 30%)
$d_{H2O2\ Conc.}$ is the density of the concentrated $H_2O_2$ (e.g. 1.11 g/mL for 30% $H_2O_2$)

Direct application of concentrated $H_2O_2$ on the stainless-steel filter is also possible if the hydrochloric acid was thoroughly rinsed from the filter beforehand. The beaker has to be covered (e.g. using aluminium foil), but not tightly closed in order to avoid the increase of pressure within the reaction vessel. Typical time for oxidative treatment by $H_2O_2$ is 12-24 h at room temperature. If the treatment is performed at higher temperature, the temperature should not exceed 60 °C and in case of a heated vessel a condenser should be fitted to return the evaporated water to the sample.

If no further processing is foreseen, the particles can be directly collected on the analysis filter, the beaker should be rinsed three times with 20 mL of 0.1% Triton X-100 and finally with 20 mL filtered ethanol. The vacuum filtration should be run for another 2-3 minutes after all liquid is passed in order to dry the filter. The fragile alumina filter is easier to remove from the filter unit when dry. It has to be placed on the top of a Petri dish turned upside down and covered with the bottom part of the dish (Annex II Figure 2). It is recommended not to wear plastic gloves during this process, as the electrostatically charged glass surface may attract particles. Filters with particles should be stored and transferred to the place of analysis in covered state.

Annex II Figure 2 Alumina filter placed on top of a Petri dish turned upside down and covered by the bottom part of the dish



*Source: own production*

### Reporting

The report about sample treatment should mention possible subsampling (volumes) and detail all the physical and chemical steps used in the process. The report should also mention if the equipment contained polymeric components (*e.g.* PTFE - in our case as part of the filtration device) or if the procedure might have compromised the quantitative analysis of certain polymer types (density separation of high density polymers, acid treatment of polyamide, etc.).

### Health and safety considerations, waste treatment

Operations including the handling of concentrated acids, and 30% hydrogen peroxide should be performed under a fume hood and wearing the appropriate personal protective equipment PPE (laboratory coat, gloves, safety goggles, closed shoes). Acid and hydrogen peroxide residuals should be collected in appropriate liquid waste containers together with other acid waste that is stored under fume hood. $ZnCl_2$ should be collected as separated waste or could be recycled by filtration through a 0.1 µm filter.

## Annex III Spiking of drinking water for recovery studies in analysis of microplastics in water for human consumption

| Version | Authored by | Date |
|---|---|---|
| V1.0 No previous history | Dora Mehn | 11.09.2023 |
| | Reviewed by | Date |
| | Douglas Gilliland | 19.09.2023 |

### Scope and limitations

The document provides detailed description of how to spike drinking water with known quantities of fluorescent, compact spherical microplastic particles for the recovery evaluation of sampling and/or sample pre-treatment.

Similar experiments can be performed with particles that are not labelled or are coloured only on the surface by non-covalently binding stain, but the readout at the end of the experiment cannot be done with a fluorescent microscope. On the other hand, the fluorescent label of the particles in this procedure interferes with the Raman based detection (532 nm excitation wavelength) of microplastics. Non compact (hollow sphere) particles were found to be non-suitable for testing the recovery of the sampling procedure because of the damage of particles due to mechanical forces under the conditions of in-line filtration.

### Principle

This procedure is based on depositing fluorescent labelled, non-porous plastic beads on a flat, solid and water soluble carrier which permits the particles to be counted using optical microscopy.

Spherical plastic beads are used for the proper control of particle size and to facilitate quantification, as irregular shaped particles are more difficult to size and distinguish when appearing in agglomerates. The fluorescent label is applied to facilitate the quantification of the particles and to make easier distinguishing them from particles that are present in the sample. Fluorescent labelled plastic microbeads might be commercially available or might be generated in house. However, beads that are stained only on their surface by non-covalent interaction between the polymer and the dye are usually not resistant to washing steps using solvents such as ethanol, thus they are not appropriate for these kind of experiments. The spiking particles also have to possess appropriate mechanical stability to pass the filtration process without mechanical damage. Some commercially available fluorescent polyethylene beads were found to be fragile, opened up and released their fluorescent content during the sampling procedure.

The water soluble, flat and solid support made of KBr allows the observation and counting of the deposited particles by using a fluorescent microscope, the easy transfer of the particles to the point of spiking and the complete dissolution of the carrier in a water-based fluid. The 13 mm pellet (at the suggested mass range) is thin enough to fit in the space available in the filter housing and does not damage the filter. KBr is transparent for both visible and IR light and - being an inorganic compound - it is not supposed to interfere with the analysis of plastic particles in any way.

