# MICROPLASTICS

*Second edition*

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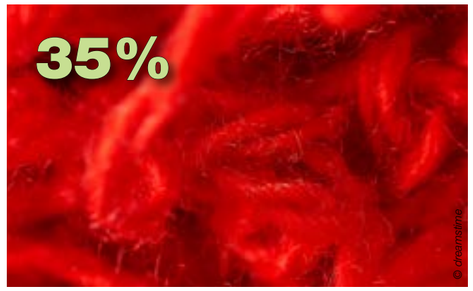
HORIBA Scientific

# What are microplastics?

## Where are they coming from?

**A study by the International Union for Conservation of Nature (IUCN)[1] identified the main sources of Microplastics and divided them into 7 main categories:**

### Synthetic textiles




Synthetic textiles are the single greatest contributors to engineered microplastics in the ocean, accounting for 35 percent of the total volume; indeed washing synthetic textiles frees engineered microplastics through abrasion and shedding of fibers from the fabrics. This is due to the mechanical and chemical stresses that fabrics undergo during the washing process in a laundry machine.
Browne et al.[2] showed that a single garment can release more than 1900 microplastics (<1mm) in each washing cycle and as there are more than 840 million washing machines globally[3] it is clear why synthetic textiles are the main source of microplastics.

### Tires


Today, about 24% of a tire consists of synthetic rubber, a plastic polymer, and 19% natural rubber. Microplastics form a matrix of the synthetic polymers, giving the tire rigidity and providing traction. The rest of the tire is metal and other compounds. Tires erode through heat and friction from contact with the road. The wind and rain spread the tire dust and wash it off the road. It enters tributaries, lakes and eventually the oceans.

### City Dust


City dust, which accounts for 24 percent of microplastics in the oceans, comes from a variety of sources. While each is a small contributor, it adds up in a opulated area. City dust includes losses from the abrasion of objects like synthetic soles of footwear, synthetic cooking utensils and of infrastructure like household dust, artificial turf, harbors and marina building coatings. It also includes particles from blasting, abrasives, weathering of plastic materials and use of detergents.

### Road Making


Crews apply road markings while building and maintaining roadways. Particularly in Europe these markings include polymer tapes and paints. These are thermoplastics that become soft and flexible at warmer temperatures, allowing weathering or abrasion by vehicles to turn them into microplastics.

1. Boucher, J. and Friot D. (2017). Primary Microplastics in the Oceans: A Global Evaluation of Sources. Gland, Switzerland: IUCN. 43pp.
2. Browne et al. ENVIRONMENT SCIENCE & TECHNOLOGY 21 p.9175 2011
3. F. Salvador Cesa et al. SCIENCE OF THE TOTAL ENVIRONMENT 598 p.1116 2017

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### Marine Coatings


Operators apply marine coatings to all parts of seagoing vessels for protection. Coating's developers use several types of plastics for marine coatings, most commonly polyurethane and epoxy coatings, vinyl and lacquers. Weathering and spills during application, maintenance and disposal of these coatings cause the release of primary microplastics.

### Personal Care Products


Many personal care and cosmetic products contain a type of engineered microplastic known as microbeads. The products include scrubbing agents, shower gels and creams.

### Plastic Pellets


Manufacturers often produce primary plastic as small pellets or powders. These producers then transport the pellets to plastic transformers that make end products. Pellets can inadvertently spill into the environment during manufacturing, processing, transport and recycling. Plastic pellets make up 0.3 percent of the ocean's primary microplastics.

# What are microplastics?

## Definition

The term microplastic was coined only in 2004 in a paper published by Thompson et all[1] in Science. In this pioneering work they observed the presence of microplastics for the first time in sediment coming from a UK beach close to Plymouth and their subsequent tests found microplastics in 17 other beaches. microplastics remained mainly an academic topic up to 2018 when the presence of microplastics was observed in bottled water[2] and human stools[3] raising a huge interest from the media.

Nowadays a universally agreed and official definition of "Microplastic" is still missing even if there is general agreement on what this term refers to within the relevant communities (Researchers, media etc.: **Microplastics are small pieces of plastic made from synthetic polymers**.

The National Oceanic and Atmospheric Administration, NOAA, defined in 2009 (Arthur et all[4]) an upper size limit in 2009: **"Piece of plastic particles smaller than 5 mm"**.

In 2015 the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP[5]), added a lower limit, including for the first time, nanoplastics (down to 1 nm): **Microplastics are particles in the size range 1 nm to < 5 mm**.

In our view, the definition which it summarizes all the others and provides an additional constraint around fibers (which are one of the main sources of microplastics in marine environments, see "where are they coming from?" section) is the one used by the European Chemical Agency in their Annex XV Restriction Report on Intentionally added Microplastics of August 2019[6].

*'Microplastic' means a material consisting of solid polymer-containing particles, to which additives or other substances may have been added, and where ≥ 1% w/w of particles have (i) all dimensions 1nm ≤ x ≤ 5 mm, or (ii), for fibr-es, a length of 3 nm ≤ x ≤ 15 mm and length to diameter ratio of >3. Polymers that occur in nature that have not been chemically modified (other than by hydrolysis) are excluded, as are polymers that are (bio)degradable.*

1. Thompson et al., SCIENCE, 304 p.838 2004
2. Mason et al., FRONTIERS IN CHEMISTRY, 6 (article 407) p.1 2018
3. Schwabl et al., ANNAL INTERNAL MEDICINE, 171(7) p.453 2019
4. Arthur, C., J. Baker and H. Bamford (eds). 2009. Proceedings of the International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris. Sept 9-11, 2008. NOAA Technical Memorandum NOS-OR&R-30.
5. GESAMP (2015). (Kershaw, P. J., ed.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP No. 90, 96 p.
6. European Chemical Agency - Annex XV Restriction Report on "Intentionally added Microplastic"

# What are microplastics?

## What are microplastics?

We understand the definition of microplastics as small pieces of solid polymer particles etc., but it is important to make a step forward and identify which are the most common types of Plastics produced globally[1].

Polyolefins (PP and PE based plastics) represent more than 50% of the global production (2015 data) as they have several advantages such as low production costs, good chemical/physical resistance, etc.; advantages that can turn into downsides when considering their lifecycle because they also degrade very slowly and can survive in the environment as microplastics for centuries, being one of the main components of city dust.

An additional differentiation of Microplastics widely used by the community, introduced first by Cole et al. in 2011[2], is the separation between:

**Primary microplastics & Secondary microplastics.**

**Primary microplastics** are directly released into the environment as small pieces of plastic. These are intentionally engineered particles, like those found in some consumer and industrial products. Cosmetics, for example, have used microplastics as abrasives and textiles use it for durability.

**Secondary microplastics** are the result of the degradation of large plastic waste, like plastic bags and bottles, into smaller plastic fragments when exposed to our environment.

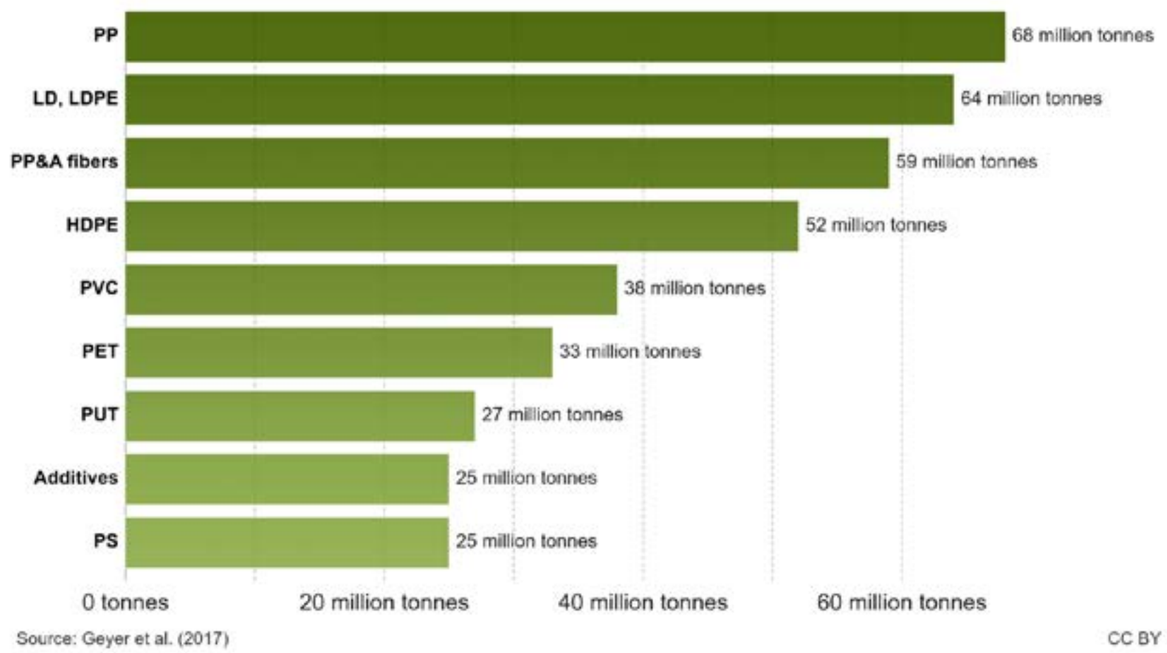



*Fig. 1. Primary plastic production by polymer type, 2015*
*Global primary plastic production by polymer type, measured in tonnes per year. Polymer types are as follows: LDPE (Low-density polyethylene); HDPE (High-density polyethylene); PP (Polypropylene); PS (Polystyrene); PVC (Polyvinyl chloride); PET (Polyethylene terephthalate); PUT (Polyurethanes); and PP&A fibers (Polyphthalamide fibers).*

1. Hannah Ritchie (2018) - "Plastic Pollution". Published online at OurWorldInData.org
2. Cole et al., MARINE POLLUTION BULLETIN 62 p2588 2011

# What are microplastics?

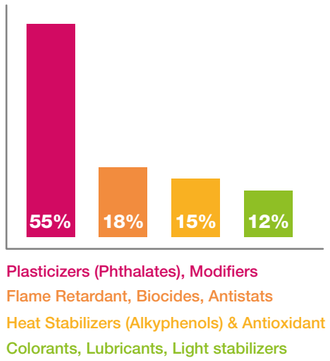
## Why are they a concern?

Microplastics are considered a great concern due to several reasons:

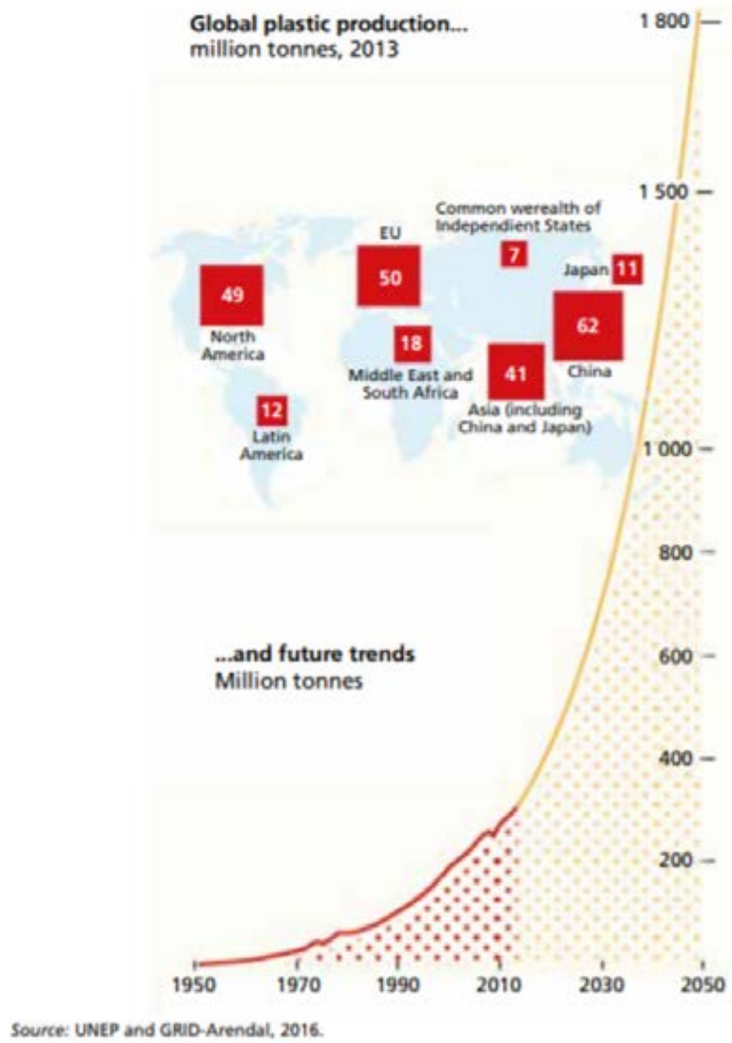
- **Plastic production** is increasing year over year and their degradation process is very slow. Plastics can remain in the environment, particularly the marine environment, for centuries*.
- Microplastics on average contain 4% by weight[1] of other substances whose **human toxicity is well-known**, including:
  - Organics such as some Persistent Organic Pollutants (POPs), Polychlorinated Biphenyls (PCBs), Polycyclic Aromatic Hydrocarbons (PAHs), Phthalates etc.
  - Inorganics such as Titanium dioxide, Barium oxide etc.
  - Remaining monomers

*Microplastics can absorb and be an aggregation center for these types of substances dissolved in water due their higher chemical affinity with respect to water (higher hydrophobicity), increasing their load and potential toxicity.*

**The plastic additives industry represents 10% (≅ 58 billion dollars) of the overall plastics value with plasticizers, modifiers and flame retardant being the main produced.**




* A high number of industries are strongly depended by plastics and several of their innovation were not achievable without them. The issue is not the Plastics but their recycling process, waste management and human behavior.




**Expected global plastics production up to 2050**

*Fig. 2. Trends in global plastic production. The projected exponential increase is the result of predictions based on increasing population and resulting demand and forecasting from the known curve*

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# What are microplastics?

## Why are they a concern?

Microplastics have been found in a huge number of species among all groups of wildlife (over 557 species[2]) and in several types of food (salts[4], fish[5], beer, honey[6], tap and mineral water[7], for example.
The consumption of these foods can transfer microplastics and their additives into the human body.


Exposure to microplastics in laboratory environment has demonstrated their potential toxicity, causing serious effects to marine animals[3] such as mortality, reduced feeding rate, body mass, and metabolic rate, decreased fertilization and larval abnormalities, neurotoxicity and others.

Recent studies have shown the presence of microplastics in human bodies:

- Schawbl et al.[8] in 2019 found microplastics in human stools, the number of samples was only 8 but each sample had a median of 20 plastic particles ranging from 50 to 500 µm in size. Nine polymer types were identified with polypropylene and polyethylene terephthalate the most abundant. The study of Schawbl demonstrates that microplastics can find a way through the human gut and potentially may move to the circulatory system,

- Ragusa et al.[9] detected plastic fragments in placenta samples collected from six patients with uneventful pregnancies, All the particles were less than 10 µm in size. The presence of microplastics in the placenta shows that they can reach the circulatory system and be transported to different organs.


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# What are microplastics?

## Why are they a concern?

Notwithstanding the potential risk associated with microplastics it is difficult to predict their toxicity for human health due to the lack of studies providing in vivo data on the absorption of microplastics. Moreover. the few in vitro studies show that particle uptake by the human body (Lusher et al.[10] and references cited within) is expected to be limited and strongly linked to the size of the particles.

Only microplastics below 150 µm may translocate from the gut epithelium and a small portion of them, with sizes below 5 µm (nanoplastics being the more dangerous), may penetrate into other organs as demonstrated by the work of Ragusa.

*Considering this, it is crucial to focus on the analytical techniques which allow the identification and characterization of the smallest particles.These include Raman Microscopy, SEM, AFM etc.*


1. Bouwmeester *et al*. ENVIRONMENT SCIENCE & TECHNOLOGY 49 p.8932 2015
2. Kühn, S., Rebolledo, E. L. B., & van Franeker, J. A. (2015). Deleterious effects of litter on marine life. In Marine Anthropogenic Litter (pp. 75-116). Springer, Cham.
3. Barboza *et al*., 2018. MARINE POLLUTION BULLETIN 133 p.336 2018
4. Yang *et al*., ENVIRONMENT SCIENCE & TECHNOLOGY 49 p.13622 2015
5. Rochman *et al*., SCIENTIFIC REPORTS 5 p.1 2015
6. Liebezeit *et al*., FOOD ADDITIVES & CONTAMINANTS: Part A 30 p.2136 2013
7. Mason *et al*., FRONTIERS IN CHEMISTRY, 6 (article 407) p.1 2018
8. Schwabl *et al.*, ANNAL INTERNAL MEDICINE, 171(7) p.453 2019
9. Ragusa *et al*., ENVIRONMENT INTERNATIONAL 146 p.1 2021
10. Lusher *et al*., FAO Fisheries and Aquaculture Technical Paper. No. 615. Rome, Italy. 2017

# What are microplastics?

## Regulatory landscape around microplastics

A good overview of the actual global regulations around plastics & microplastics is given by the United Nations Environment Programme (UNEP) which in 2018 published a review on the national laws and Regulations on the legal limits for single-use plastics and microplastics[1].

This global review covers all the national legally-binding instruments, including bans and restrictions, and it separates them into three main sections:

1) plastic bags,
2) other single-use plastics,
3) microbeads.

**Plastic bags**.

127 out of 191 countries have adopted plastic bag legislation but only 91 imposed some kind of ban and/or restriction on production, importation and distribution of plastic bags. Europe and Africa are the continents with most legislative activity.