In order to study the recovery of particles of various sizes in the same process, it is possible to deposit multiple particle types on the same KBr pellet. If particle sizes are similar, it is recommended to use different fluorescent label that allows the better identification of particle types. At the end of the series of treatment steps, particles are collected on a small (13 or 25 mm) alumina filter appropriate for spectroscopic analysis. The number of spike particles is counted then on this filter by fluorescent microscopy. Recovery (for each particle type separately) is calculated according to the equation 1 below:

Eq1

$$Recovery = \frac{Number\ of\ particles\ collected\ on\ filter\ for\ analysis}{Number\ of\ particled\ deposited\ on\ KBr\ pellet} * 100\%$$

Equipment and reagents

Graseby Specac Manual Hydraulic Press, 15 ton capacity

KBr pellet die, ICL 13 mm

Stainless steel spatula

Agate mortar (small, 3 cm diameter) with pestel

Micro-balance

Glass filtration device for 25 mm filters

Microwave digester, CEM SPD 35

Microwave digester vessel, CEM 35 mL, 908035

PTFE coated magnetic stirrer bar (8-10 mm)

Fluorescent microscope (Axio Imager M2, Zeiss) +software (ZEN 2.3, blue edition)

Stainless steel tweezers (pointy and round tip)

Ultrasonic bath

Nitrogen spray gun

Aluminium oxide Anodisc filters, 0.1 µm pore size, 25 mm

10 µL pipette, Eppendorf Research

Glass petri dish, 10 cm diameter

Glass bottle with screw cap, PTFE liner (Sigma Aldrich)

Pipette tips 10 uL, Eppendorf

Eppendorf tubes, 1.5 mL

MilliQ water

Ethanol (≥99.5%, Merck 1070172511)

Acetone (≥99.5%, Merck 904082)

KBr (Uvasol® for IR spectroscopy, Merck B1954607)

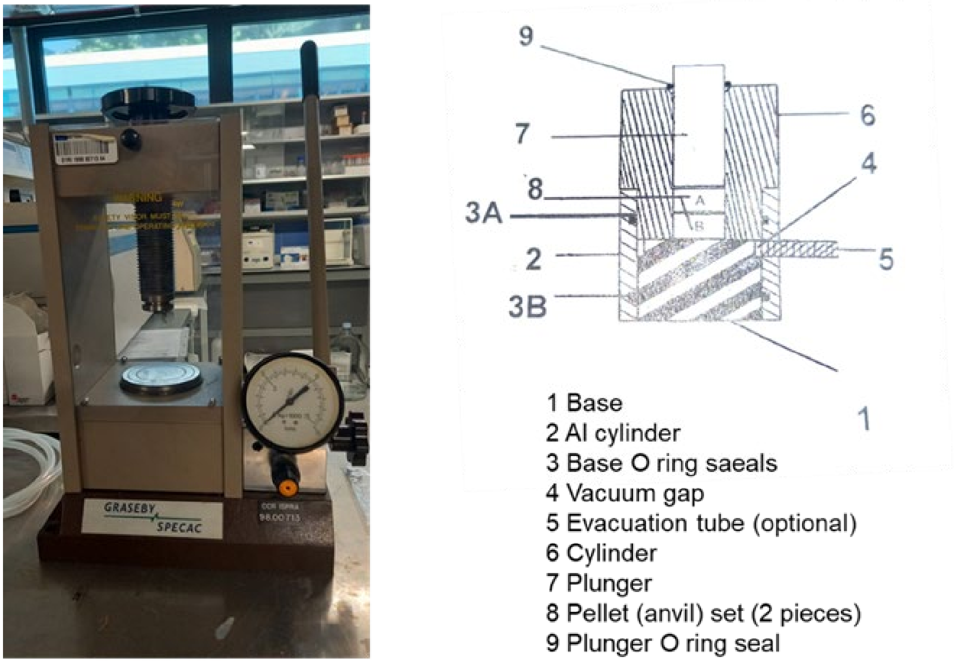
Polymethyl methacrylate (PMMA) particles, 30 µm (Supelco, Merck 77529) stained with Nile red (for microscopy, 72485, Merck)

Fluo-Max fluorescent polystyrene (PS) particles, 143 µm (ThermoScientific 35-13)

KBr pellet preparation

The figure below (Annex III Figure 1) is a schematic drawing of the die and a photograph of the press for KBr pellet production.

Annex III Figure 1 Left: Photograph of the press, right: schematic drawing of the pellet die



*Source: own production*

Follow the operating instructions of the die (ICL's macro/micro KBr die Instructions ICL Part No. 0012-2477) to generate a pellet.

Use a clean, stainless-steel spatula to measure 0.15-0.20 g of KBr in an agate mortar placed on a microbalance. Grind it with the pestle to fine powder and load it in the die to create a pellet. No air suction is needed, press the die at 8-10 tons for 30s. Stop pressing, wait another 30s and release the die. Dismount the die, turn upside down, and release the pellet from die. Clean the die by washing it with distilled water and drying it carefully by paper and nitrogen stream.

Inspect the resulting pellet for smooth surface and even thickness. Transparency of the pellet is not so important here, cloudy area close to perimeter is not excluding the pellet from use. On the contrary, smooth surface and thickness are crucial because particles to be visualised by microscope should stay on the same focal plane. Thinner pellets dissolve faster in water.

KBr pellets are hygroscopic. If the pellet is not used immediately it is recommended to store it in a glass Petri dish in desiccator or in sealed glass container.

### Nile red staining of PMMA particles

Fluorescent particles can also be generated in-house by deep staining of particles using a hydrophobic dye. The Nile Red staining of 30 µm PMMA particles is performed as follow.

Prepare 1 g/L Nile Red solution in acetone. Filter it using 0.1 µm Anodisc filter and glass filtration device into a glass bottle with screw cap that is equipped with PTFE liner. Prepare 10 mL of 50% water - 50% ethanol mixture in a round bottom 35 mL microwave digester tube. Place a small PTFE stirrer in the tube. Add 100 µL of the original PMMA suspension. Add 100 µL of the concentrated Nile Red solution. Sonicate the mixture for 30 s. Place the tube on the rack of the microwave digester and follow the working instructions of the equipment to run a heating program for 10 minutes at 120 °C and consecutive cooling down of the mixture. Remove excess Nile Red in the solution from the particles by filtration using a 10 µm polyamide filter, rinse particles with 20 mL ethanol and resuspend them in 10 mL ethanol.

During the heating and cooling process, Nile red penetrates the PMMA particles and remains entrapped in the more hydrophobic environment even in an ethanol-based suspension. Ethanol wets well the particles and evaporates fast from the surface of KBr pellets. The concentrated spiking suspension generated in this way can be used at least for 2 months without visible loss of the staining or aggregation of the particles.

**Spiking procedure**

The total number of particles to be used for spiking might depend on experiment, typical number is 50-300.

1 Start up the fluorescent microscope, run stage calibration. Switch on lamp for fluorescent detection. If commercial, concentrated particle suspension is used, add 1 mL of ethanol in an Eppendorf tube and mix it with 10 µL of the concentrated spiking suspension. Sonicate the suspension for 10 s in a water bath sonicator.

2 Place a clean microscope slide on the stage of the fluorescent microscope. Place a KBr pellet on the microscope slide using stainless steel tweezers. Homogenise the particle suspension and deposit 1 µL of it on the KBr pellet. Wait that the drop dries and inspect the particles under the microscope using the ocular and bright field illumination. In case of too low numbers, additional drops can be added, preferably at a separate location to avoid the generation of multi-layer particle aggregates, where individual particles in bottom layer(s) are hard to identify. The same is true when depositing different kind of particles on the same KBr pellet. Avoid to deposit mixture of different size particles, as small particles adsorbed on big ones are more difficult to identify.

3 Collect image of the deposited particles by using a 5x objective, appropriate fluorescent illumination and image stitching, covering the full area of the drop(s). The borders of the dried drop are usually better visible in bright field, but particles are better visualised using the fluorescent mode.

4 Count the total number of particles deposited on the pellet by using the Event tool of the microscope and selecting each particle. Add scale bar to the image – this tool will also add the total number of counted particles. Generate picture from the image and save it in jpeg format. Alternatively, the counting can be performed on an exported image by means of different tools, such as the ImageJ software.

In case of too high number of particles deposited on the pellet, the pellet has to be discarded, and the spiking suspension has to be diluted before repeating the procedure with less particles in the same deposited volume.

5 Move the glass slide holding the pellet carefully in a glass Petri dish and cover it during transfer from the microscope to the spiking point.