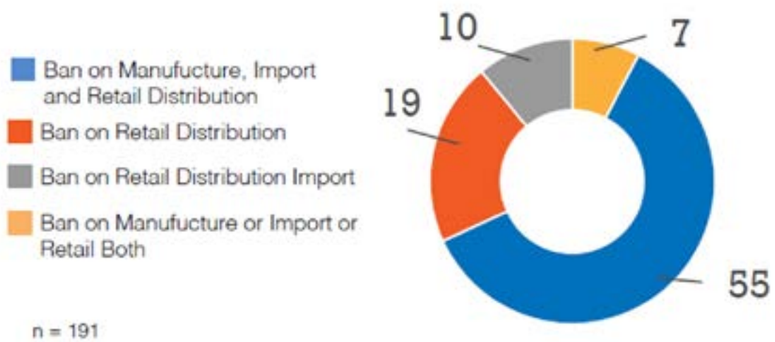
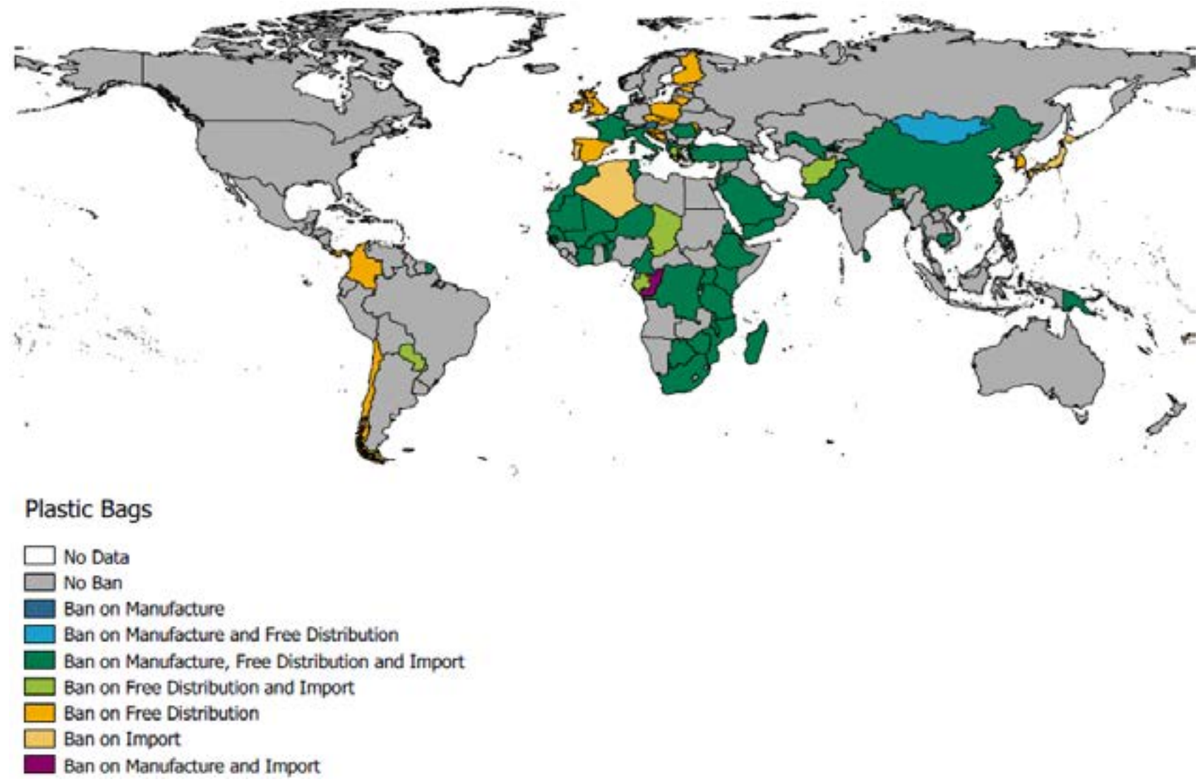



*Fig. 3. Ban of plastic bags*

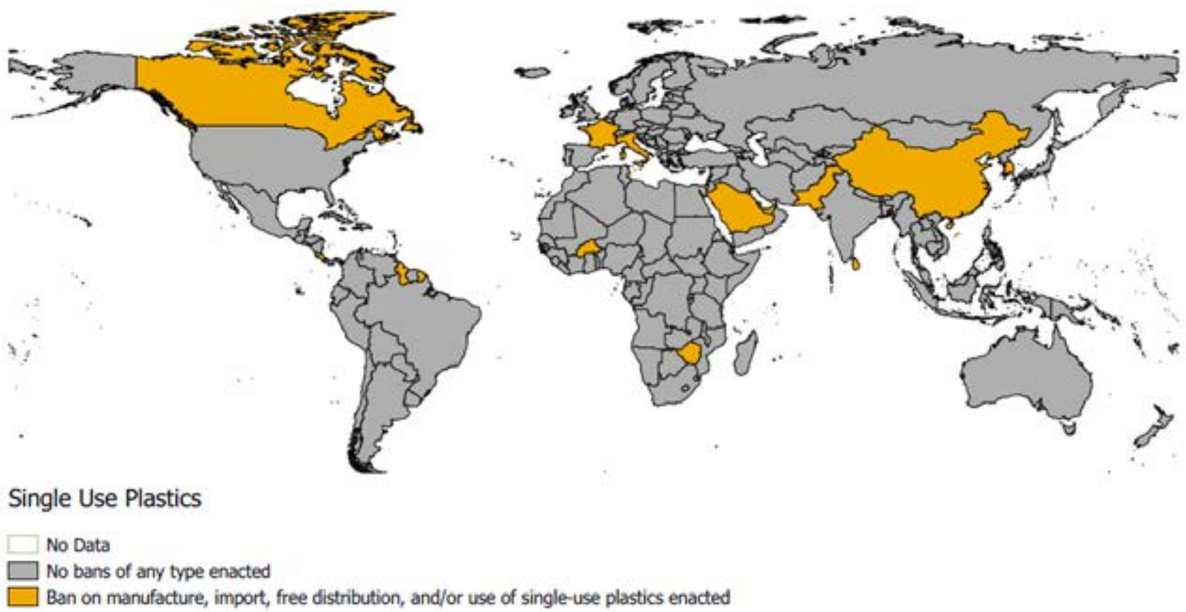



*Global overview of countries with bans on the manufacture, free distribution, and importation of plastic bags*

# What are microplastics?

## Regulatory landscape around microplastics

**Single-use Plastics**.
For single use plastic the situation is different. Only 27 out of 191 countries have adopted single-use plastic legislation, imposing some kind of ban and/or restriction on production, importation and distribution of plastic bags.




*Bans on Single-Use Plastics*

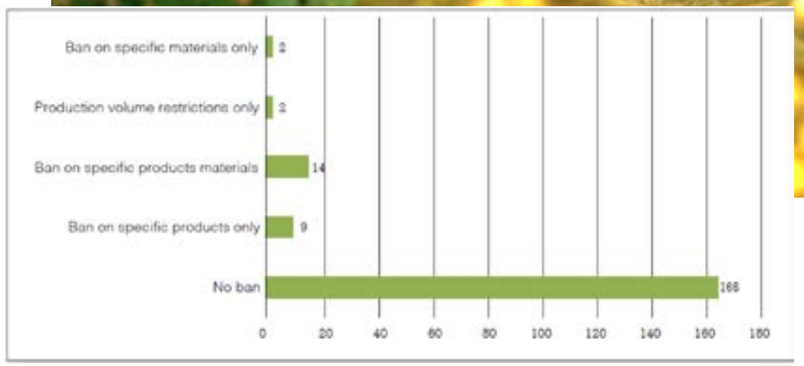



*Fig. 4. Number of bans by type*

# What are microplastics?

## Regulatory landscape around microplastics

**Microbeads.**
Only 9 countries out of 191 have adopted microbead legislation, imposing some kind of ban and/or restriction on production, importation and distribution of microbeads.

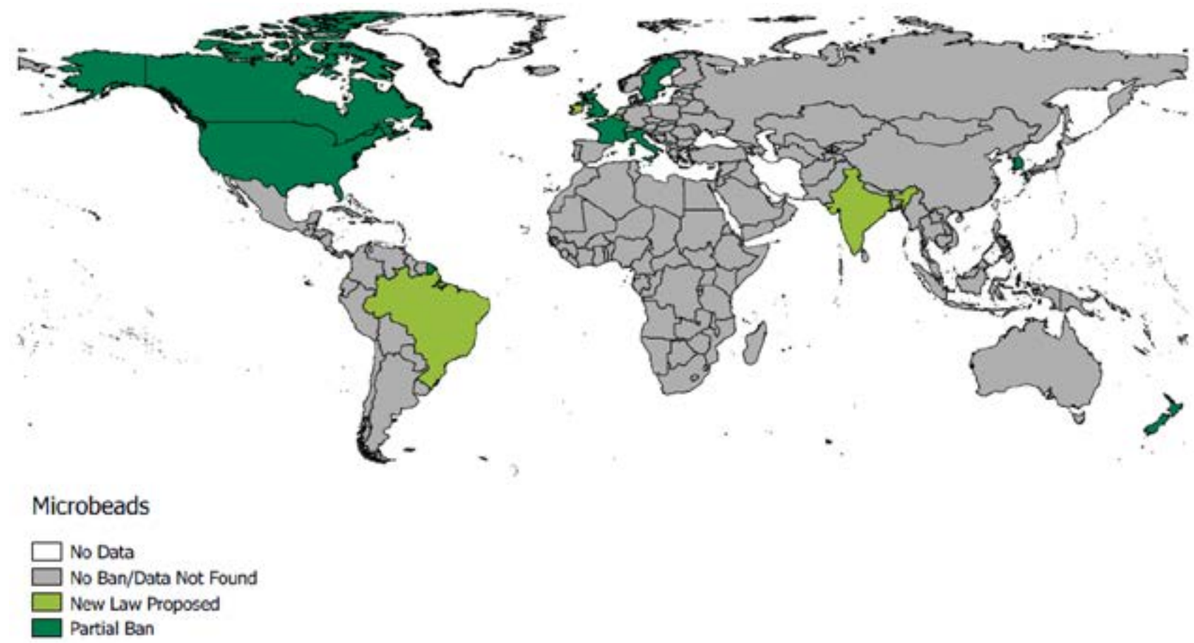
Microbeads are a primary microplastic, designed and intentionally engineered to be small, and are used for example in cosmetic products.

All countries define microbeads within their legislation, but it is possible to summarize all the different definitions with the following one: a “plastic microbead” is defined as any solid plastic particle that is — 5 millimeters or less in size.

The Canadian legislation, microbeads in toiletries regulations (SOR/2017-111) of 2017, is the first to mention molecular spectroscopy techniques as a testing method.

*Table 1. Regulatory landscape*

| Country | Law or Regulations Name |
|---|---|
| Canada | Microbeads in Toiletries Regulations (SOR/2017-111), June 2 2017 |
| France | Reclaiming Biodiversity, Nature and Landscapes Act No 2016-1087 of 8, Article 124, August 2016 |
| Italy | General Budget Law 2018: Law No 205 of 27, Art.1, Sections 543 to 548, December 20172 |
| Republic of Korea | Regulations on safety standards for cosmetics [Annex 1] No. 2017-114, Notice, Article 3, Dec. 29, 2017 |
| New Zealand | Waste Minimisation (Microbeads) Regulations 2017, under section 23(1)(b) of the Waste Minimisation Act 2008. |
| Sweden | Regulation amending Regulation (1998: 944) prohibiting etc. in certain cases in connection with handling, import and export of chemical products |
| UK | |
| England | The Environmental Protection (Microbeads) (England) Regulations 2017 |
| Scotland | The Environmental Protection (Microbeads) (Scotland) Regulations 2018 |
| Wales | The Environmental Protection (Microbeads) (Wales) Regulations 2018 |
| Northern Ireland | The Environmental Protection (Microbeads) (Northern Ireland) Regulations 2018. |
| US | Microbeads-Free Waters Act of 2015 |




*Number of Countries with bans on Microbeads*

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# What are microplastics?

## Regulatory landscape around microplastics

In the last few years, the European Community has made progress in this area and is developing a plan that intends by 2021 to regulate to restrict the use of intentionally added microplastics particles to all kinds of consumer and professional use products of all. Mineral and tap water regulation is in the scope of this plan.

The document (Annex XV1) released by the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) of the European Chemical Agency (ECHA) in 2019 was a first step towards final adoption.

In Annex XV it was identified that 'intentionally added' microplastics are used in various products such as consumer, agricultural and industrial and they include:

- Agriculture and horticulture (in fertilizers and plant protection products)
- Cosmetic products
- Detergents and maintenance products
- Paints, coatings and inks
- Chemicals used in the oil and gas sector
- Construction
- Medicinal products
- Medical devices
- Food supplements and medical food

**Intentionally added Microplastic, i.e. Primary Microplastic, UPDATE**

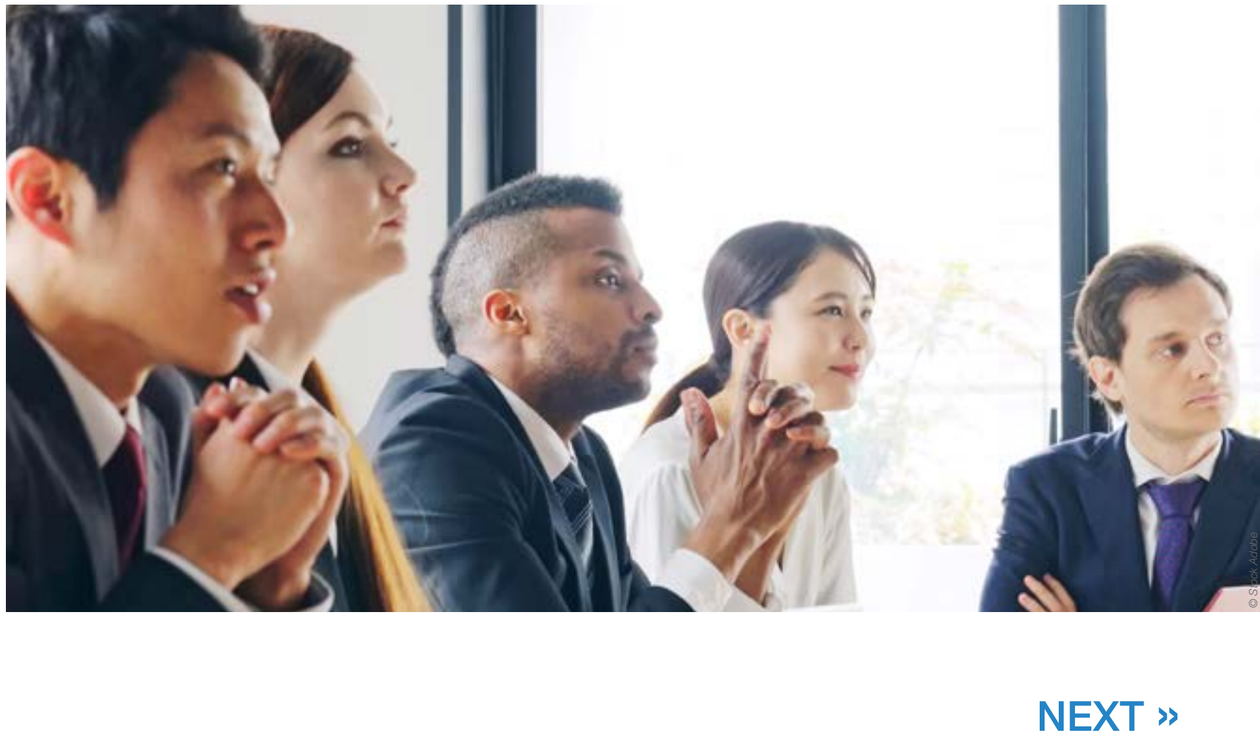
The European timetable related to the restriction of intentionally added Microplastics delays respect its original plan fixed for the end of 2021 as targeted in the REACH. The target date now is for the end of 2022 and this delay opens up several concerns because the sooner a ban enters into force, the less microplastics will end up in the environment. Indeed 1 year delay means, as ECHA estimated in its document Annex XV, a quantity of microplastics released into the environment close to 36,000 tons.

HORIBA France is actively involved in a group of experts within the French Standardization Association (AFNOR) currently working on establishing a regulation on the analysis of microplastics in drinking water, through spectroscopic techniques (µFTIR and Raman). This group, part of the T91M "Organic micropollutants" Commission, gathers various governmental, academic and industry organizations, including the Standardization Bureau for Plastics and Plastics Engineering (BNPP), with the objective of drawing up a new norm by the first half of 2021.

**AFNOR Update**

Recently in November 2021 this norm, i.e. **PR NF T90-968-1**, entered in its second step becoming available for public consultation and the official publication has been done for June 2022.

https://www.echa.europa.eu/documents/10162/05bd96e3-b969-0a7c-c6d0-441182893720



# What are microplastics?

## Regulatory landscape around microplastics

The AFNOR, T91M "Organic micropollutants" committee, is not the only standardisation body working on a norm related to microplastics; at the international (International Organization for Standardization - ISO) and European level (European Committee for Standardization - CEN) there are a few technical committees working on the same project: **prEN ISO 16094**.

The sub-committee (SC from hereon) 14, responsible for the Environmental aspects, and part of the Technical Committee (TC from hereon) 61, related to Plastics (ISO/TC61/SC14) built a joint working group together with the SC2 responsible for the physical, chemical and biochemical methods, and part of the TC147 related to water quality (ISO/TC147/SC2), to address and standardise the analysis of plastics (including microplastics) in waters and related matrices.

The project prEN ISO 16094 aims to define general principles for microplastics analytics and to provide guidance on the design of sampling techniques for the sampling of plastics in waters with low contents of total suspended solids. In the scope of this project are waters from different sources, for example drinking water, ground water, precipitation water, surface water and water resulting from water treatment processes. This project is divided into three parts:

- Part 1 (16094-1) ➡ General and sampling
- Part 2 (16094-2) ➡ Method using vibrational spectroscopy
- Part 3 (16094-3) ➡ Thermo-analytical methods for waters with low content of natural suspended solids

As a part of the TC147 related to water quality considering the Microplastic topic it's important to mention the **ISO/CD 5667**. The SC6 of the TC147, responsible for the general methods of sampling, started a project/document in mid 2021: The ISO/CD 5667-27. In this document we describe a methodology for the sampling of suspended microplastic particles in water (drinking water, surface waters, freshwater, seawater, wastewater-treated effluents, and untreated wastewater) for their subsequent characterization with spectroscopic and chromatographic techniques.

Moving away from the ISO/CEN environment but remaining in the water matrix for Human consumption, is critical to the new **DIRECTIVE (EU) 2020/2184** of the European Parliament of 16 December 2020 (https://eur-lex.europa.eu/legal-content/IT/TXT/?uri=CELEX%3A32020L2184) which amended and updated the **DIRECTIVE 98/83/CE** of 3 November 1998.

in this new directive, Microplastics are mentioned as new emerging compounds of concern for the human health and in **Article 13**, related to the Monitoring, paragraph 6 stated the need to measure them:
Article 13 – Paragraph 6 ➡ "By 12 January 2024, the Commission shall adopt delegated acts in accordance with Article 21 in order to supplement this Directive by adopting a methodology to measure microplastics with a view to including them on the watch list referred to in paragraph 8 of this Article once the conditions set out under that paragraph are fulfilled."

Anticipating the prEN ISO 16094 mentioned above, the **CEN/TC248** related to Textiles has worked on a project to address and standardise the analysis of microplastics from textile sources in the textiles sectors; the project was presented by the Italian standardisation body (UNI) under the guidance of Aquafil S.p.A and the institute of intelligent industrial technologies and systems for advanced manufacturing part of the Italian National Research Council (CNR). This project is the **prEN ISO 4484** and it's divided in three parts:

- Part 1 (4484-1) ➡ Determination of material loss from fabrics during washing
- Part 2 (4484-2) ➡ Qualitative and quantitative evaluation of microplastics
- Part 3 (4484-3) ➡ Measurement of collected material mass released from textile end products by domestic washing method

The three parts are proceeding separately and have reached (at the time of publication of this booklet) different stages. The most advanced is the part 1, prEN ISO 4484.1, which reached the stage of FDIS (Final Draft International Standard).

Dr. Tiziano Battistini from Aquafil and prof. Raffaella Mossotti from the CNR accepted to submit a contribution to outline the project.