6 Use the full KBr pellet carrying the particles for spiking. It can be added to a water-based liquid or placed in the sampling device. Handle the pellet with stainless steel tweezers and keep it horizontal with the spiked side facing up during transfer. In case it is used for testing the sampling procedure, it has to be placed on the first (100 µm, (a)) filter of the sampling device before mounting it to the upper part of the filter housing and closing it with the 3 screws (see details of sampling procedure, Annex I). In this case, it is recommended to let the water first flow slow (1 L/min) for about 10 minutes.

7 At the end of the series of treatment steps, particles are collected on a small (13 or 25 mm aluminium oxide filter appropriate for spectroscopic analysis. The number of spike particles is determined by fluorescent microscopy.

## Reporting

Data registered at spiking should include the

- date of spiking
- number of particles and nominal size deposited on the KBr pellet
- number of particles counted on the analysis filter
- recovery calculated according to the equation 1 of the present Annex
- any observation worth to note.

## Health and safety considerations, waste treatment

The KBr press and sampling device is built from stainless steel elements that are heavy. The operator should wear proper, closed shoes to avoid any damage in case of falling heavy pieces.

The pressure at the KBr pellet production procedure should not exceed 10 tons and the Plexi window should be kept in down position when applying pressure to avoid being hit by accidentally released, flying items.

Microwave digester has to be operated under a closed fume hood. Always ensure that there is appropriate nitrogen supply, the wash bottles are filled, and flow is not blocked. The digestion vessel always has to contain a magnetic stirrer, the lack of stirrer might lead to uneven heating and explosion of the vessel.

Organic solvent residuals (including their suspensions containing microplastic particles) have to be disposed in the non-halogenated organic solvent liquid waste container.

Wear appropriate PPE (nitrile gloves) when working with acetone.

## Annex IV Analysis of microplastic particles by FT-IR microscopy and chemical mapping

| Version | Authored by | Date |
|---|---|---|
| v1.1 | Dora Mehn | 05.12.2023 |
| | Reviewed by | Date |
| | Claudia Cella | 15.01.2024 |

### Scope and limitations

The document provides detailed description on how to collect transmission FTIR spectra about samples potentially containing microplastics particles deposited on aluminium oxide filters by using the Bruker Hyperion 3000 FTIR microscope equipped with a 64x64 pixel focal plane array (FPA) detector and how to analyse data and recognize polymeric particles by using the software from Purency.

Similar measurements can be performed using other instrumentation and analysis software, but the actual details are specific for the above-mentioned combination. Plastic recognition requires reference spectra. The ones used for identification were collected under similar analytical conditions (in transmission mode, using aluminium oxide filters) between 4000 and 1300 cm$^{-1}$. Importing data from different data format than the ones generated by Bruker acquisition software (OPUS) might require modification of the analysis software. The analysis software has limited availability due to licencing strategy of the company.

The method is suggested to be used for the analysis of particles in the 20-100 µm range. Below 20 µm size the number of FPA pixels collecting information about the particle becomes too low and signal to background ratio is not sufficient for identification, while above 100 µm spectra might appear saturated.

### Principle

This procedure is based on (i) depositing the particles to be analysed on an aluminium oxide filter, (ii) collecting an optical image by image-stitching with a lower resolution objective (4x) followed by (iii) collecting spectra about the area of interest in transmission mode by a Bruker Hyperion 3000 FTIR microscope using the 15 x FTIR objective and the FPA detector. The collected data are then exported in a format that can be imported in the analysis software.

The analysis software allows the superposition of the optical image with the spectral hypercube. Recognition is not based on comparison with individual spectra of a database, but with searching similarities between the collected spectrum and sets of spectra corresponding to 20 polymer types and an additional “Other” category. The large database of the software contains many spectra corresponding to a single polymer type, including weathered, small and large particles. On the other hand, the identification is limited to 20 polymers and there is no information shared about the chemical identity in the “Other“ category (*e.g.* cellulose, mineral, etc.). In addition, the software considers that absorption spectra might appear truncated when particles are too thick, spectra might appear noisy when particles are too small and when materials might be weathered.