# MEASURING if you want to know, KNOWING if you want to manage and improve

## Textile Microplastics: a standard tailored method

**Tiziano Battistini[1], Raffaella Mossotti[2], Giulia Dalla Fontana[2], Anastasia Anceschi[2]**

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2. STIIMA-CNR Sistemi e Tecnologie Industriali Intelligenti per il Manifatturiero Avanzato- Consiglio Nazionale delle Ricerche, C.so G. Pella 16, Biella, 13900, Italy

The term "plastic" comes from the Greek word "plastikos" and means suitable for moulding, referring to the malleability or plasticity shown by the material during production and processing. These properties allow plastics to be cast, pressed or extruded into a wide variety of shapes such as films, fibres, sheets, tubes, bottles and boxes, thus finding use in different industries. To date, 389 million tonnes of plastics [1] have been produced, generating a large amount of waste. Despite the current policy of good practice and awareness raising for the reuse and recycling of waste at least in the more developed countries, the presence of plastic accumulations in particular in our oceans is still large and unsightly with repercussions on tourism, shipping, fishing and aquaculture. When plastic reaches the marine environment it does not degrade completely, but fragments into smaller and smaller debris, called microplastics.

Microplastics are divided into primary and secondary. Primary microplastics are specifically produced to be microscopic in size while secondary microplastics are formed during the degradation of larger plastic objects under environmental conditions. In addition, microplastics that leak into the air, soil and water can come from different sources such as the tyre, coating, paint and textile industries.

For this reason, microplastics have been found in significant quantities in the world's oceans with effects on biota that can be physical and/or chemical. In fact, microplastics can be ingested by biota and accumulate in organs and tissues, causing inflammation, or they can become vehicles for toxic chemicals, such as persistent organic micropollutants (POPs), and enter the food chain, compromising our health.

https://orcid.org/0000-0003-0877-5444

According to the European Chemical Agency (ECHA), microplastics are defined as a material composed of solid particles containing polymers, to which additives or other substances may be added.

The family of microplastics includes synthetic-based particles such as polypropylene (PP), polystyrene (PS), polyamides (PA), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyacrylonitrile (PAN), polymethylacrylate (PMA), elastomers and silicone rubber with particles ranging from 1 nm to 5 mm. In addition to particles, fibres with a length between 3 nm and 15 mm and a length-to-diameter ratio that must be greater than 3 [2] also fall within the definition of microplastics. Synthetic fibres account for about 60% of total global fibre production and the most common are polyester, polyamide and polypropylene [3]. Updated data have estimated that washing synthetic garments contributes about 35% of the global release of primary microplastics, making textiles a major source of pollution to aquatic ecosystems. Over the whole life cycle of a garment, microplastics may be released during washing, within a textile process, in certain processing steps, or during the recycling of the garment. Finally, when the garment at the end of its life is accumulated in landfills as waste with a serious impact on the environment [4 5 6]. Fibres, due to their elongated shape and average size, are not fully retained by Waste Water Treatment plants. For example, in a city of 100,000 people, considering 0.35 $m^3$ of sewage per person per day and assuming a sewage treatment plant that retains 94.8% of microplastics, about 1.02 kg of fibres would be produced per day [7].

To complicate matters further, some authors have noted that the data on the amount of microplastics retained by wastewater treatment plants vary considerably depending on the sampling, sample preparation and microplastic identification methods used by the scientific community.

Various methodological approaches for the quantification of microplastics have been carried out to date, using different analytical techniques such as light and electron microscopy, gravimetric and thermogravimetric analysis, chromatography and molecular spectroscopy.

The most commonly used technique is based on a morphological classification and characterisation by light microscopy of microplastics, but it is not precise and can lead to an error of more than 70% in recognition [8]. Considering the only use of the microscope, small fragments (< 50 µm) are underestimated, while long fibres (< 200 µm) create false positive results [9]. To increase the accuracy of the technique, some protocols involve the use of dyes such as Nile red [10]. However, for some polymers the method does not reach the quantification capabilities comparable to other analytical approaches and can therefore only be used for rapid evaluation [11 12].

# MEASURING if you want to know, KNOWING if you want to manage and improve

In the field of microscopy, the use of the scanning electron microscope (SEM) provides extremely clear, high-magnification images of microplastics and, thanks to the related EDS (energy dispersive X-ray spectroscopy) microanalysis, offers the elemental composition without, however, providing complete information on the molecular and bond structure that characterises polymers [13].

Recently, a new approach to monitor, identify and count microplastics has been presented that is based on the principles of digital oleography. The non-invasive method combines 3D imaging with artificial intelligence through Machine Learning (ML) In particular, this technique has been used to automatically detect the presence of microplastics in marine samples, distinguishing them from microplankton [14].

Alternatively, some destructive analytical techniques are used to characterise microplastics, such to differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA), which measure changes in the physical and chemical properties of polymers depending on their thermal stability [15 16]. In the same field, pyrolysis coupled with gas chromatography and mass spectrum thesis (pyrolysis-GC/MS) and TGA-MS liquid chromatography coupled with high resolution mass spectrometry are also used to assess the chemical composition of microplastics by analysing the products of thermal degradation [17 18]. Another method of identification and quantification of microplastics used by some authors is liquid chromatography [19] in which samples are previously dissolved in selected solvents and the mass distribution of the polymers is performed by size exclusion chromatography (SEC).

However, the latter techniques are limited to microplastics with a size of more than 500 mm so they are not applicable for the identification of particles, but mainly fibres with a smaller size. Recently, gas chromatographic thermal desorption mass spectrometry (TED-GC-MS) has been used as a method of fast identification of microplastics; it is a procedure that does not require special pre-treatment.

To overcome the potential loss of microplastics with small size, another approach requires the use of sieves [20] and gravimetry is used for quantification. This method has been used to analyse wastewater from the washing of various standard synthetic fabrics as well as machine-finished garments [7 21 22 23].

Currently, a valid protocol on the identification and quantification of microplastics relies instead on spectroscopic measurements that provide information on specific chemical bonds. These techniques allow the identification of small plastic particles and are therefore also appropriate for fibre detection.

The techniques are Fourier transform infrared and near-infrared spectroscopy (FT-IR; μFT-NIR) and Raman spectroscopy. In particular, by coupling an optical microscope with μ-FTIR and μ-Raman [24] molecular spectroscopy, it is possible to identify very small plastic particles with spatial resolution below 50 μm and 1 μm respectively [9]. These techniques are fast, non-destructive and reproducible.

Therefore, to date, many analytical procedures can be extrapolated from scientific publications for the quantification and identification of microplastics, but there are still no precise guidelines or standardised methods to follow for the preparation and quantification of samples containing micrometric sized fibres.

On the basis of these premises, Aquafil, a European leader and one of the first players worldwide in the synthetic fibres sector, has decided to invest in a multi-year research project developing a standard method for identifying and quantifying the fibrous microplastics released by the textile sector. From the outset, Aquafil wanted to create a standard protocol for "measuring" the release of microplastics in order to obtain indicators (number of microplastics, size, area, weight) useful for the subsequent design of new products with new eco-design and low environmental impact processes.

In order to better develop the project, Aquafil has been collaborating for three years, also financing a scholarship for young researchers, with the Institute of Intelligent Industrial Systems and Technologies for Advanced Manufacturing of the National Research Council located in Biella (CNR STIIMA), which is dedicated to research and innovation on manufacturing technologies and materials in the textile industry.

In 2019, thanks to this collaboration a first draft with the proposal of a standard method was presented to the Italian standardisation body (UNI-Italia) within WG 046 sustainability. The standard body decided to support and present together with Aquafil S.p.A and CNR-STIIMA the joint project to the international commissions ISO (International Organisation for Standardisation) and CEN (European Committee for Standardisation). In 2020, the method was positively evaluated by the textile commissions (CEN TC248 and ISO TC38) and in 2021 it was submitted for analysis and approval by the CEN and ISO worlds, receiving requests for technical comparisons to "harmonise" it with other methods developed in the plastics, drinking water and environmental sectors.

In this respect, three methods for the determination of textile microplastics have been proposed within the ISO CEN WG 37 and are referred to as standard method 4484:

- 1: Determination of fibre loss from textiles during a washing cycle

- 2: Qualitative and quantitative determination of microplastics

- 3: Measurement of the mass of collected material released from final textiles by the household washing method

Methods 4484 -1 and 3 use a gravimetric method for the determination of the material released during a simulated washing cycle or in a washing machine.

Method 4484-2 allows collecting information about the type of microplastics (fibres, particles) physical parameters such as (size, area, volume, mass) and characterisation of the polymer using analytical techniques such as optical microscopy coupled with molecular spectroscopy (µ-FTIR and µ-Raman). It is a versatile method that can be used for the determination of textile microplastics from a solid sample, e.g. the residue from methods 4484-1 and 4484-3, or from an aqueous sample such as from process wastewater, or from an aero-dispersed sample (fibres collected in a working environment).

The proposed method involves pre-screening the samples and determining certain parameters such as the amount of total suspended solids (TSS) and conductivity to assess the need for pre-treatment (oxidative and/or acidic) to purify the synthetic material from organic protein or salt. The sample is then filtered through suitable filters for subsequent spectroscopic analysis. The results of the spectroscopic investigation are processed by means of image analysis, which allows for correct identification from a chemical point of view, but also from the point of view of physical parameters such as count and size (diameter and length). Acquiring the physical parameters of microplastics can potentially be more important than their concentration in the environment, as their size is very significant in determining their ecotoxicological impact. Indeed, only particles/fibres of a certain size can enter the biota and then the food chain. In addition, having small particle sizes results in an increase of the absorption/adsorption area.

Furthermore, a protocol for the preparation of standard suspensions of microfilaments was designed and defined within the method to facilitate the monitoring of microplastics in different matrices and the verification of the steps of the procedure in laboratory tests. It is known that standard fibrous microplastics are rarely used in laboratory studies because they are not available for purchase. Existing methods for the preparation of microfilaments are limited to cutting or cryogenic grinding of the synthetic yarn [25 26 27 28] and with the latter technique, fibres of different lengths are obtained. Furthermore, cryogenic grinding can only be used on certain polymers. Within the method, in the section concerning standards, a new and simple procedure is proposed for the preparation of standard suspensions of fibrous microplastics with a length of about 200 µm. [29] The procedure for microtome cutting of standard synthetic yarns is in accordance with IWTO standard 8-97 and UNI EN ISO 137.

Currently, as requested by the ISO and CEN commissions, the validation of the method with the Round Robin Test (RRT) is being organised involving public and private national and international laboratories. All laboratories equipped with µ-FTIR or µ-Raman analytical instruments can participate in these tests.

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It is expected that standardisation of the method can be completed by Q2/Q3 2022 in line with the directives of the European Commission (State of the Union 2021, Letter of Intent), which has confirmed its intention to present a legislative proposal aimed at reducing the release of microplastics into the environment and limiting the addition of microplastics in products. In particular, one of the topics will be focused on the adoption of policies and good practices to reduce the release of microplastics from different sources including textiles.

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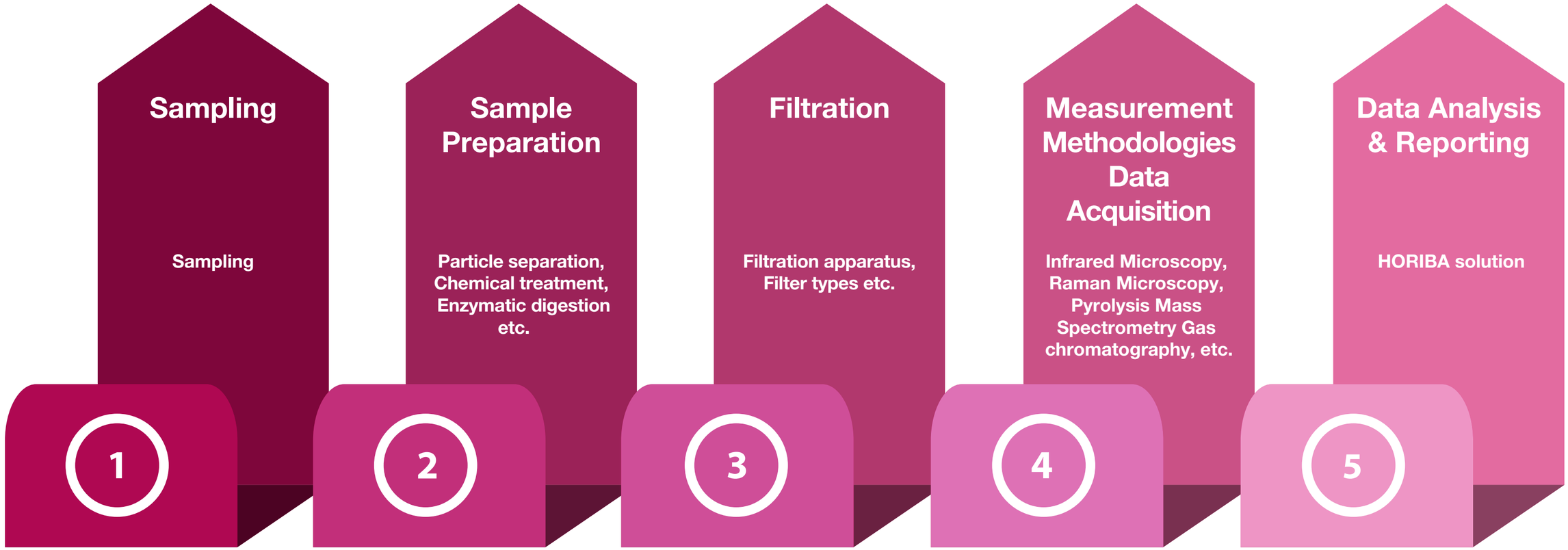
# Analysis Workflow

**Analysis Workflow**
Sampling
Sample Preparation
Protocols
Filtration
Measurement methodologies

A common analysis workflow for microplastics separation, counting and identification requires 5 main steps: Sampling; Sample preparation or sample pre-treatment ; Filtration, measurement methodologies and data acquisition; and finally, data analysis and r eporting.



# Analysis Workflow

## Sampling

The appropriate sampling step is highly dependent on the matrix to be investigated/analyzed for the presence of microplastics. Considering the number of possible matrixes, it is tough to provide a complete picture, but we will touch the most important ones.

**Water Sampling**.
Most important point for water (but also valid for sediment and other matrixes) is the representativeness of the sample collected. Ocean, sea and river water samples must come both from the water surface and the water column. Several studies (review of Hidalgo-Ruz et al.[1]) have demonstrated that the water surface has a higher number of microplastic items than the water column: Microplastics ranks from 0.022 to 8,654 items $m^3$ at the surface and from 0.014 to 12.51 items $m^3$ in the column.

The most common tools for water withdrawal are manta trawls (surface water) and plankton nets (water column) (Stock et al. [2] and papers cited within); the mesh of the net can vary between 50 to 3000 µm but the most common is 300 or 330 µm. Due to the mesh size most microplastics under 300 µm are lost. A mesh size net of 80 µm has also been used but the risk of clogging is high. A flow meter is usually used to measure the amount of water flowing through them for comparative and quantitative measurements.

DR

Plankton Net

DR

Manta Trawls

An alternative tools are: Continuous flow centrifuge which can collect particles down to 5 µm without clogging, but with a longer sampling time (1 hour for 1 $m^3$ of water); Filter cascade with a fractionated pressure filtration, which guarantees fast measurement times and direct separation of the particles into size classes.

Some general guideline for water sampling, and also sediment and biota, in seas can be found in Guidance on Monitoring of Marine Litter in European Seas[3] by the Marine Strategy Framework Directive (MFSD).

# Analysis Workflow

## Sampling

**Sediment Sampling.**
For marine and freshwater sediment, the golden rule of ensuring the representativeness of the sample is still key. Some guidelines are provided in the MSFD document of the MSFD. Important are; the amount of the sample collected (often measured in volume L, weight kg or areal extension $m^2$, analyzed); The location and the repetition for each location. The sampling depth is also an important parameter and can vary depending on the aim of the study but in many publications the upper 5 cm or less, is where most of microplastics are concentrated, and therefore has been used for microplastics monitoring. Sediments contain more microplastics than water, ranging from 0.21 to more than 77,000 items per $m^2$.

Marine sediment, a part of the shoreline (beaches), can be differentiated by the location where they are collected in 3 different zones: Tideline or supralittoral, intertidal or eulittoral and sublittoral. In freshwater ecosystems the same differentiation does not apply, due to the minimal effects of tides. The tools for sediment collection are mainly mechanical, such as tweezers, table-spoons, hand picking and grabbers for deep sediment.

**Biota Sampling.**
It's important to define the term Biota as a common starting point: Biota is the animal and plant life of an ecosystem.

Sampling methods are highly diverse and depend on the target and type of habitat: water column, sea surface, aquaculture etc. Lusher et al.[4] wrote a wide and exhaustive review on this field, underling as the most important points: the avoidance of plastic contamination and handling of animals.

Handling stress can result in a loss, and therefore underestimation of microplastics due to gut evacuation. The safest methods of storage of the organisms, before their analysis are desiccation and freezing.

**Food Sampling.**
Foods is more straightforward than sediment, water and biota sampling, They are readably available thorough the commercial chain; the key sampling factors in food sampling are the number of samples and repetitions, even if a recognized protocol is still missing. Below we summarize some examples of food sampling.

**Honey and Sugar.**
Liebezeit et al.[5] collected mainly from Germany, 19 types of honey, both solid and liquid plus 5 types of sugar directly from the producers or supermarkets. Honey samples were filtered with a 40 µm sieve (the solid one after melting it), sugar was dissolved in deionized water and then filtered with a 0.8 µm cellulose filter.

# Analysis Workflow

## Sampling

**Salts.**
Several studies on salts have been conducted since the first one of Yang et al.[6] but the one of Kim et al.[7] in 2018 is the most exhaustive. Kim collected 39 brands of table salt from supermarkets in 17 different countries over 6 continents, Salts were selected to provide a diverse range of sources (seawater, lake water) and manufacturing methods (solar-dried, refined or un-refined). A minimum of 500 grams for each salt were tested and duplicated.

**Tap water.**
A recent study of Kosuth et al.[8] published in 2018 is a good example of tap water sampling. Kosuth collected 159 samples from 14 different countries. Samples were collected by running the tap water for 1 minute and then, while the water source was running, a bottle of 500 ml was filled and dumped twice before the final filling.

**Bottled water.**
Manson et al.[9] in 2018 conducted a study on bottled water selecting 259 bottles from 11 brands in 27 lots, including leading global brands from various bottled water producers, purchased in 9 different countries. Bottled water came in bottles of different capacities (from 0.5 L up to 2 L) and several bottles were analyzed to reach a volume close to, or above, 5 L for each lot.

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# Analysis Workflow

## Sample Preparation

Sample pre-treatment is the most important step for microplastics analysis because, if done correctly, it eliminates all types of possible organic contaminants that can affect microplastic chemical identification when using various techniques: Infrared Microscopy, Raman Microscopy, Pyrolysis Gas Chromatography/ Mass Spectrometry (GC/MS) etc. These contaminants are always present (even when analyzing bottled water) and their amount depends on the matrices analyzed.

There is extensive literature on pre-treatment protocols which vary depending by the type of matrix under investigation. There are some key aspects that must be considered independent of the analysis method :

**Integrity of the microplastic**
Chemical treatment can modify both chemistry and sizes of microplastics if too aggressive. These two aspects are important to determine the potential toxicity of microplastics, so treatment must be carefully chosen to avoid changing the sample.

**Plastic contamination**
Sample manipulation can cause additional plastic contamination from the laboratory environment. A blank, or reference, sample of just filtered deionized water is essential to understand plastic contamination and to avoid over-estimation of the microplastic content. Preparing samples under a laminar flow hood is highly recommended.

This section provides a general overview of the different methodologies and also gives detailed suggestions for some of the most common matrices. Due to the absence of standardized methods, the protocols suggested are the combination of our experience and the literature.

Apart from the organic contaminant removal protocols, additional treatments must be mentioned for sediment analysis. The first step is **physical separation** using various sieves to isolate Microplastics and inorganic materials depending on their size. The second step is **extraction**. Sediments contain other inorganic materials, such as quartz sands and silicates. These must be separated from the microplastics to avoid interference during chemical identification. Extraction is done by means of density separation, exploiting the different densities of plastic and inorganic materials; the majority of polymers possess a lower density (usually from 0.8 to 1.6 see Table 1) than the inorganic constituents of the sediment. As an example, silicates density range from 2.196 for amorphous to 2.648 for $\alpha$-quartz.

# Analysis Workflow

## Sample Preparation


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Commonly density separation involves 4 steps as highlighted in the review of Hanvey et al.[1]:

- Introduction of an aqueous solvent with a specific density
- Mixing for defined periods of time
- Settling, or equilibration time
- Filtering to specific size fractions

By using an aqueous solvent with a higher density than plastics, they will float on the surface allowing them to be separated from inorganic materials. It is important to vigorously mix the solution to ensure that the microplastics can separate out during the settling step. It is highly recommended to repeat these steps at least two times.

The addition of salts increases the density of the aqueous solution and varying the types of salts allows the density to be tuned to meet specific requirements. Several salts (Hamm et al.[2] and references within) have been used in literature and the most common ones are listed in the following Table.

*Table 2. Density values for the most common polymers. The ones highlighted in grey represent more than 80% of global plastic production.*

| Polymer type | Density (gr/cm$^3$) |
|---|---|
| *Poly(propylene), PP* | *0.861* |
| *Poly(ethylene), PE (Low to High density)* | *0.854-0.96* |
| *Poly(vinyl chloride), PVC* | *1.388* |
| *Poly(ethylene terephthalate), PET* | *1.333* |
| *Thermoplastic Polyurethane PUR* | *1.23-1.35* |
| *Polystyrene, PS* | *1.052* |
| *Polytetrafluoroethylene, PTFE or Teflon* | *2.2* |
| *Poly(amide) 6, PA6* | *1,06-1,16* |
| *Poly(vinylidene fluoride), PVDF* | *1.675* |
| *Polychloroprene, Neoprene* | *1.243* |
| *Poly(methyl acrylate), PMA* | *1.224* |
| *Poly(isobutene)* | *0.864* |
| *Poly(caprolactam)* | *1.084* |
| *Poly(Bisphenol A carbonate), PC* | *1.206* |
| *Polylactic acid, PLA* | *1.248* |
| *Poly(ethylene glycol), PEO, PEG* | *1.128* |
| *Poly(methyl methacrylate), PMMA* | *1.159* |
| *Poly(vinyl alcohol), PVOH* | *1.300* |
| *Poly(vinyl acetate), PVA PVAC* | *1.190* |
| *Poly(ethylene-vinyl acetate) PEVA* | *0,92 - 0,94* |

# Analysis Workflow

## Sample Preparation

This table also provides the cost, an important consideration for sediment analysis, and the potential safety issues correlated with the handling of some of them.

*Table 3. List of salts for density separation process.*

| Salt | | Maximum density ρ (g/cm³) | Amount (Kg) | Cost (euro) | Amount (gr/L) for Maximum ρ | Safety |
|---|---|---|---|---|---|---|
| **Sodium Chloride** | **NaCl** | 1.2 | 1 | 35,9 | 311 | no effect |
| **Sodium Iodide** | **NaI** | 1.8 | 0,5 | 287 | 797 |  |
| **Zinc Chloride** | **$ZnCl_2$** | 1.9 | 1 | 116 | 1373 |  |
| **Zinc Bromide** | **$ZnBr_2$** | 1.7 | 0,5 | 139 | 1125 |  |
| **Calcium Chloride** | **$CaCl_2$** | 1.4 | 0,5 | 25 | 558 |  |
| **Sodium Polytungstate** | **3 $Na_2WO_4$ 9$W0_3$** | 3 (1.55) | 0,1 | 216 | 5671 (798) | no effect |

 Eye, skin and respiratory tract irritation;

 Corrosive;

 Possible burns.

Sodium Chloride is the readily available and safe solution but the maximum density achievable is only 1.2 g/cm³ which does not allow separation of high-density plastics such as PVC and PET. A minimum density of 1.5 to 1.55 is needed to recover more than 90% of the plastics.
Sodium Polytungstate is probably the best technical solution because the density can be easily tuned up to 3 g/cm³, it has a low viscosity, it can be reused and additionally it has no safety constraints, but its main drawback is the cost which is more than 2000 euros per kilograms. According to Coppock et al.[3] and considering all the aspects, we suggest as the best balanced solution Zinc Chloride; care must be taken with handling, but the price is reasonable and densities up to 1.9 g/cm³ can be reached.

*After this overview of physical separation and extraction methods we can move on to organic contaminant removal protocols or the digestion step. Most of the digestion protocols envisage the use of concentrated acids and alkali solution which destroy proteins, carbohydrates and fats (the main constituents of organic residues in sediment and marine water samples and also in foods). These are the main interreference agents for microplastic chemical identification using the common analytical methods e.g. Fourier Transform Infrared/ Raman Microscopy and Pyrolizer GC-MS.*

# Analysis Workflow

## Sample Preparation

The review of Hamm et al.[2] of 2018 (and papers cited within) provides an exhaustive picture of digestion protocols and a summary of their efficiency vs. their unwanted ability to degrade Microplastics. A visual representation of this summary is depicted in figure here after.

Acid treatments are highly effective for removing organic residues reaching an efficiency above 80% in several cases, but they can easily damage microplastics preventing their chemical identification.

**Alkali treatments can have different effects:**

1. 30% and 35% aqueous solutions of $H_2O_2$ are an effective treatment but they can chemically damage some types of plastics (such as PVC and Polyamide 6-6/6 – Nuelle et al.[4]) and moreover they can also modify the shape and size of the particles. Temperature and incubating time are also important parameters to be considered, increasing them we have a positive impact on the digestion efficiency but a negative one on the particle chemistry/shape/size.


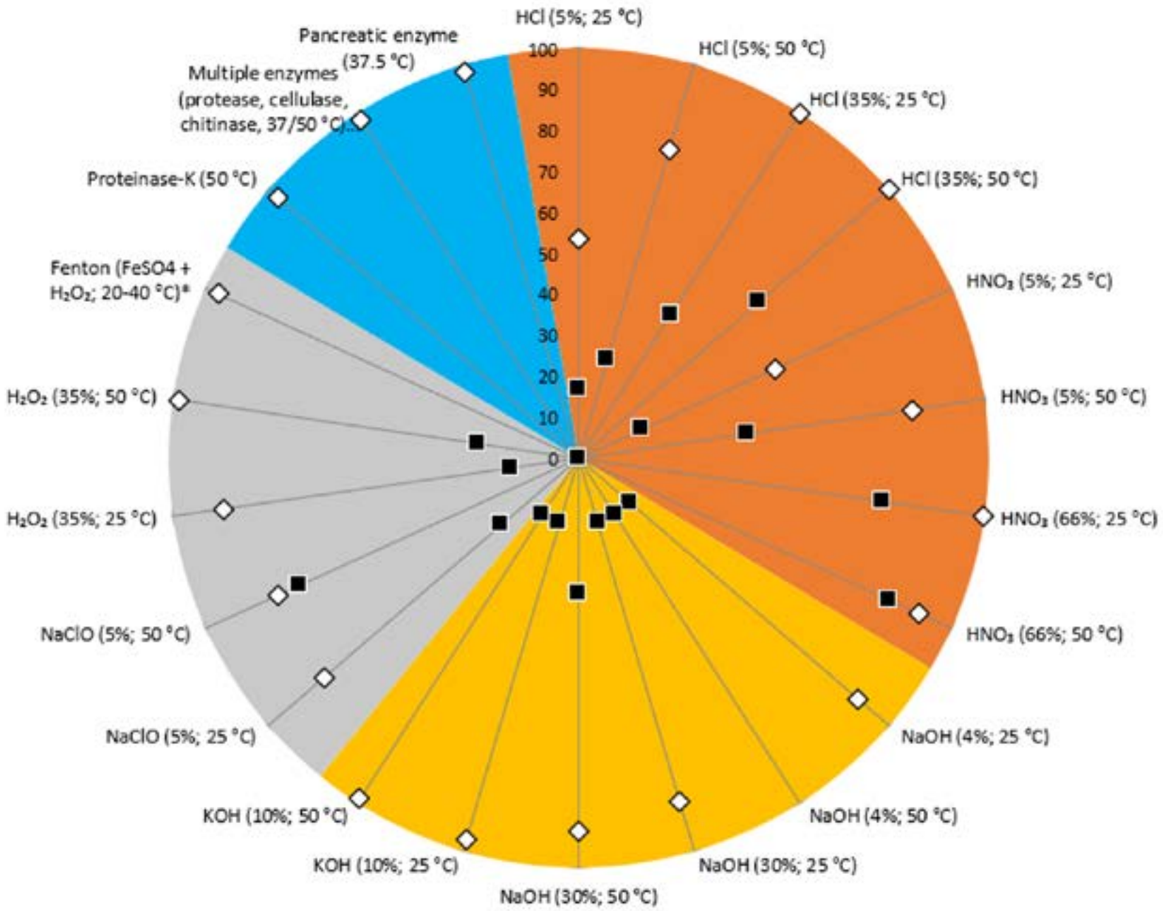


*Fig. 5. (■) Max. % of Microplastic negatively affected by treatment; (◇) Effectiveness of the treatment in %. (Image provided by Claudia Lorenz, University of Aalborg)*

# Analysis Workflow

## Sample Preparation

2. 10% aqueous solution of KOH provides better results than $H_2O_2$ as demonstrated by Karami et al.[5]. Karami tested this solution at different temperatures and incubation times, the table below summarizes the results.

*Table 4. Treatment efficiency in function of conditions*

| | Temperature (°C) | Incubation Time (hours) | Efficiency / Recovery Rate |
|---|---|---|---|
| **Digesting Solution 10% KOH** | 25 | 96 | 97.1 |
| | 40 | 48 | 98.6 |
| | 50 | 36 | 98.9 |
| | 60 | 24 | 97.61 |

The treatment efficiency (obtained by averaging the values for all the polymers tested) is above 97% with all combinations but at 50°C and 60°C, Karami observed some degradation of PVC, PET and Polyamide 6-6/6. The best condition balancing temperature / speed (i.e. incubation time) was at 40°C for 48 hours, where only PVC shows a recovery rate below at 93%.

Three additional digestion processes are important to mention:
Fenton's Reagent (Tagg et al.[6]); Mono-Enzymatic treatment (Cole et al.[7] used Proteinase-K, while Courtene-Jones et al.[8] used Trypsin); Basic and Universal Enzymatic Purification Protocol (BEEP-UEEP) which combines a Multi-Enzymatic treatment with an oxidizing agent ($H_2O_2$) and a detergent Sodium Dodecyl Sulfate (SDS), this protocol is usually employed for protein denaturation (Loder et al.[9])

**Fenton's reagent** is prepared by mixing solutions of 30% $H_2O_2$ and $FeSO_4 \cdot 7H_2O$ to reach final $FeSO_4 \cdot 7H_2O$ concentrations of: 3.33, 6.67 and 10 mg/ml. Its efficiency was demonstrated with infrared microscopy and even PVC and Polyamide didn't undergo to any modifications. The major advantages of Fenton's reagent stressed by Tagg et al. is the rapid digestion time of only around 10 minutes, much faster than Alkali treatments and the fact that it works at room temperature.

We observe only one issue connected to Fenton's reagent digestion that can affect microplastic identification by Raman microscopy (the key technique for analyzing microplastics below 5/10 microns): the presence of Iron leads to the formation of fluorescence compounds that interfere with the chemical identification of polymers by Raman.

**Enzymatic treatment**. Both the mono-enzymatic digestions were tested on marine biota, bivalve species for Cole and mussel for Courtene-Jones, and they show very high efficiency and no degradation of any plastics. Efficiency was 97% for Proteinase-K and 88% for Trypsin.

# Analysis Workflow

## Sample Preparation

As some biogenic material remains undigested, using Trypsin, Courtene-Jones et al.[8] proposed the use of an additional enzyme such as chitinase to remove the residual parts. The protocol developed by Cole[7] for bivalves differs on one aspect: the sample was homogenized with a solution of 400 mM Tris–HCl buffer, 60 mM EDTA, 105 mM NaCl and 1% SDS before adding the enzyme while Courtene-Jones[8] used a solution of Trypsin, made with deionized water, directly on the sample.

These enzymatic protocols are particularly useful for marine biota and marine sediments, their main drawback is the relatively high cost of the purified enzymes.

**Basic and Universal Enzymatic Purification Protocol (BEEP-UEEP).** Loder et al.[9] starts from the approach of Cole[7] and Courtene-Jones[8] but develops a complete protocol (BEEP), including a density separation step, combining multi-enzymatic digestion (Protease, Cellulase and Chinase) and oxidative treatments. Moreover, he successfully evaluated the protocol for its efficiency and applicability for infrared microscopy and for Microplastics with dimensions down to 20 µm.

UEEP is a further optimization of the BEEP protocol that widens its versatility for different environmental sample matrices (BEEP was developed first for seawater samples) by adding two additional enzymes (Lipase and Amylase). Loder[9] developed one of the most complete sample pre-treatment protocols (particularly suited for marine environments – biota, sediment and water) where all the possible interferents (such as chitin-containing materials, plant residues, planktonic organisms and cell residues) for microplastics identification are selectively attacked and, notwithstanding the use of enzymes, he was able to reduce the cost compared to Cole[7] by using technical grade enzymes.

One potential drawback of the BEEP/UEEP protocols is the incubation time needed for all the enzyme steps which bring the overall pre-treatment time to between 10-12 days. Furthermore, the presence of several steps is an additional potential source of unintended plastic contamination and/or particle loss.


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# Analysis Workflow

## Protocols

Following the overview of the sample pre-treatment workflows, in this section we will propose protocols for various matrices, starting with bottled and tap water. This section will be updated twice a year and new detailed protocols will be added for different matrices as a result of advances in the literature and HORIBA experience.

**Bottled Tap Water.**

Bottled water sample can be analyzed without any pre-treatment, but we recommend the protocol developed by Oßmann et al.[1] as the treatment is rapid and the removal of many non-plastic particles can reduce the total measurement time. The same treatment can also be used for tap water.

The method uses:

- Ethylenediaminetetraacetic salt (EDTA): EDTA is well-known to reduce the water hardness by complexing metal ions such as $Ca^{2+}$ and $Mg^{2+}$;
- Sodium Dodecyl Sulfate (SDS): SDS is an anionic surfactant that improves plastic suspension and provides better homogeneity.

EDTA (250 g/L solution) is added in an equimolar amount depending on the content of Calcium and Magnesium ions indicated on water bottle label.

EDTA must be left for 15 minutes. This treatment reduces the number of Calcium and Magnesium carbonate particles speeding up the full analysis time; since Raman and infrared microscopy identify plastics by analyzing each particle individually (see “Measurements Methodologies” section) removing the inorganic ones in advance reduces the overall acquisition time.

Following EDTA, 3 ml of SDS (100 g/L) is added per litre of water. After the filtration step, SDS, must be removed with a solution of 50% ethanol (ultrapure ethanol) in deionized water. This SDS step is optional.

Prior to using any solution, filtering using a 0.1 µm mesh will limit plastic contamination from the lab environment.

1. Oßmann et al., WATER RESEARCH 141 p.307 2018

## Marine water samples

### Mitigating unwanted microplastic contamination

Unwanted contamination is a relevant issue affecting the quality of data related to microplastic analysis. It is hence of paramount importance to avoid or at least to try to limit contamination throughout the whole workflow.

Specifically, all glassware used must be flushed with filtered MilliQ water three times before usage! Moreover, it is good practice to cover all sample containers with a glass lid and aluminum foil. As much as possible, the sample preparation should be done in a fume hood, or better, inside a laminar bench.

During the sample preparation, always flush the 'previous' sample container three times after transferring the sample into a new container. This way, sample loss between steps will be limited. For the same reason, use the same filter(s), beaker and magnet for each of the filtration steps (keep the filter(s) and magnet in a petri dish).

Filtration times can be significantly reduced by allowing the particles to settle at least 1 hour (the more a sample stays still, the lower the filtration time will become) and by handling the sample with great care not to disturb the settled particles.

Filtered demineralized water (0.7 µm GF) and MilliQ water can be both used during the sample preparation (it is just important to use pre-filtered water). On the contrary, it is important to use Milli-Q to prepare reagents to avoid to contaminate them with either organics molecules or inorganic ions dissolved in the water (e.g. when you prepare buffer solutions). A similar approach must be adopted when adding water during the preparation of Fenton's reaction (use filtered Milli-Q water).

It is possible that, in certain steps, the particles from a sample will be stuck on the side of the beaker. To detach the particles, fill the beaker up with MilliQ (or filtered water) and sonicate for a couple of minutes.

### Preparing reagents for microplastic sample preparation

Sample preparation for MP analysis is a complex procedure involving multiple steps and to use of several reagents. A brief chapter illustrates how to prepare the reagents needed for the sample processing.

5% w.v SDS solution (NaC12H25SO4) (1 L)

Sodium dodecyl sulphate is an anionic surfactant which is present in small quantities in many cosmetics and soaps. In this framework it is used to start degrading the matrix of the sample, preparing the substrate for the enzymatic treatment.

The materials, glassware and equipment needed to prepare SDS solutions is summarized in this bried list: measuring cylinder (1 L); glass bottles (1 L - glass cap); beacker (2 L); pre-muffled glass fibre filters (GF-F 0.7 µm or GF-C 1.2 µm); magnetic stirrer and stirrers (Teflon stirrers); vacuum filtering equipment (glass vacuum flask, filtering unit 47 mm diameter, funnel, clamp); vacuum pump; sodium dodecyl sulphate in pellets (better than powder); balance.

Measure 1 L of filtered Milli-Q water using the measuring cylinder and pour it into the beaker. Weigh 50 g of sodium dodecyl sulphate (SDS) and add it into the beaker together with a magnet. Place the beaker on a stirring plate and stir vigorously. When the powder is completely dissolved (and the foam has disappeared), filter the solution using a GF filter (0.7 µm or 1.2 µm) and transfer the solution in a glass bottle.

### Buffer solutions for enzymatic treatment

Enzymathic treatmets is nowadays widely used in microplastic science to process environmental samples, because they proved to be effective without damaging the MPs contained in the sample. Enzymes work at their best in specific ranges of pH, so it is important to use buffer solutions when dealing with enzymes to ensure their optimal activity.

### TRIS buffer (pH 8.2) for enzymatic treatment with Protease

Tris buffer is used with proteolytic enzimes, such as protease. These enzymes show their best activity with a slightly basic pH. In this specific case, the TRIS buffer has to be prepared at pH 8.2. The material, equipment and glassware needed to prepare buffer solutions is summarized in this brief list: measuring flask (1 L); glass bottles (1 L - full glass); glass fibre (GF) filters (0.7 µm or 1.2 µm pore size); tris(hydroxymethyl) aminomethane; hdrochloric acid (HCl, 37%); sodium hydroxide (NaOH); filtered demi-water or Milli-Q water; glass filtering equipment (glass vacuum conical flask, filtering unit, funnel, clamp); vacuum pump; balance.