## Equipment and reagents

Bruker Hyperion 3000 FTIR microscope with 4x visible and 15x FT-IR objectives + software (OPUS v. 8.8.4)

Imagelab, Purency Microplastics Finder v. 4.17

Stainless steel tweezers (round tip)

Aluminium oxide Anodisc filters, 0.1 µm pore size, 13 mm

Glass petri dish

Liquid nitrogen

## Data collection

1 The instrument is always kept switched on in order to decrease the amount of adsorbed water in the spectrometer. Switch on the computer. Start instrument software. Check if conditions of the spectrometer are right. If humidity is too high, heat-treat / replace adsorbent. If needed, purge with nitrogen using the connections entering the spectrometer on the back side.

2 Place grids (attenuator, low pass, between spectrometer and microscope) in the way of light by pulling them in upper position.

3 Check cleanness and if needed clean the motorised stage of the instrument from particles. Carefully load both liquid nitrogen containers of the instrument with liquid nitrogen. Wait 15 min till the FPA detector cools to the working temperature range.

4 Place the dry Anodisc filter in the appropriate sample holder on the motorised stage. The sample has to be completely dry as water signal appears in the spectra disturbing spectral recognition. Minimise the reload of the liquid nitrogen containers while sample is on the stage or cover the sample while reloading as particles might be moved by the pressure of evaporating nitrogen.

5 Start Wizard on the OPUS software for video guided measurements. Select Imaging device Hyperion 3000 – FPA. Allow calibration of the stage if the computer was switched off.

6 Select manually the 4x visible objective. Change to transmission mode with microscope control button. Search for focus position. Adjust the condenser position by closing the hole with the wheel behind the motorised stage, set light in central position with the dedicated screws and sharpen the image of the hole. Reopen the hole. Check light position on video image and adjust if needed. The red square on the screen defines the size of a single tile. Open Video camera settings (right click in image, Hardware settings) and remove the tick mark of automatic setting from exposure, gain and white balance.

7 Select 4 x objective in the Objective control drop down menu of the software. Define overview image by moving the stage with the joystick and adding new border points at the top, bottom, left and right margins of the filter. Collect defined image.

Warning: the collected image is stored at a lower resolution than the image actually captured. It is an unfortunate feature of the software that gives still acceptable resolution images for 13 mm filters, but might compromise image resolution if larger areas are imaged.

8 Select manually the 15x FTIR objective. Re-adjust focus and centre light with the condenser adjustment screws. Select 15x IR objective in the Objective control drop down menu of the software.

9 Go to Next step and search for an appropriate area without particle on the filter to collect background. The yellow square on the screen defines the area covered by the FPA detector. Measure background ones at the current position. Adjust settings of the background measurement considering that spectrum collection should be done with the same settings. Suggested values: 4x4 pixel binning, 4 scans, 6 $cm^{-1}$ resolution, Method: "Hyperion 3000-FPA_TRANS Anodisc.xpm", Acquisition and data saving 1300-3950 $cm^{-1}$, absorbance spectra. Look at signal in live mode, monitor background and set the gain in the appropriate range. Collect background.

10 Go to Next step and select the area on the image to be mapped. In case of 13 mm Anodisc filters it is worth scanning 1/3 of the filter at a time. This allows to decrease the area that is outside the filter and reload liquid nitrogen between scans if needed. Click on Next when measurement is finished. After this step the Chemical image file together with the collected optical images are automatically saved.

11 Remove the sample from the microscope stage. Clean the stage with wet paper towel. Decrease illumination of the microscope. Do not switch off the spectrometer.

## Data analysis

1 Load the Opus chemical imaging file on the OPUS software. Export optical image by right click on it, then "Export image" "to file" by keeping resolution as high as possible. Save file as ENVI data file by selecting ENVI in the Mode menu point and taking care of proper file naming (i.e. remove .img from file name). Copy data parameters by: right click on file name, Show parameters, opening Data Parameters AB/multiple, right click on right panel, Copy to Clipboard.

2 Open the Imagelab Purency Microplastics finder. The file saved as ENVI file appears with .hdr extension in the file list. Import the ENVI data file (File/Import Data) by selecting Data file, IR spectrum type and marking the Mirror Y Axis option.