The first step is to prepare the stock solutions. The Tris (hydroxymethyl) aminomethane (Solution A) is simply prepared by weighing 24.2 g of Tris (hydroxymethyl) aminomethane, transfer them into a measuring flask (1 L) and fill with Milli-Q to the mark. The hydrochloric acid (0.2 M HCl) (Solution B) is prepared by diluting 16.6 mL of HCl 37% in 1 L of Milli-Q water (measuring flask – 1 L). It is very important to add first most of the water and then the concentrated acid, then adjust the volume to the mark with more water. Diluting concentrate acid is very dangerous. The preparation of the actual buffer solution (1L; pH 8.2) is done as follow: add 250 mL of A and 109.5 mL of B in a 1 L measuring flask, then dilute to 1 L with Milli-Q. Mix the solution turning upside down the flask several time. Filter the solution over a 0.7 µm or 1.2 µm GF filter and transfer it to a 1 L glass bottle.

**Acetate buffer (pH 4.8) for enzymatic treatment with Cellulase**

Acetate buffer is used with cellulolytic enzymes, such as cellulose or blends of cellulase. These enzymes show their best activity with a slightly acid pH. In this specific case, the acetate buffer has to be prepared at pH 4.8. The material, equipment and glassware needed to prepare buffer solutions is summarized in this brief list: measuring flask (1 L); glass bottles (1 L - full glass); glass fibre (GF) filters (0.7 µm or 1.2 µm pore size); acetic acid (CH3COOH); Sodium acetate (C2H3O2Na or C2H3O2Na * 3H2O); Filtered Milli-Q water; glass filtering equipment (glass vacuum conical flask, filtering unit, funnel, clamp); vacuum pump; balance.

The first step is to prepare the stock solutions. The 0.2 M solution of acetic acid (Solution A) is prepared by diluting 11.55 g of acetic acid (just weigh the CH3COOH on a balance) in 1 L (measuring flask). The 0.2 M sodium acetate solution is prepared by diluting 16.4 g of C2H3O2Na or 27.2 g of C2H3O2Na * 3H2O in 1 L of Milli-Q. To prepare 1 L of buffer solution (pH 4.8), add 200 mL of A and 300 mL of B in a 1 L measuring flask, then dilute to 1 L with Milli-Q. Mix the solution turning upside down the flask several time. Filter the solution over a 0.7 µm GF filter and transfer it to a 1 L glass bottle.

**Preparing solutions for Fenton oxidation**

Catalyzed oxidative reactions are widely used in sampling preparartion for microplastic analysis nowadays. Although there are different recipes, these reactions, called Fenton oxidation, use Iron (II) as catalyst to reduce reaction time and enhance reactivity. The material, equipment and glassware needed to to perform a Fenton reaction (AAU recipe) is summarized in this brief list: measuring flask (500 mL); glass fibre (GF) filters, 0.7 µm or 1.2 µm; iron sulphate heptahydrate (FeSO4 * 7H2O); filtered Milli-Q water; measuring cylinder (10 mL); concentrated sulphuric acid (H2SO4); glass filtering equipment (glass vacuum conical flask, filtering unit, funnel, clamp); vacuum pump.

**0.1 M Iron Sulphate (FeSO4 + H2SO4)**

Find a 500 mL measuring flask, fill it half way with Milli-Q water. Measure 15 g of iron sulphate heptahydrate and add it into the measuring flask, then mix the until it is completely dissolved. When all powder is dissolved remove the magnet and fix the volume to 500 mL with Milli-Q water. Transfer the 0.1 M iron solution to a glass flask and add 6 mL concentrated sulphuric acid using a small measuring cylinder. Filter the solution using GF filter (0.7 µm or 1.2 µm.

**0.1 M Sodium hydroxide (NaOH)**

Find a 500 mL measuring flask, fill it half with MilliQ. Measure 2 g of sodium hydroxide and add it into the measuring flask and mix it until the powder is dissolved Fix the volume to 500 mL with filtered demi-water or filtered Milli-Q water. Filter the solution through a 1.2 or 0.7 µm GF filter. Transfer the solution to a glass flask.p

**Preparing high density salt solution for sample flotation**

Flotation by using high density salt solution is widely used in microplastic sample preparation when extracting solid matrices to isolate less dense materials (including plastics) from the denser inorganic materials, but also to remove remaining inorganic solids and digested organic matter later on in sample prep. (also for liquid matrix samples). Different options are available when it comes to high density salt solutions. Here we report the procedure to prepare a solution of Sodium Polytungstate and a solution of Zinc Chloride (ZnCl2). The material, equipment and glassware needed to perform a Fenton reaction (AAU recipe) is summarized in this brief list: 2 L beaker; glass bottles (full glass); glass fibre (GF) filters, 0.7 µm or 1.2 µm; SPT (powder); filtered demi water or Milli-Q water; stirring plate; magnetic stirrer (Teflon); glass filtering equipment (glass vacuum conical flask, filtering unit, funnel, clamp); vacuum pump.

**SPT (density 1.75 g/cm$^3$) (1 L)**

This solution is prepared assuming that the density of water is 1 g/cm$^3$ at room temperature (this is a simplification). Weigh 927 g of SPT powder (Sodium polytungstate) into a 2 L beaker. Add 823 mL filtered Milli-Q water. Add a magnet to the beaker, stir the solution and wait until powder is dissolved. Filter the solution over a 0.7 or 1.2 µm GF filter, then transfer the solution in a glass bottle. Measure the density of the obtained solution by weighing 3 times 1 mL of SPT-solution on a scale (use a calibrated 1 mL micropipette). The density should be around 1.75 g/cm$^3$.

**Preparing 50% v/v Ethanol for sample evaporation and deposition (1 L)**

The last step of sample treatment for MP analysis involves a sample transfer inside a 10 mL headspace vial after mixing it with an ethanol solution (50% v/v) (39.5% w.w). The material, equipment and glassware needed to prepare a Fenton reaction (AAU recipe) is summarized in this brief list: glass measuring cylinder (1 L or 0.5 L); glass bottles (plastic lid); glass bottles (full glass) (pre-muffled at 500°C); glass fibre (GF) filters, 0.7 µm or 1.2 µm; ethanol absolute (CH3CH2OH ≥ 99.8% for HPLC); filtered demi water or filtered Milli-Q water (0.7 µm GF filtered); stirring plate; magnetic stirrer (Teflon); glass filtering equipment (glass vacuum conical flask, filtering unit, funnel, clamp); vacuum pump.

Measure 0.5 L of ethanol absolute using a measuring cylinder. Transfer the liquid into a glass flask. Do the same with 0.5 L of Milli-Q water (or demi-water). Put a plastic lid and mix the two liquids until you obtain a homogeneous solution. Filter the solution using a GF filter (0.7 µm or 1.2 µm) and transfer the filtered solution into a pre-muffled glass bottle (glass cap).
NOTE: Absolute ethanol is used in all the steps excluding the evaporation, where HPLC grade ethanol is used instead (it prevents to have unwanted residues in the samples).

**Sample preparation of marine water samples**

**Sonication and SDS treatment**

Place the filters (the enriched filters from the AAU UFO) in a crystallizer, cover all filters with 5% SDS solution, and incubate them by placing the crystallizer on a heating plate for at least 24h. Sonicate the filters separately into enough 5% SDS solution to cover the filter for 5 min (each filter) in an additional glass crystallizer. Remove, flush and scrape the filters using enough SDS (up to 700 ml), and then pour all the liquid into a 1 L beaker. If you have access to an orbital shaking water bath place the sample into a water bath (set 50°C and 100 rpm) for at least 24 hours. If you have a stirring water bath, add stirring (glass stirrer) and a glass lid and place the sample into a water bath (set 50°C and 100 rpm) for at least 24 hours. Alternatively use a heating plate with the same settings. The water level in the water bath should be approximately the same than in the sample and this could cause the sample's beaker to float. Add a weight to the glass watch to secure the sample.
After this step, filter the content of the beaker onto a 10 µm steel filter, taking care of rinsing thoroughly with filtered demi-water (0.7 µm or 1.2 µm GF filtered).

**Enzymatic treatment: Protease**

The glassware and equipment required to carry out a Fenton reaction is listed here: 1 L beaker (use the same used for the previous steps); 100 mL cylinders class A (x2), 250 mL cylinder class A (x1); glass syringe (luer lock with aluminium joint needle); micropipette (1 mL) and tips; TRIS buffer (pH 8.2); Protease enzyme; Viscozyme; shaking water bath or stirring water bath.
Measure 200 mL of TRIS Buffer solution (pH 8.2), use a fraction of it to sonicate and flush the filter containing the sample (save the steel filter for the next filtration steps by placing it into a glass Petri dish). The beaker from the previous step can be used. Add the rest of the TRIS buffer, and then add 0.5 mL Protease to the sample. Place it into the water bath (set 50°C and 100 rpm) and leave it for at least 40 hours.
After this step, filter the content of the beaker onto a the same 10 µm steel filter used previously, taking care of rinsing thoroughly with filtered demi-water (0.7 µm or 1.2 µm GF filtered).

**Enzymatic treatment: Cellulase, Viscozyme**

The glassware and equipment required to carry out a Fenton reaction is listed here: 1L beaker (use the same used for the previous steps); 100 mL cylinders class A (x2), 250 mL cylinder class A (x1); glass syringe (luer lock with aluminium joint needle); micropipette (1 mL) and tips; Acetate buffer (pH 4.8); Cellulase enzyme blend; Viscozyme; shaking water bath or stirring water bath.
Measure 200 mL of Acetate Buffer solution (pH 4.8), use a fraction of it to sonicate and flush the filter containing the sample (save the steel filter for the next filtration steps). The beaker from the previous step can be used. Add the rest of the Acetate buffer, and then add 0.5 mL of Cellulase enzyme blend and 0.5 mL of Viscozyme.
Place the sample into a water bath (set 50°C and 100 rpm). Incubate the sample for at least 40 hours.
After this step, filter the content of the beaker onto a the same 10 µm steel filter used previously, taking care of rinsing thoroughly with filtered demi-water (0.7 µm or 1.2 µm GF filtered).

**Fenton oxidation**

The glassware and equipment required to carry out a Fenton reaction is listed here: 1 L beaker (use the same used for the previous steps); 100 mL cylinders class A (x2), 250 mL cylinder class A (x1); glass syringe (luer lock with aluminium joint needle); bucket containing ice (storage); large buckets to use as water/ice bath.
Measure 200 ml of Milli-Q use a fraction of it to sonicate and flush the filter containing the sample (save the steel filter for the next filtration steps by placing it into a glass Petri dish) in a 1 L beaker and fix the volume to 200 mL. Cool the sample to ca. 15-20°C and add 145 mL $H_2O_2$ (50%), 62 mL of 0.1M $FeSO_4$ and 65 mL of 0.1M NaOH. Place the sample on an icy water bath and keep the temperature between 20-30°C for at least 4 hours (add ice in the water bath when necessary). Let it stand overnight.
(Use the ice bath to cool your sample down. If the sample gets too cold (< 20°C), then remove your sample from the ice bath and place it on the table for some time.). Keep a close eye on the oxidation, as the temperature can increase even after a couple of hours from the start of the reaction.

**Size fractionation**

The glassware and equipment required to carry out a size fractionation is listed here: metal sieve (10 cm diameter; 500 µm or 300 µm mesh); glass funnel with large opening (to place the sieve) 1 L beaker (use the same used for the previous steps); 100 mL cylinders class A (x2), 250 mL cylinder class A (x1); glass syringe (luer lock with aluminium joint needle); bucket containing ice (storage); large buckets to use as water/ice bath.

MP analysis using FTIR technology requires to use different approach depending of the size range of the particles targeted. The fraction between 5 mm and 500 µm (or 300 µm) is analysed via ATR-FTIR spectroscopy, while the fraction < 500 µm (or 300 µm) is instead analysed via µFTIR-Imaging spectroscopy. Therefore, it is necessary to size-fractionate the sample at this stage of the sample preparation.

Place a 500 µm (or 300 µm) steel sieve on top of a filtration unit containing the 10 µm filter used for sample filtration in the previous steps. The sieve can be accommodated on top of a glass funnel with large opening. Pour the liquid through then thoroughly flush the previous beaker (containing the sample after Fenton reaction). Flush the particles on the sieve abundantly with Milli-Q. Remove the sieve, backflush the particles into a beaker and save them for further treatment (then proceed with step K.). The fraction < 500 µm (or 300 µm) which passed through the sieve is filtered onto the 10 µm steel filter.

Flotation in separation funnel with high density liquid (SPT or ZnCl2)

The glassware and equipment required to carry out a flotation is listed here: glass separation funnel (from 100 mL to 250 mL) with Teflon stopcock; glass lid for the funnel; lab stand and clamps to secure the funnel; 150 mL glass beaker; glass syringe (luer lock with aluminium joint needle); nitrogen/compressed air intake; silicon pipes and piping joints.

Transfer the filter enriched with the sample (< 500 µm or < 300 µm) to a pre-cleaned 150 mL beaker containing around 25 mL of SPT (or Zinc Chloride). Sonicate for three minutes. Remove the filter (or filters in case you have to use more than one) and flush it with SPT. (Use the appropriate density, check the density before usage, because it can change during storage). Save the filter for the next step by placing it into a glass Petri dish.

Transfer the liquid to the separation funnel (choose an appropriate funnel volume according to the amount of solids in the sample); flush the beaker with SPT into the funnel. Fill the funnel 3/4 with SPT. Apply air for 15 minutes from the bottom of the funnel (filtered compressed air or nitrogen) by connecting a silicon pipe to the funnel's bottom opening. Do this by slowly opening the air valve, then the funnel's stopcock. If you do not have compressed air or nitrogen available, simply shake the funnel manually for a couple of minutes (close it with a glass lid before shaking).

After bubbling/shaking the funnel, flush the inside walls with SPT. Add SPT until the level reaches the largest aperture of the separation funnel. Leave it to settle overnight. Remove the settled matter using the funnel's stopcock. Wait 30 minutes, then remove the settled matter again. Repeat this until there is nothing settling. In case a relevant amount of particulate is still present, repeat all these flotation steps once more. After removing the settled particulate, proceed with filtering the top part of the flotated sample through the 10 µm steel mesh (! Filter the top part of the liquid ↧), flush 1 L of warm (50°C) Milli-Q water, then 100 ml of EtOH through the filter. Transfer the filter containing the sample to a 150 mL beaker with 50 mL of 50% v/v ethanol and incubate overnight at 50°C to clear out any SPT residue that may be attached to the particles. Filter the liquid through a new 10 µm steel mesh, flush with 4 L cold and 1 L of warm (50°C) Milli-Q water (the large volume are used to further flush the sample to remove any SPT residue), then 100 mL of EtOH through the filter.

**Evaporation**

The glassware and equipment required to carry out the sample evaportation is listed here: 10 mL headspace vials; vials lids with Teflon septa; glass syringe (luer lock with aluminium joint needle); ethanol (EtOH) 50% v/v (HPLC grade); metal spatula; warm pre-filtered demi-water; evaporation bath (biotage XXXXX).

Add the filter containing the sample to a new muffled 150 ml beaker, use 50% ETOH (HPLC grade) to flush (used a glass syringe with luer-lock attachment and needles with aluminium joint) and sonicate. Use as little EtOH as possible. Add the liquid from the beaker into a 10 mL headspace vial. Fill the vial ¾ and evaporate it into an evaporation bath before adding more liquid (the evaporation bath is a water bath operation at 50 °C using a gentle stream of nitrogen directed inside each vial to speed-up the evaporation process). After every aliquot is transferred into the vial for evaporation, flush the 'pouring'-side of the beaker, so particles do not get stuck to the glass wall. When the beaker is empty, flush it three times to ensure the whole sample is transferred into the vial.

Be careful not to dry out the beaker while waiting to transfer a new aliquot to the vial; the bottom of the glass must always be covered with some 50% EtOH.

After all the sample has been transferred into the vial and dried, remove the vial from the evaporator, and add 5 mL of 50 % EtOH (HPLC grade) to the vial using a calibrated 5 ml glass pipette. Sonicate the vials for three minutes. The sample is now ready to be analysed. The known volume allows to analysed sub-samples and re-calculate the particle conc. in the whole vial.

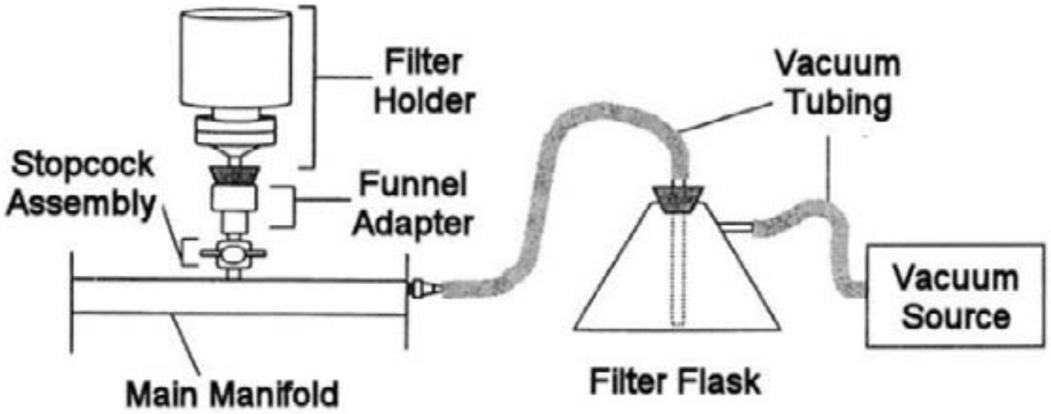
SPECIAL THANKS to Alvise Vianello and the group of prof. Jes Vollertseen for sharing this protocol

# Analysis Workflow

## Filtration

**Filtration apparatus.**

There are several choices of set-up but the main point to keep in mind is to avoid, as far as possible, plastic parts since they can be an unintended source of microplastics. The HORIBA choice (the parts depicted below are offered in our "Microplastic package" see HORIBA Solution section) for the filtration apparatus is:





DR

Stainless steel manifolds: These can be selected depending on the workload but we do not include any of these in our package where only the flask with the side arm is proposed.

*Filtration is the last step prior to the identification of the microplastics by the technique of choice (FTIR microscopy, Raman microscopy and optical microscopy) and two points must be addressed in this section:*
*Filtration apparatus and filter types.*

We have selected Sterlitech (https://www.sterlitech.com/) as our preferred filtration apparatus supplier, and the parts are: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Glass funnel (available up to 1 L, 100 ml in our package), a sintered glass support base with 13 mm available filtration area, a silicone stopper and a 1 L borosilicate glass flask with side arm.


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A diaphragm vacuum pump chemically resistant and completely oil-free.

# Analysis Workflow

## Filtration

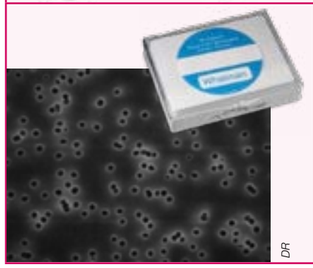
**Filters.**

There is a wide choice of filter/membrane and several of them have been tried and tested microplastics analysis. The three important characteristics are: filter size (13, 25, 47, 55 mm in diameter), filter material (polycarbonate, polytetrafluoroethylene PTFE, alumina, silicon etc. and pore size (0.2, 0.7, 1.6, 4.2 µm etc.). Of course, these features must be tuned depending on the microplastic sizes of interest and also on the techniques that will be used to identify them.

Our focus for filter choice is on the microscopy techniques (FTIR, Raman and optical microscopy) which are the most commonly used and seem to provide the most complete microplastics picture allowing: Chemical identification (true for Raman and FTIR), counting (number and size distribution) and quantitative estimation (number and mass).

The most commonly used filters are: Borosilicate glass fibers, Alumina, Polycarbonate (un-coated and coated with various metal layers) and Silicon. The table below summarizes the pros and cons of each of them including: **optical quality** (for microscope visualization); **mechanical resistance and handleability; interference** for microplastic chemical identification with Raman and Infrared Microscopy; and price.

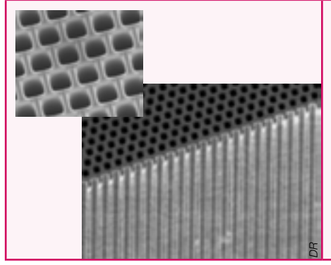
*Table 5. Filters pros and cons (part 1)*

| Filter Type | | Optical Quality | Handleability | Interference | Unit Price per filter (euro) |
|---|---|---|---|---|---|
|   | **Borosilicate Glass Fiber** (no binder) available with different pore sizes (lowest 0.6 µm) | Rough surface can reduce ability to identify microplastics (most significant for small particles, below 10 µm). White membrane ➡ low contrast for transparent plastics | No issue | Possible interference signals for Raman and Infrared Microscopy. | 0,25 to 14 Depending on filter size (up to 257 mm in diameter available) and grade |
|  DR | **Polycarbonate Uncoated** available with different pore sizes (lowest 0.2 µm) | Flat surface. White membrane ➡ low contrast for transparent plastics | Issue in case of Alkali tretament (KOH) | Strong interference with Raman and Infrared Microscopy. Polycarbonate shows strong bands both in Raman and Infrared. Not usable for Transmission Infrared Microscopy | 0,6 to 13 Depending on filter size (up to 142 mm in diameter available) |

# Analysis Workflow

## Filtration

*Table 6. Filters pros and cons (part 2)*

| Filter Type | | Optical Quality | Handleability | Interference | Unit Price per filter (euro) |
|---|---|---|---|---|---|
|  | **Polycarbonate Coated** available with different pore sizes (from 0.2 to 5 µm) and different metal coating: gold,silver | Flat surface and high relectivity and good contrast (Highly textured surface for Silver) | Issue with Alkali treatment (KOH) | Less interference than uncoated, but still present if metal is thin and for particles below 5 µm. Not useable for Transmission Infrared Microscopy | 8 to 23 for silver and 18 to 30 for gold Depending on filter size (up to 47 mm in diameter available for both) |
|  | **Alumina (Anodisc)** supported (surrounded by a polypropylene ring) and unsupported available with different pore sizes (from 0.02 to 0.2 µm) | Flat surface. White membrane low contrast for transparent plastics | Highly fragile, careful handling required | Low interference for FTIR (peak intensity change over the filter) and for Raman (broad spectral feature) - Useable for Transmission Infrared Microscopy but no signal below 1250 $cm^{-1}$ | 5 to 15 Depending on filter size (up to 47 mm in diameter available) |
|  | **Silicon** with different pore sizes (from 1 to 18 µm) | Flat surface. High relectivity and good contrast | Easy handling, possible fragility along crystalline direction. Squareshaped (dedicated holder needed) | Raman (silicon peaks do not interfere with plastic peaks) - FTIR (possible interference from silicon oxide) - Useable in Transmission Infrared Microscopy | 14 to 24 Depending on volume |

Regarding polycarbonate filters one research group[1] tried different metal coatings, not commercially available, and obtained very good results with Raman microscopy and Aluminum coated polycarbonate. Aluminum can enhance Raman scattering by a factor of 4[2], thus improving detectability.

Alternatively, it is possible to use $CaF_2$ and/or ZnSe windows (usually with a diameter of 13 mm); these are not filters but windows widely used in Infrared and Raman microscopy. A solution of microplastics can be concentrated to few millilitres by evaporating the solvent and then it can be poured onto the window and left to dry before spectroscopic analysis.

A last important point, it is simple but is key to getting good results, is the amount of microplastics in the solution to be analyzed. The filter must not be tightly packed with material otherwise optical identification and further analysis of the particles will be complicated if they overlap. In this case just prepare a more dilute solution before filtration as was done in the literature[3].

1. Oßmann *et al.*, WATER RESEARCH 141 p.307 2018
2. Kamemoto *et al.*, APPLIED DPECTROSCOPY 64 p.255 2010
3. Bergmann *et al.*, ENVIRONMENTAL SCIENCE & TECHNOLOGY 51 p.11000 2017

# Analysis Workflow

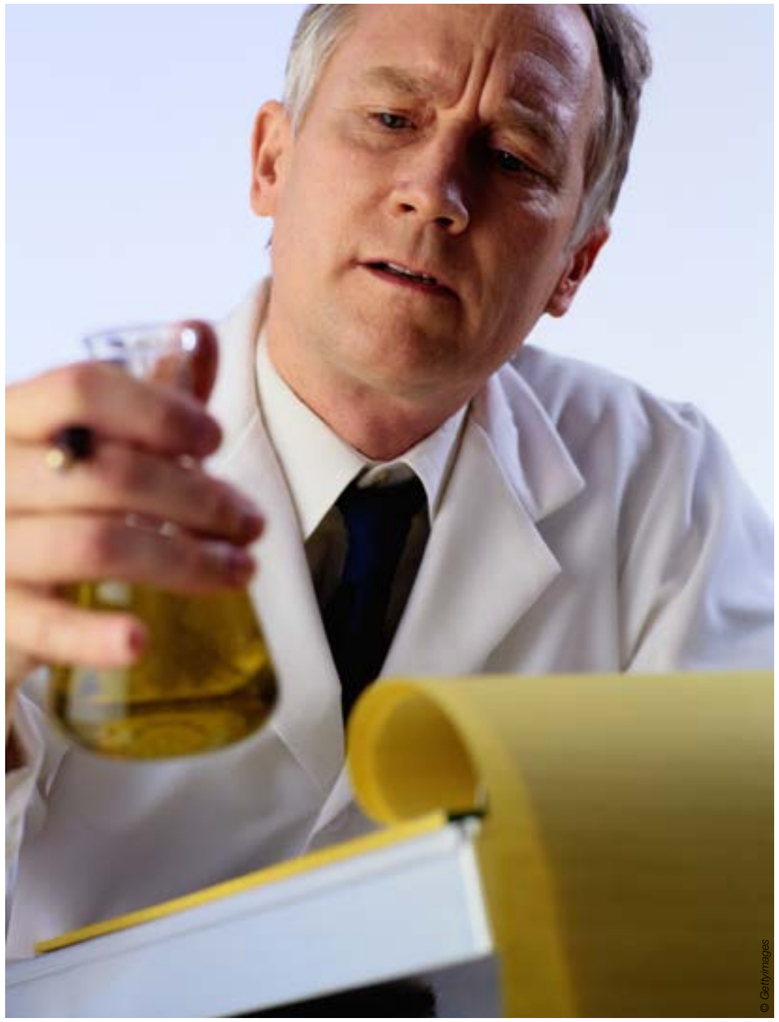
## Measurement methodologies

The last step following sampling, sample preparation and filtration is identification of the microplastic using one or more different techniques.

Five main techniques are used for this purpose:

1. Fluorescent staining with Nile Red, coupled with Fluorescence microscopy
2. Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDX)
3. Infrared Microscopy
4. Raman Microscopy
5. Pyrolysis Gas Chromatography Mass Spectrometry (Pyr-GCMS)

They are complementary with each other but Infrared and Raman provide a more detailed picture. Raman being more flexible (all sizes of plastics can be analyzed) since it is able to detect microplastics below 10 µm. These represent the main threat/concern for Human health (Ragusa et al.[1] observed plastic pieces below 10 µm in the placenta).



**Nile Red staining/Fluorescence microscopy**.
Nile Red is a fluorescent dye (see absorption curve, dashed line, and emission curve, below) widely used to localize and quantify lipids but it can also selectively bind to most plastics, allowing them to be identified by looking at the fluorescence in both the green as well as in the red.

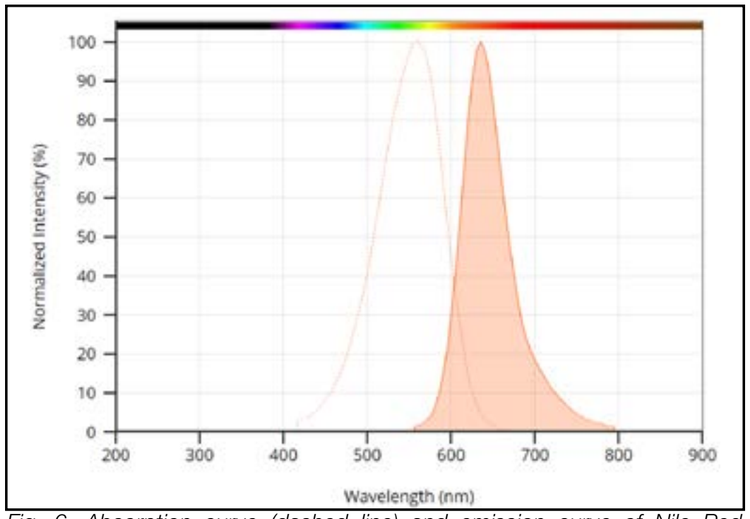



*Fig. 6. Absorption curve (dashed line) and emission curve of Nile Red fluorescent dye*

Since Nile Red also binds to lipids, environmental samples, careful sample preparation (complete digestion of the biogenic material[2]) is key to successful analysis, as the presence of biological residues can lead to an overestimation of the amount of microplastics.

# Analysis Workflow

## Measurement methodologies

For non-environmental samples such as bottled/tap water[3] where digestion is not needed Nile Red can be used directly. After staining microplastics can be easily and automatically counted by looking at the green or red colored particles with a fluorescence microscope. The best approach, as demonstrated in the literature[2], is to use green / yellow fluorescence (excitation/emission 450–490/515–565 nm) as this avoids natural lipids which emit deeper into red (higher wavelength). A Nile red concentration of ranging from 0.1 and 2 µg/mL is typically used.

*The advantage of this technique is speed and simplicity, the main drawbacks are the lack of chemical identification of the polymer types and the possible presence of false positives. Erni-Cassola et al.[3] validated this method by also using Raman microscopy, which notwithstanding the presence of the dye, can be still used, to chemically identify the polymer.*

**SEM-EDX.**

Scanning Electron Microscopy stand alone allows the complete morphological characterization of the particles down to the nanometer range (which is a strong advantage in comparison to the other techniques) but it is not able to provide chemical information and samples, in almost all cases (Fries et al.[4]), must undergo to additional treatments due to the high vacuum in the test chamber and to avoid charge accumulation. Moreover, the filters suitable for Infrared and Raman microscopy cannot be used, instead the microplastics must be dried and then transferred onto double-sided adhesive carbon tabs on aluminum SEM stubs.

SEM combined with the energy dispersive X-ray spectroscopy can give additional information by providing the elemental composition of the sample. Elemental information allows:

- Inorganic and carbon-based material to be distinguished (the full digestion of organic contaminants is essential to assign carbon-based material to plastic);
- Some polymer types to be identified, such as PVC due to the presence of Chlorine[5];
- Identification of the presence of other elements[6] such as Al, Ca, Mg and Si on the plastics which can be the signature of polymer additives.

Summarizing, SEM-EDX is a technique which unlike can provide detailed morphological information down to nanometer range (morphology may influence the diffusion of microplastics within the human body) but it cannot be used alone as it does not provide comprehensive chemical information.

**Infrared Microscopy.**

Infrared microscopy is currently the most widely used technique for microplastic analysis and all the commercial instruments are also combined with optical microscopy. In some instruments the optical microscopy is limited by the use of infrared objectives only, which cannot provide the flexibility and high magnification of standard visible objectives and this limits the identification of small particles.

Infrared microscopy is a non-destructive technique and can provide morphological information (by the analysis of the optical and/or chemical image), quantitative analysis (in terms of number of particles) and chemical identification of the microplastic (by comparing the collected infrared spectra with the ones in commercial libraries). Like Raman microscopy one of the most complete techniques. The main drawback/limitation of Infrared microscopy is its inability to identify particles smaller than 10 µm. Zhu et al.[7] in a recent review of June 2020 mentions that the smallest particle size determined with infrared microscopy is 20 µm.

# Analysis Workflow

## Measurement methodologies

There are two main approaches to analyze microplastic with infrared and both start with an optical image acquisition of the filter used in the filtration step:

1. In the first approach, the particles' size, shape and their location on the filter are determined by analyzing the optical image, the location is then used to individually measure each particle by moving the infrared beam to the required location;
2. In the second approach, the particles' size and shape are determined by the analysis of the optical image and then the whole filter is chemically imaged with the infrared beam (This approach collects many spectra so needs a specialized detector such as a Focal Plane Array (FPA) or a linear array, to reduce the acquisition time as much as possible). The size and shape of the particles can also be determined by analyzing the chemical image, but accuracy can be limited by the resolution of the image.

**Raman Microscopy.**

Raman microscopy is the second most common technique and like Infrared it also includes standard optical microscopy. Raman instruments use visible objectives that are available with a full range of magnifications, so image quality and morphological information is uncompromised, Raman microscopy is a non-destructive, non-contact technique that provides morphological information (by analyzing the optical image), quantitative analysis (number of particles) and chemical identification of the microplastic (by comparing the collected Raman spectra with the ones in commercial libraries).

The biggest advantage of Raman microscopy compared to infrared is the ability to measure and identify particles of 1 µm[8] and below in size. This point is crucial since the biggest concern for human health seems to come from particles below 10 µm because they can migrate within our body[1].

One drawback of Raman microscopy is the interference caused by fluorescent materials such as environmental and/or plastic pigments, additives and pollutants; fluorescence which can overlap with the Raman spectrum, limiting the ability to identify the microplastic. The presence of fluorescent material does not always limit plastic identification (Enri-Cassola et al.[2] successful measured Nile red stained particles) and additionally several excitation wavelengths are available to assist avoiding fluorescence interference (the use of a near infrared excitation source, i.e. 785 nm, often limits the fluorescence signal).

The main approach to analyze microplastics with Raman microscopy is:

*First step is the acquisition of an optical image of the filter from which the particles' size, shape and location are determined; second step, is to move the laser beam to each identified location, and acquire a Raman spectrum of each particle.*

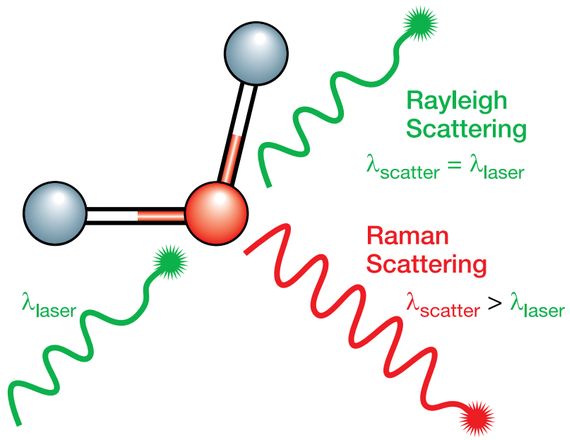



*Fig. 7. Raman principle*

**Pyr-GCMS.**

Pyrolysis combined with Gas Chromatography Mass Spectrometry can determine the chemical composition of the microplastic by analyzing their pyrolysis products (Pyrograms). Similar to Infrared and Raman, which use spectral libraries for chemical identification, the pyrograms obtained are compared with reference ones of known polymers. The chemical identification is not as detailed as for vibrational spectroscopy techniques, in particular, the polymer[9] sub-type (such as Low density vs. High density polyethylene) cannot be discriminated and, in case of complex matrices, the identification can be misleading.

The main advantages of Pyr-GCMS are the quantitative analysis of Microplastic in terms of weight per polymer type for polymers which exceed the quantification detection threshold, and the low amount of material needed (5 µg can be enough) although this small quantity may not be representative for complex environmental matrices.

The drawbacks of Pyr-GCMS are:

- Destructive technique: Samples cannot be re-analyzed;
- Lack of information on particle morphology: size and shape, which are well known to influence the risk assessment of microplastics;

*Table 7. In the table, we have summarized the main advantages and disadvantages of the different techniques.*

| Technique | Nile Red & Fluorescence Microscopy | Scanning Electron Microscopy & Energy Dispersive X-ray Spectroscopy (SEM-EDX) | Infrared Microscopy | Raman Microscopy | Pyrolysis Gas Chromatography Mass Spectrometry (Pyr-GCMS) |
|---|---|---|---|---|---|
| **Pros** | Fast and Simple<br>Low-cost<br>Morphological information<br>Quantitative analysis (Number of Particles) | Particle down to nanometer size<br>Discrimination between Inorganic and carbon-based material<br>Elemental analysis | Non destructive<br>Morphological information<br>Chemical identification<br>Quantitative analysis (Number of Particles) and Quantitative per polymer type | Non contact and non destructive<br>Morphological information<br>Chemical identification<br>Quantitative analysis (Number of Particles) and Quantitative per polymer type<br>Particles down to 1 micron and below | Quantitative analysis (weight of particles)<br>Partial Chemical identification |
| **Cons** | False positives<br>No chemical identification<br>No Quantitative analysis per polymer type | Additional preparation needed<br>No chemical identification<br>High cost | Sensitive to particle dimension (bigger particles cannot be analyzed in transmission)<br>Smaller particles (<10 micron) cannot be analyzed | Interference by fluorescent material | No Morphological information<br>Destructive |

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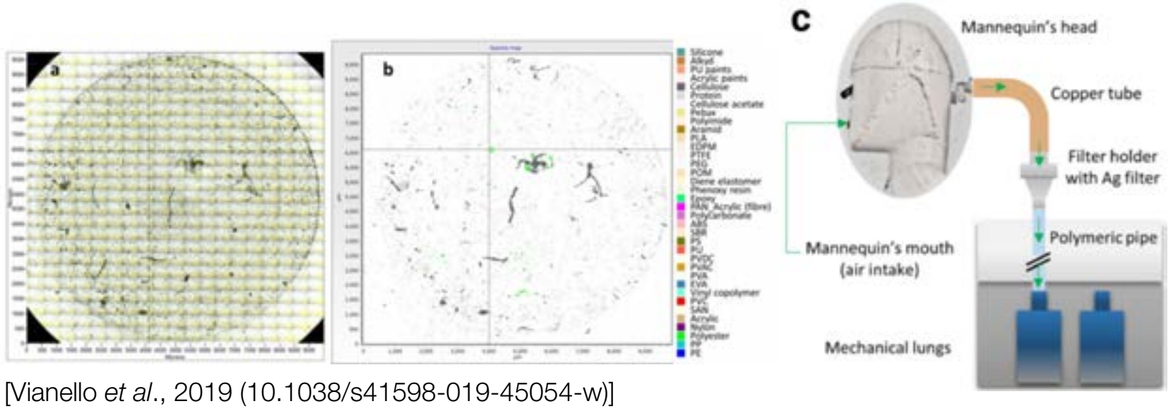
# Humans and Plastic

## Microplastic and humans: proofs of exposure and hypothesis of danger

Considering the increasing concern about the human and animal exposure to Microplastics we decided to have a dedicated chapter on this topic and as first and introductory contribution we asked Valentina Notarstefano to write a short review to explain the most common routes of human exposures to microplastics and show the evidence of their accumulation and translocation in the human tissues. Valentina is a post-doc researcher at the Department of Life and Environmental Sciences at the Polytechnic University of the Marche (Ancona, Italy) and is working in the team of Prof. Elisabetta Giorgini (head of the Laboratory of Vibrational Spectroscopy). Valentina has focused her research on the use of Infrared and Raman spectroscopy to study biological systems such as cells, tissues, fluids and is providing in this review a clear picture of the actual state of the art about Humans&Microplastic without reaching hasty conclusion.