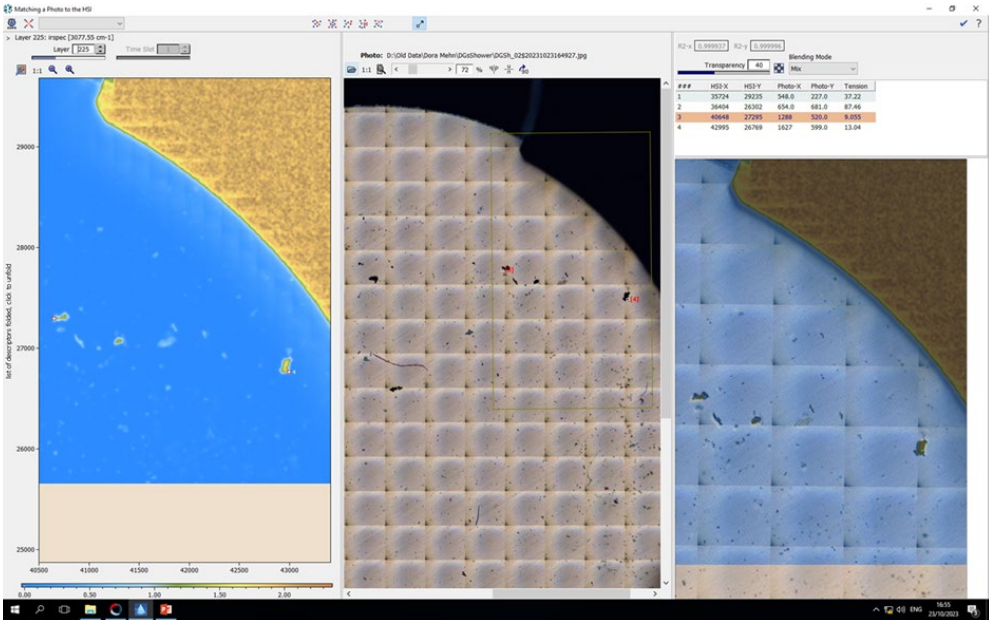
3 Import the measurement parameters at Tools/Recalibration/Import opus calibration data/Paste data from Clipboard. Add the saved optical image to the HIS data with the menu button "Add photo…". Select the image in the list of attached photos and calibrate the photo in the appearing "Matching a Photo to the HSI" window (see Annex IV Figure 1). It allows the user to select 4 points on both the optical and hyperspectral image (HSI) and to pull them to the same position, until the two images become perfectly overlaid. Best option is to select 4 particles with well recognizable shape or sharp edges. Selecting a layer in the HSI that belongs to the C-H stretching region (around 300 $cm^{-1}$) improves the visibility of particles. Accept the overlay in the right upper corner using the "take over the current calibration" button. Close the List of attached photos window. Save the file.

4 Run particle analysis by pressing the dedicated button under the menu bar. Analysis time depends on the size of the image, but usually it is less than 10 min. Inspect particles one by one, compare with optical image, add or remove pixels if needed for correction of particle size. The spectrum of added pixels can also be compared to that of the neighbouring ones.

When complete, save the particle analysis file.

Warning: Critically inspect the spectrum of polyamide (PA) particles. The Purency ImageLab software was found to often misinterpret natural polyamide (protein) spectra as polymer particles.

Annex IV Figure 1 Screenshot illustrating the overlay process of HSI layer (left) and optical image (center) of the same filter with particles. Combined image appears in the right



Layer 225: irspec [3077.55 cm-1]

Layer 225

Time Slot 1

Photo: D:\Old Data\Dora Mehn\DGsShower\DGSh_02$20231023164927.jpg

72 %

R2-x 0.999937 R2-y 0.999996

Transparency 40

Blending Mode Mix

| ### | HSI-X | HSI-Y | Photo-X | Photo-Y | Tension |
|---|---|---|---|---|---|
| 1 | 35724 | 29235 | 548.0 | 227.0 | 37.22 |
| 2 | 36404 | 26302 | 654.0 | 681.0 | 87.46 |
| 3 | 40648 | 27295 | 1288 | 520.0 | 9.055 |
| 4 | 42995 | 26769 | 1627 | 599.0 | 13.04 |

*Source: own production*

## Reporting

Data has to be exported and area of the particles ($\mu m^2$) has to be transformed to area equivalent circle diameter according to the following equation.

$D=(A/\pi)^{1/2}$

Data should be reported after classification of the particles on the basis of their polymer types, size bin and shape - irregular shaped particles or fibres.

Reporting has to consider the filtered water amount, report the concentration of particles in filtered volume and scale the reported numbers accordingly.