[Vianello *et al*., 2019 (10.1038/s41598-019-45054-w)]

**Valentina Notarstefano**

Postdoc at Università Politecnica delle Marche - DiSVA - Ancona, Marche, Italy

The presence of microplastics in the environment has been widely documented and their ubiquitous nature makes the human exposure inevitable. The three so-called exposure routes are ingestion, inhalation and dermal contact. However, we have to pinpoint that dermal absorption is a minor exposure route, since only particles smaller than 100 nanometers can likely pass the dermal barrier [1].

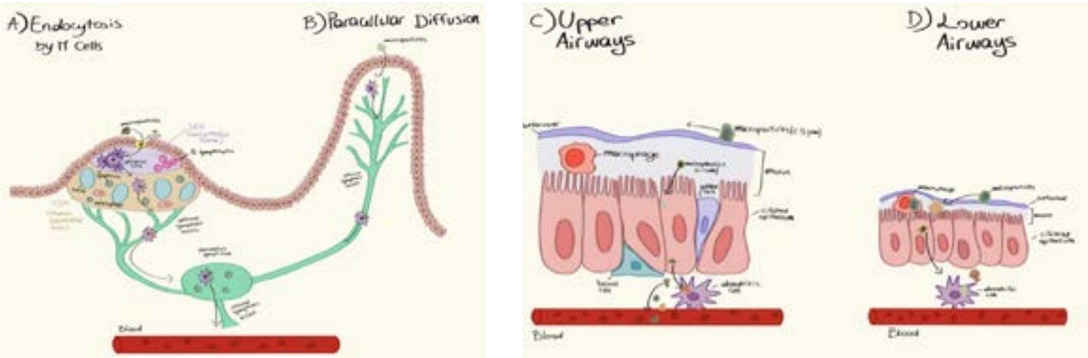
Ingestion is considered the principal exposure route, with an estimated intake of about 39 to 52 thousand ingested particles per person per year. These particles can be ingested from contaminated food, like fish and mussels, but also from table salt, sugar, honey, milk and bottled water. Moreover, food can be contaminated from our use of plastic packaging and plastic kitchen utensils [1].

Besides ingestions, it has been demonstrated that microplastics also contaminate the air we breathe. These microplastics mainly derive from synthetic textiles, but also from the abrasion of plastic materials. In particular, it has been estimated that outdoor concentrations range from 0.3 to 1.5 microplastics per cubic meter, while the indoor ones can also reach 56.5 microplastics per cubic meter. Moreover, a study demonstrated that a person can inhale up to 272 particles per day, with a tangible possibility that these particles reach alveoli and enter the bloodstream, obviously according to their dimensions [1–3].

# Humans and Plastic

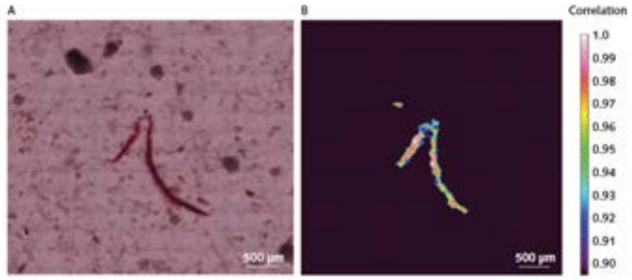
## Microplastic and humans: proofs of exposure and hypothesis of danger

Once the routes of exposure have been identified, researchers focused on elucidating what happens to the internalized particles. Obviously, the fate of the inhaled or ingested microplastics strongly depends on their features, first of all their dimensions, that cannot exceed 10-15 microns, making it possible for the particles to travel in the bloodstream. In fact, the interest towards the toxic effects of microplastics in terms for example of oxidative stress, inflammation, and immune response is everyday greater, however these phenomena can only happen after the passing of microplastics through cellular membranes and their accumulation within tissues. Some hypotheses have been suggested to explain the penetration of microplastics through human tissues, until their arrival into the bloodstream from which, hence, they can potentially reach numerous body districts [4,5]. 1) endocytosis by the M cells (which are mucosal cells of the intestine, placed next to the lymphoid nodules called Peyer's Patched, with the role of modulating the immune response); the M cells act by endocytosing solid particles and transferring them to the dendritic cells; when these soldi particles are microplastics, we know that they cannot be destroyed by the action of the lytic enzymes and hence they would be transported by the dendritic cells to the lymphatic stream and then to the blood stream. 2) paracellular diffusion: microplastics may also penetrate the organism by passing the intestinal lumen if there are points where the junctions are more loose; this possibility arises when there are inflammation states, for example; even in this case, the dendritic cells would transfer microplastics to the lymphatic and then blood streams. 3) at the level of the Upper airways, the mucus layer is thick and allows a successful clearance of the foreign bodies; moreover, the mechanical movement of the ciliated epithelium prevents particles from spreading through the epithelium and reach the circulation. (D) conversely, at the level of the Lower airways, the mucus layer is thinner, thus facilitating the diffusion of particles which have reached the respiratory tract. Once penetrated, the MPs can spread into the general circulation by cellular uptake or diffusion.



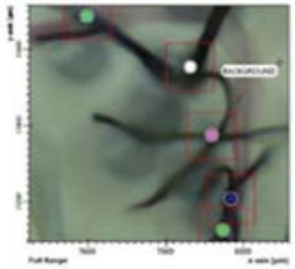

[Ragusa *et al*., 2021 (10.1016/j.envint.2020.106274)]

The proofs of human exposure are growing in literature. The first study in this sense is from 2019: the authors reported the presence of MPs in human stool. This is not a proper evidence of accumulation in tissues, but for the first time the researchers demonstrated that ingestion really is an exposure route for humans [6].




[Schwabl *et al*., 2019 (10.7326/M19-0618)]

A step forward has been made by another research group, who found MPs in human colectomy samples, ultimately proving that not only MPs are ingested and excreted, but also a part of the mis also internalized through the intestinal tissue [7].



[Ibrahim *et al*., 2021 (10.1002/jgh3.12457)]

Then, MPs have been found in lung tissues by exploiting Raman spectroscopy: this result confirms that inhalation is an exposure route and that inhaled particles can accumulate in human tissues [8]. Recently, this study has been confirmed by another one, quite similar, but performed by exploiting IR spectroscopy [9]. In 2021, some Italian researchers found microplastics in human placenta samples, by using Raman microspectroscopy; in particular, 12 MPs were found in 4 out of the 6 analysed placenta samples [5]. We obviously have to mention a very recent paper where researchers report the presence of MPs bigger than 700 nanometers in blood, with an average concentration of about 1.6 micrograms per ml. The main identified polymers where polyethylene, polyethylene terephthalate and various styrene-based polymers. Obviously, this result is crucial, since it indirectly confirms all the others, proving that MPs really reach the bloodstream, by which then they reach numerous other body districts. For this study, authors did not exploit a vibrational spectroscopy, pyrolysis double shot - GC/MS [10]. Finally, the same Italian team who found microplastics in human placenta recently published their discovery of microplastics in human breastmilk; in particular, microplastics ranging from 2 microns to 12 microns, mainly made of PE, PVC, and PP, were found in 26 out of the 34 analyzed samples [11].

# Humans and Plastic

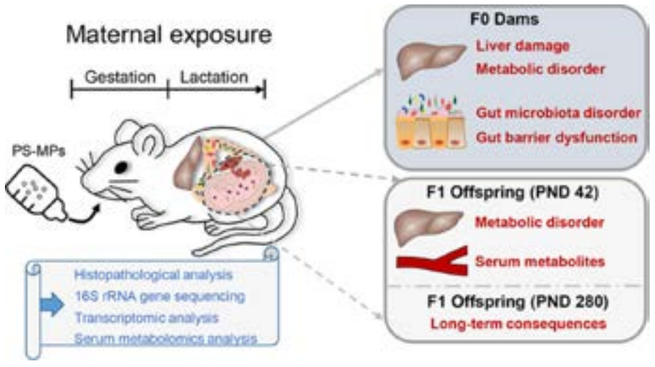
## Microplastic and humans: proofs of exposure and hypothesis of danger

So far we have seen how microplastics are able to enter the human organism, and travel through the bloodstream, potentially accumulating in almost all districts. The next question is: once internalized and accumulated, can microplastics exert a toxic effect? Or are they just inert material? Several studies have reported clear toxic effects of various types in animal models, marine organisms and human cell lines, and these results are precious in understanding what can happen to humans. It has been reported that microplastics, in addition to translocating to other tissues, can cause for example oxidative stress, cytotoxicity, neurotoxicity, immune reaction, etc. As regards cytotoxicity and immune modulation, it has been demonstrated, for example, that PP-MPs exert a cytotoxic effect on immune and blood murine cells, mainly by inducing an increase in ROS, in a size-dependent and concentration-dependent manner [12]. Moreover, the direct contact of the polypropylene particles with the immune cells did not in itself lead to toxicity, but induced an increase in the production of cytokines and histamine.

Oxidative stress can derive from an overcrowding of antioxidant responses, generated by the high surface area of microplastics, from the release of oxidizing species adsorbed on their surfaces, such as metals, or from the inflammatory response. Microplastics have been shown to alter some biomarkers of oxidative stress and to trigger the production of reactive oxygen species. For example, the tissue uptake and accumulation of polystyrene microplastics in zebrafish were investigated in this study [13]: the authors reported that, depending on their size, microplastics accumulate in the gills, liver and intestines of fish, also inducing inflammation and accumulation of lipids in the liver, with an increase in the activities of superoxide dismutase and catalase, signals of oxidative stress. Also in mice, fluorescent polystyrene microplastics added to the water showed to accumulate in the liver, kidneys and intestine; moreover, the authors highlighted a disturbance of energy and lipid metabolism, together with oxidative stress [14].

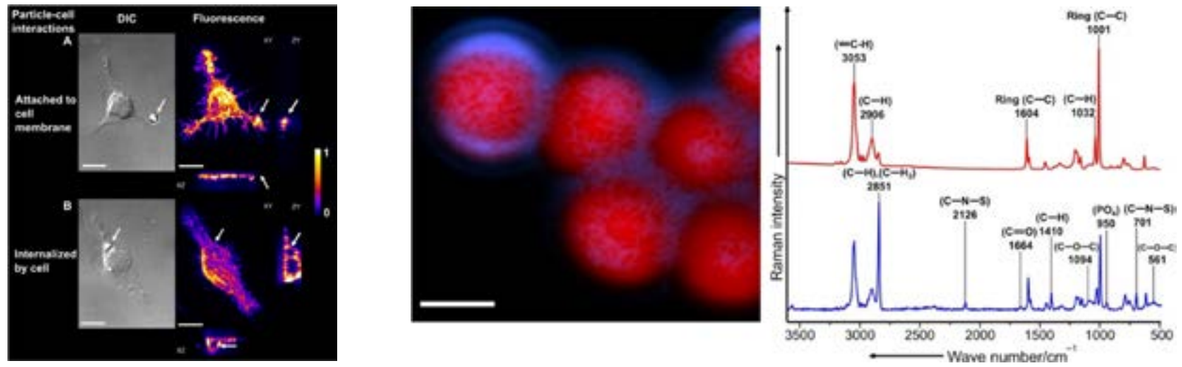
The immune system also appears to be strongly influenced by the absorption of microplastics: in this regard, microplastics act like other environmental particles which, once internalized, provoke local or systemic immune responses. However, the evidence in this area is still limited. For example, a study performed on the mussel *Mytilus galloprovincialis*, showed that the exposure of the mussels to microplastics led to the interruption of global homeostasis, with the production of immune-related proteins; the removal of microplastics showed to activate apoptotic processes and to upregulate stress-related proteins, in an attempt to compensate for the stress associated with exposure to microplastics [15]. Notably, repeated exposures to microplastics have suggested that mussels may be able to create some sort of memory about exposure to microplastics.

There is also evidence of detrimental effects on reproductive abilities. A very alarming study reported transgenerational effects due to the exposure to microplastics [16]. Rodents who were given contaminated water during gestation and lactation showed liver changes and altered intestinal microbiota; in addition, the F1 generation showed similar hepatic alterations and altered levels of metabolites in serum; negligible effects were highlighted in generation F2. Another study reported that the exposure to polystyrene microplastics is able to cause a thinning of the endometrium and severe deposition of collagen fibres in female mice, finally leading to uterine fibrosis [17].




[Luo *et al.*, 2019 (10.1021/acs.est.9b03191)]

All these works are inevitably conducted in the laboratory, under very controlled conditions and with microplastics produced for the very specific purpose. A very interesting study has highlighted how the erosion and aging of microplastics caused by their environmental exposure play a fundamental role in the internalization process in cells [18]. In fact, the researchers have identified biomolecules that form an eco-corona on the surface of the microplastic particles that appear to significantly increase the internalization of microplastics after environmental exposure.




[Ramsperger *et al.*, 2020 (10.1126/sciadv.abd1211)]

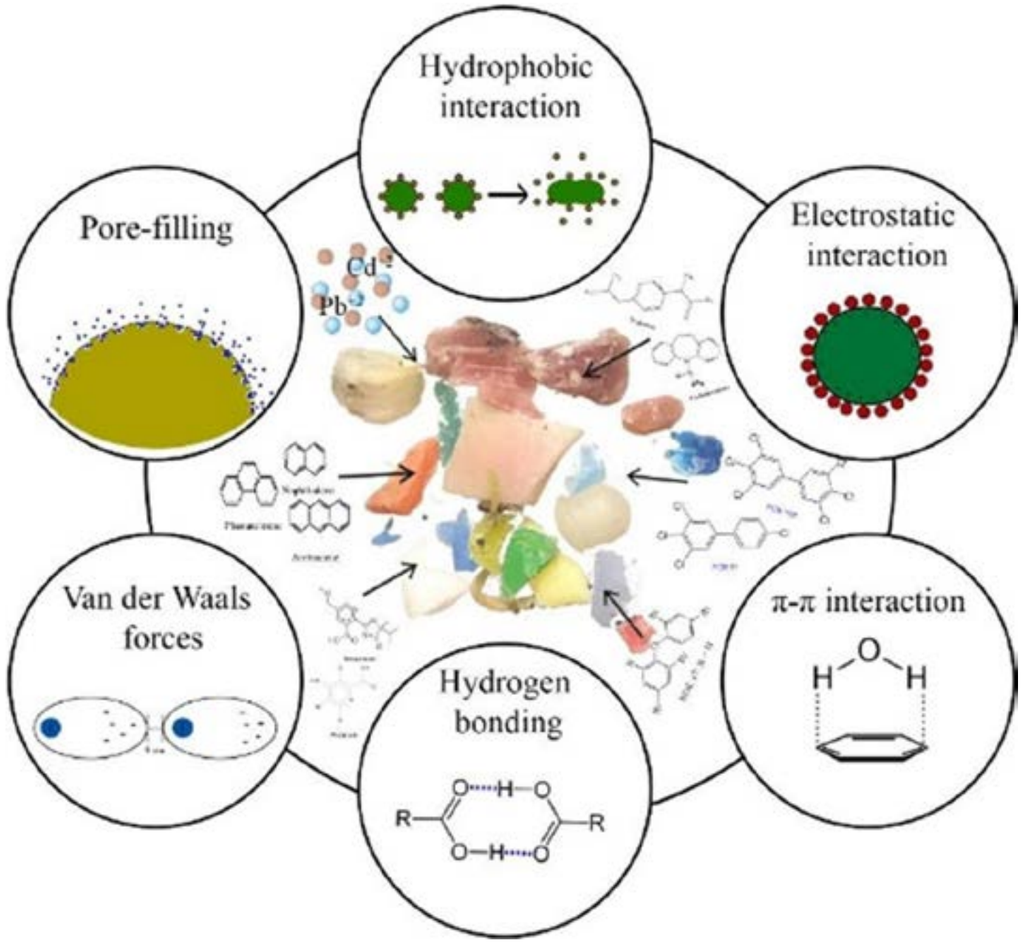
# Humans and Plastic

## Microplastic and humans: proofs of exposure and hypothesis of danger

Another aspect must be taken into account when considering the interaction between microplastics and the external environment: it has been widely demonstrated that microplastics display the ability to act as carriers of other possibly toxic and dangerous chemicals, metals and microorganisms, concentrating them several orders of magnitude respect to the levels in the surrounding environment [19–21]. The research presented in the previous examples was conducted in the laboratory, under controlled conditions and with pristine microplastics, while it can be assumed that environmental exposure to microplastics also involves contact with other chemical and biological species. Microplastics may act as efficient vectors for the transport of other potentially toxic and even carcinogenic chemicals, including persistent organic pollutants (POPs) has emerged, including pyrene, benzo (a) pyrene, phenanthrene, polychlorinated bisphenyls, DDT, and polycyclic aromatic hydrocarbons. This absorption is finely regulated by various factors, both related to the polymer (type, colour, size, state of aging) and to the environment (pH, salinity, temperature). It has to be considered that the real evidence of the delivery and desorption of toxic compounds by microplastics is difficult to prove: most of the effects described for pristine microplastics and toxic chemicals are similar and it is difficult to discriminate between the two pollutants. For this reason, the debate on the vector role of microplastics is still ongoing, with favourable and unfavourable arguments: for example, it is not clear whether the absorption of POPs on the surface of microplastics makes them more or less bioavailable to the body once internalized.

[Torres *et al*., 2021 (10.1016/j.scitotenv.2020.143875)]

Furthermore, microorganisms can colonize the surface of microplastics, which act as vectors, carrying microorganisms to tissues, protecting them from the immune system and creating tissue damage that can promote infection. Microorganisms can form fully grown biofilms on the artificial substrate of microplastics, which allowed Yang and colleagues to describe microplastics as new microbial niches in the aquatic environment. For example, Vibrio bacteria are normally characterized by low concentrations in water, but several studies have highlighted their presence on the surface of microplastics with the highest concentration of all microorganisms found. Surely the microplastics that carry bacteria and viruses are more biotoxic and can trigger, among all the effects previously considered, even infections [1,22,23].