For example, if $V_{tot}$ [$m^3$] of water was filtered, it was resuspended in a volume of $V_{conc}$ [mL] but only $V_{proc}$ [mL] was processed and transferred to a filter, where x% of the filter area was analysed, and n particles of a certain type was found on the filter, the original concentration of particles is calculated as:

$$c\ [1/m^3]= n*(100/x)*(V_{conc}/V_{proc})/V_{tot}$$

## Health and safety considerations, waste treatment

Follow the specific working instruction about the handling of cryogenic liquids and wear appropriate PPE when working with liquid nitrogen.

Filters after analysis (including microplastic particles) should be disposed in the solid laboratory waste container.

## Annex V Analysis of microplastic particles by Raman microscopy

| Version | Authored by | Date |
|---|---|---|
| v1.1 | Dora Mehn | 05.12.2023 |
| | Reviewed by | Date |
| | Claudia Cella | 18.01.2024 |

### Scope and limitations

The document provides detailed description on how to collect Raman spectra from particles deposited on aluminium oxide (Anodisc) filters using the Renishaw in Via Qontor Raman microscope with the help of the WiREParticleAnalysis software.

Similar measurements can be performed using other instrumentation and analysis software, but the actual details are specific for the above-mentioned combination. The method does not require the use of a specific spectral database, but spectral databases containing both polymeric and non-polymeric material spectra is recommended. This procedure was tested using the 532 nm laser of the above-mentioned instrument. Other laser (785 nm) of the same instrument can be applied, but laser power and illumination time have to be tested in order to avoid damaging the microplastic particles due to high transmitted laser power.

### Principle

This procedure is based on depositing the particles to be analysed on an Anodisc filter and collecting an optical image by image-stitching using a 10x (bright-field, N PLAN) objective in transmission mode. Both optical image and spectra are collected using a Renishaw in Via Qontor Raman microscope. The WiREParticleAnalysis software is applied for image analysis and for controlling spectrum acquisition from the selected particles at single positions by the same micro-spectroscope using the same 10x objective combined with the Live Track function of the microscope. This function allows to set the focus before measurement by optimisation of the Raman signal intensity arriving to the detector. Measurements for the various particles do not happen on the same focal plane but are individually optimised for each particle. The collected data are processed using the WiREParticleAnanlysis software.

This software allows to run a pre-recorded chain for data treatment that includes truncation, baseline subtraction and comparison with spectral database(s). As a result the image of each analysed particle is associated with a spectrum, a material name and the hit quality of the match with the database reference spectrum.

### Equipment and reagents

Renishaw in Via Qontor Raman microscope equipped with 532 nm laser, WiRe software v5.6

Renishaw Particle Analysis software v5.6.30846

Stainless steel tweezers (round tip)

Aluminium oxide Anodisc filters, 0.1 µm pore size, 13 mm

Sample holder for 13 mm Anodisc filters (from FTIR microscope)

Glass petri dish

### Data collection

1 Switch on the instrument. Launch the WiRE software. Reference all motors. Switch on the 532 nm laser, select 1800 l/mm optical grating. Perform fast calibration using the internal Si standard of the instrument. Launch the Particle Analysis software.

2 Place the sample (Anodisc 13 mm) using an appropriate sample-holder in middle of a hole on the Raman microscope motorised stage. As an example, the 13 mm sample-holder of the FTIR microscope can be used by placing it on the Raman microscope stage. Change manually to objective 10x bright field (BF). Check if objective selection, laser wavelength and optical grid selections are right in the WiRE software. Switch on camera and light (both for BF and transmission). Bring the light to the centre of the filter. Set 0,0 position in the software at sample centre. Switch off light for BF keep light for transmission on.

3 Adjust focus and camera settings to get sharp image of particles with good contrast compared to brighter background. Send camera to 0,0 to check if everything was properly set: if yes, the stage should not move. Collect image using the menu point Live video/Snap montage. Set top, right, bottom, left positions on the filter perimeter and press Run.

4 Reduce your image by selecting an area of interest. In order to minimise the amount of non-valuable information processing, it is recommended to cut all dark corner areas as much as possible around the filter before saving. Save image without axes, keeping original ratio and size.

5 Switch off the light used for transmission imaging. If transmission light is on, Raman spectrum collection will be compromised.

6 Open WiREParticleAnalysis. Start a new project, fill details, add new name, add image with no axes. Set binarisation and particle separation on a partial image and analyse the image. When settings allow to get acceptable identification of particles with not many artefacts added from filter optical features, enlarge the frame, and analyse again. In order to process only the selected region of interest and remove dark corners, select “partial”. The analysis is time consuming; it might take more than 15 minutes to complete and might overload memory if there are too many particles to be analysed, or dark corners are interconnected. In case of restarting from WiRE image export, a new project has to be created.

7 Order particles according to size by clicking on “Area”. Select particles to be analysed (for example larger than 200 $\mu m^2$) and place them in the “Box”.

8 For data collection select measurement template. This template is constructed in WiRE earlier and saved in the folder C:\ProgramData\Renishaw\WiRE2\WiREParticleAnalysis\Measurement Templates as .wxm files. Suggested method: “532_DoraMP_LiveTrack”. This method considers the use of a 532 nm laser combined with the 1800 optical grating, spectral range of 100-3200 $cm^{-1}$ and applies the move of the grating to cover the required range with a single pixel overlap. Automatic cosmic ray removal is foreseen. Laser power and illumination time are free to adjust in the ParticleAnalysis software. For microplastics identificaton, set laser power to 50%, illumination time to 0.5 s, 3 repetitions, intelligent measurement position selection. The latter will select a measurement point among the recognised pixels of the particle image. (Selecting “Centroid” might be useful in case of the artificial situation of measuring spherical particles, where the central part of the particles appears to be transparent in the optical image.) Allow the use of livetrack by selecting the corresponding option.

9 Start the Live Track function in WiRE software using the menu point Life video/LiveTrack Raman. Select standard signal mode among options, adjust focus manually until you see the smallest laser spot on the screen, set focus, and turn on the Raman signal tracking.

10 In Particle Analysis software, order particles according to ID (chemical identity). This will allow to measure the particles in the order of their position on the filter. The result is less stage movement time that might have a large impact on total measurement time if many particles have to be analysed.

Start collecting spectra. Time estimation will show what to expect for remaining measurement time and spectra will appear in the table next to the particle images, ID and Area.

(Troubleshooting: if spectrum collection does not start, check if measurement Queue is empty in WiRe software)

## Data analysis

1 When the measurement is finished, a data processing chain has to be run. This chain – similarly to the method – has to be pre-registered in WiRE and saved in the folder C:\ProgramData\Renishaw\WiRE2\WiREParticleAnalysis\ChainFiles as .xml files. The suggested chain for microplastics analysis is: “ChainDoraMP”. The chain will truncate the spectra at 300 $cm^{-1}$, perform baseline correction and a spectral library search using commercial polymer and inorganic compound libraries.

2 At the end of the analysis the name of the first hit (hit with highest similarity) will appear in the table next to the spectrum. Particles can be reorganised now according to their size or material and with right click on table header hit quality can be added to the table. It is a value between 0-1, with 1 representing perfect match.

3 Clicking on material name helps to organise particles according to their chemical composition as materials appear in ABC order, thus polymers appear under letter P. Each spectral identification has to be carefully examined. Particles that are not intended to be included in data export can be unboxed. Data report will include the boxed particles and can be customised also regarding size bins.

4 Spectra and evaluation is saved automatically when returning to the main menu. On the other hand, spectra will be overwritten if new measurement is started without generating a new project.

## Reporting

Data have to be exported, and equivalent circle area can be selected as size descriptor. The diameter is calculated from the particle area based on the number of pixels corresponding to the particle in the binarised image. The area of the particles based on pixels might underestimate the real size if particles are (partially) transparent or in case of fibres where the particle might not lay with its full length on the filter, in the same focal plane. Therefore, each particle identified as polymeric material needs individual inspection regarding shape and – if needed - manual correction of size. Data should be reported collecting particles that were identified as polymers in size bins and mentioning if they are irregular shaped particles or fibres. Reporting has to consider the filtered and analysed water amount. In order to be able to report the concentration of particles in the original filtered liquid, the microplastic numbers identified in the analysed filter area have to be properly scaled. For example if $V_{tot}$ [$m^3$] of water was filtered, it was resuspended in a volume of $V_{conc}$ [mL] but only $V_{proc}$ [mL] was processed and transferred to a filter, where x% of the filter area was analysed, and n particles of a certain type was found on the filter, the original concentration of particles is calculated as:

$$c\ [1/m^3] = n*(100/x)*(V_{conc}/V_{proc})/V_{tot}$$

## Health and safety considerations, waste treatment

The instrument has powerful laser sources, but no interaction of laser light with the eyes is possible due to safety mechanisms.

Filters after analysis (including microplastic particles) should be disposed in the solid laboratory waste container.

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