In this brief chapter, the identity of microplastics, their sources of contamination, the routes of exposure by which they contaminate animals and humans, and the reasons why they are a concern, have been investigated. In particular, the routes of human exposure, mainly ingestion and inhalation, were explained, together with the published evidence on the translocation and accumulation of microplastics in human tissues. The reported selection of the studies clearly supports the theory that microplastics are not inert particles, but have various effects once internalized: oxidative stress, cytotoxicity, altered immune responses, neurotoxicity and so on; moreover, microplastics are potential and effective vectors of other toxic chemicals and microorganisms: however, evidence of all these aspects has been partly found in marine organisms and animal models, and some results have been achieved in the laboratory, under controlled conditions, which are not always comparable to what actually happens in the environment. Based on all these considerations, it must be stated that the results presented are valuable for understanding what can happen to humans, although we must be cautious in translating this information into alarmism.

# Humans and Plastic

## Microplastic and humans: proofs of exposure and hypothesis of danger

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# Nanoplastics

## A growing interest

Nanoplastics, as discussed on chapter 1 (i.e. Microplastics) are the smallest piece of plastics and they are less than 1 micron in size. The interest on plastics below 10 microns and down to nano size range, if we consider publications in scientific peer reviewed journals, grew almost 70% year over year in the last 5 years. One of the main drivers of this growth is connected to the capability of plastics of these sizes to enter in the human body through different routes such as ingestion, inhalation and dermal contact (indeed dermal absorption is possible only for nanoplastics smaller than 100 nanometers) posing a big concern about their effect on the human's health (see chapter Humans and Plastic).

The growing interest on Nanoplastics can also be measured by looking at the increased number of grants financed on this topic in the last three years (see picture 2). These data are considering a limited number of all the financing entities, but they can be representative of a global trend.

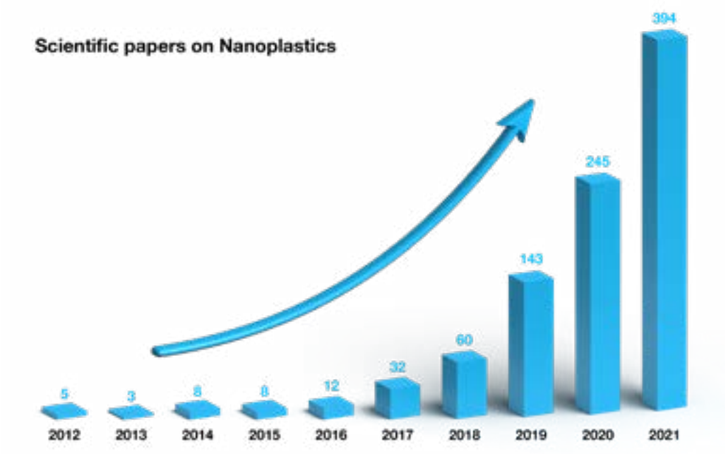



*Fig.1 Scientific peer reviewed papers on Nanoplastics in the last 10 years*

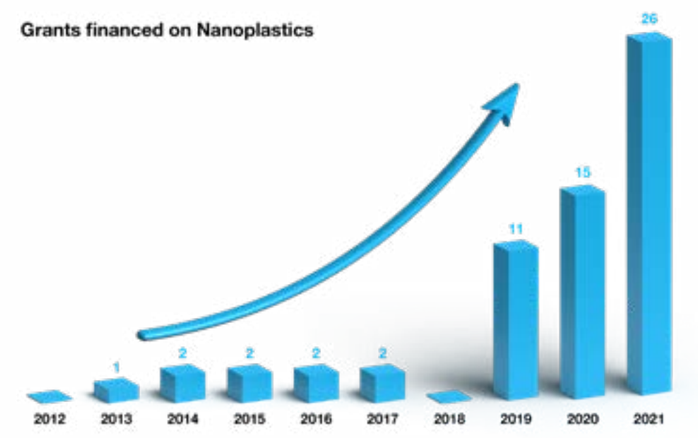



*Fig. 2 Financed grants around Nanoplastics in the last 10 years*

*(information collected using SCITODATE engine by different sources Swiss National Science Foundation – SNSF; Deutsche Forschungsgemeinschaft – DFG; National Science Foundation – NSF; UK Research Innovation – UKRI; Community Research and Development Information Service – CORDIS; French National Research Agency – ANR; Russian Science foundation – RSF; National Institute of Health – NIH etc…)*

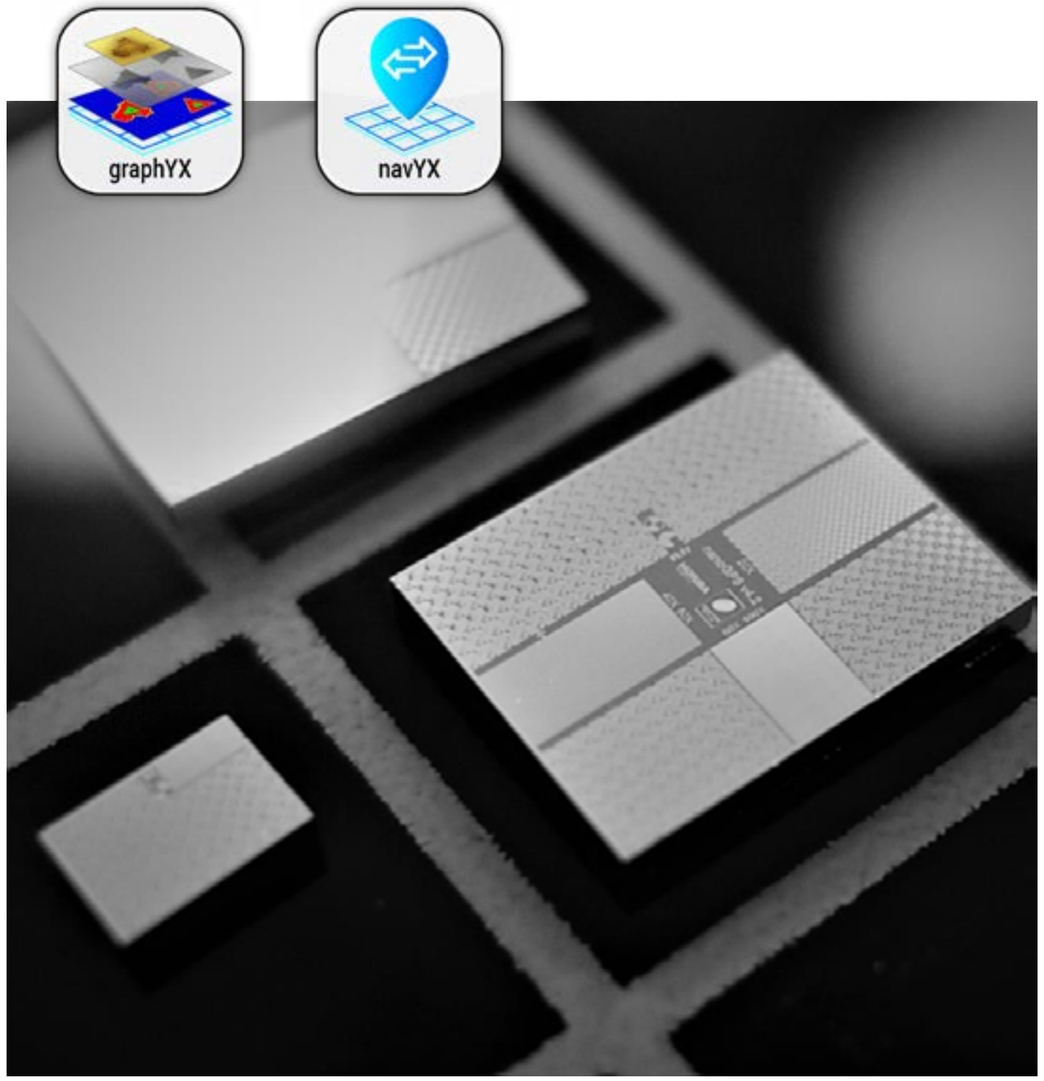
# Nanoplastics

## A growing interest

In HORIBA we are working on testing and optimising different solution to analyse Nanoplastics, such as co-localized Raman-Atomic Force Microscopy analysis, Tip-Enhanced Raman microscopy, cathodoluminescence combined with Scanning Electron Microscopy (SEM) and in the future edition of the booklet we will provide more insights in these different approaches but on this we would like to focus on the HORIBA nanoGPS for correlative microscopy. HORIBA nanoGPS is a patented technology which allow to best leverage the correlative microscopy approach since it permits to quickly localize small objects of interest ($\mu m^2$) on large samples ($cm^2$) and analyse them across multiple microscopy techniques. This technology combines two softwares for navigation (navYX™) and superimposition of cartographies (graphYX™); and hardware tags (Fig. 3). It guarantees re-localisation of points of interest between multiple modalities with micron accuracy, whatever the magnification, the sample orientation, and the brand of the instrument.

Coming to Nanoplastics the nanoGPS and the correlative approach guarantees the Raman analysis of plastics in the nanometers range by combining SEM and Raman microscopy; indeed the SEM image allows to locate the nanoplastics (which are not clearly visible with the optical microscope of a Raman instrument) on the sample determining as well if they are well separated or aggregated with others and the Raman allows the chemical characterization thank to the precise re-localization of them under the laser spot granted by the nanoGPS technology. Moreover, the nanoGPS tags are particularly suitable for nanoplastics characterization because they are small (1.4 x 2 mm) and can be affixed very easily to any type of filter design, from polymeric to silicon membranes, and are compatible not only with SEM but also with Atomic Force Microscopy, Fourier Transform Infrared Microscopy.

In the contribution of George Sarau et al. entitled “Context microscopy and fingerprinting spectroscopy of micro- and nanoplastics and their effects on human kidney cells using nanoGPS and ParticleFinder” we will see the nanoGPS technology applied in a real case.



# Nanoplastics

## Human kidney cell and Nanoplastics

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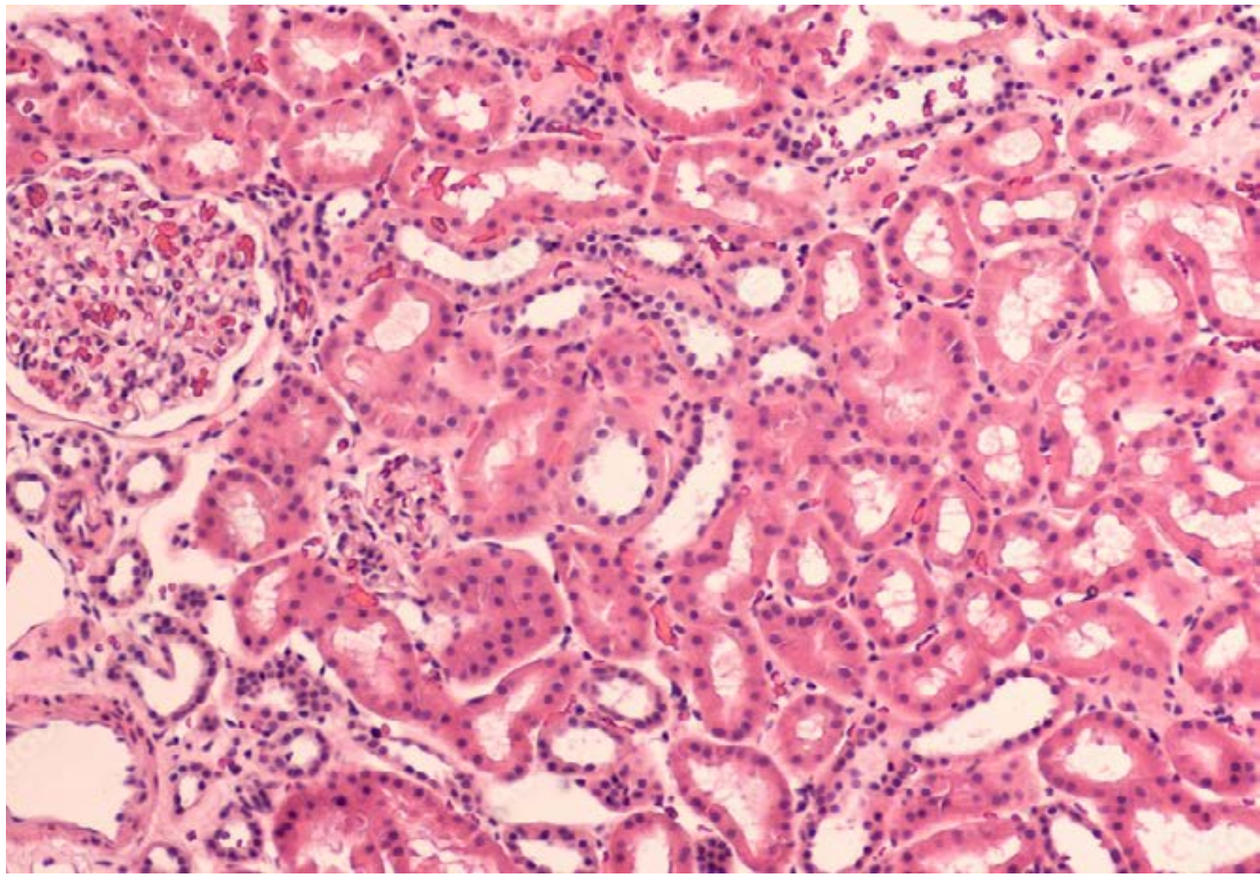
**Key words:** microplastic, nanoplastic, tyre wear, podocytes, kidney, nanoGPS, ParticleFinder, SEM, Raman, correlative workflow, microscopy, spectroscopy

Nowadays humans are almost continuously exposed to micro- and nanoplastics (MNPs) through food and air, but very little is known about the exposure level and impact on our health. Here, we focus on bottled mineral water and cultured human podocytes as representative kidney cells prone to accumulation of particles. It is demonstrated that identical MNPs and cells can be precisely relocalized and extensively characterized down to nanoscale in independent instruments using nanoGPS and ParticleFinder technologies developed by HORIBA. Reference particles and particles contained in mineral water were detected, enabling statistical distributions of their mean number, size, and type depending on the bottle and label materials. The primary effects of MNPs (three standards and tyre wear) on human podocytes were assessed using a cell viability test followed by correlative microscopy and spectroscopy investigations of the same cells. We observed changes in the biological features of MNP treated cells compared to non-treated controls, attributed to cell damage through surface adhesion and uptake of plastic particles. The integration of automatic relocalization and detection of identical objects in a multi-instrument workflow represents a novel analytical approach that can be applied beyond this topic.



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## Introduction

Production of plastics has dramatically increased over the last decades and with it the plastic waste in the environment.[1] Plastics are nowadays used almost in all products including packaging, construction, textiles, tires, cosmetics, and so on.[2-4] The major issue is the mismanaged plastic waste that is not collected at all or improperly filtered and recycled, which significantly contaminates thea environment on a global scale through the transfer between terrestrial, river, and ocean compartments.[5] Once left in the environment, plastic debris persists and degrades continuously into smaller fragments down to micro- and nanoplastic (MNP) particles, attributed to size classes of < 5 mm and < 1 µm or ≤ 100 nm, respectively.[6,7].With time, these MNPs are assumed to develop into toxic chemical cocktails by increased adsorption of hazardous pollutants and pathogens from the environment given their larger surface areas due to fragmentation, in addition to additives and pigments added during manufacturing of plastics. Moreover, the smaller the plastic particles become (< 1.5 µm), the higher the probability to enter by ingestion and inhalation into human organs and subsequently to accumulate and leach chemicals with still unknown toxicological effects on our health.[8-10]

Microscopy- and spectroscopy-based methods are commonly used to monitor MNPs in environmental samples usually after filtering as well as in various biological matrices and organisms. The employed techniques mainly include optical microscopy with stereozoom, scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDS), pyrolysis gas chromatography coupled with mass spectrometry (py-GC-MS), Fouriertransform infrared (FT-IR) and Raman microspectroscopies, each method with its benefits and drawbacks.[11-13] Recently, we showed that a correlative approach is needed to avoid overestimation of particles' size and underestimation of particles' number for clustered MNPs as well as to measure Raman without optically visualizing the plastic nanoparticles by overlapping SEM and optical images of high (< 10 nm) and low (~ 1 µm) spatial resolution, respectively. This was achieved by a correlative microscopy and spectroscopy workflow applied to identical MNP particles on large-area filters using an optical zoom microscope and a hyphenated SEM-Raman instrument (with a bright field optical objective for micro-Raman inside the SEM vacuum chamber).[14] However, such combined systems are limited with respect to the number of measurement techniques available on one instrument compared to stand-alone, method-specific instruments from different manufacturers, in which finding the same micro- and nanosized objects is still a challenge.[15-19]

In this work, the first application of a newly developed relocalization technology for a detailed characterization of MNPs and their effects on human kidney cells in independent instruments is demonstrated. This technology is based on a patented position encoder tag (from HORIBA), called nanoGPS tag, with lithographically defined patterns. These patterns are used to translate the sample coordinates corresponding to the regions of interest (ROIs) into the stage coordinates of different instruments (from HORIBA, Zeiss, Leica in this study), regardless of the sample orientation. Furthermore, the applicability of the ParticleFinder software module (from HORIBA) for automatic detection of microplastic (MP), pigment, and additive particles on large-area filters is shown. Context microscopy and fingerprinting spectroscopy approaches were applied to standard MPs, microparticle contamination of bottled mineral water, and human podocytes that were either untreated or incubated with MNPs. The podocytes exposed to MNPs were under stress and started to die gradually, indicating an overall effect of particle exposure on cell viability.

## Experimental

The samples investigated in this study can be divided into three categories: reference micro-sized plastic particles, mineral water from different bottle types bought in Bavarian food stores, and human podocytes cell cultures exposed to MNPs.

### Standard MP particles

Commercially available standard plastics (see Table 1) were selected to match the polymer types routinely encountered in the environment.[13,20,21] A mixture of polyethylene (PE), poly(vinyl chloride) (PVC), polyamide-Nylon 6 (PA), polystyrene (PS), and polypropylene (PP) particles were suspended in a solution (ultrapure water and sodium dodecyl sulphate (SDS)) followed by vacuum filtration through polycarbonate (PC) membrane filters (diameter 25 mm, pore size 0.4 µm) previously coated with aluminum (Al thickness 100 nm) as detailed in our previous work.[22] These reference materials were used to evaluate the nanoGPS relocalization technology (hardware and software) and its integration in a correlative microscopy and spectroscopy workflow applied to identical MNP particles (see Figure 1). The nanoGPS tag (4×5 mm²

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silicon piece) is firmly attached next to the filter, which is rigidly stretched and flattened between two metal rings fixed on a SEM holder, to avoid any thermal drift and ensure precise relocalization in different instruments. Along with the corresponding NaviGo software, the instruments' stages involved in the workflow are calibrated and the coordinates of ROIs are recorded.

## Mineral water particles

Real mineral water samples packaged in reusable bottles made of poly(ethylene terephthalate) (PET), in single use PET bottles, and in glass bottles (single and reusable) were analyzed for microparticle contamination, taking also into account bottle age as well as label and cap type. Before suspension in SDS solution and vacuum filtration through Al coated PC membranes, calcium and magnesium carbonate particles were dissolved with ethylene diamine tetraacetic acid tetrasodium salt (EDTA) to reduce the number of non-plastic particles.[21] To obtain statistically relevant data given the complexity of bottled mineral water contamination including microplastic, pigment, additive, and mixed particles, we employed an automatic particle detection approach. This is based on the ParticleFinder software that transforms large-area (1 $mm^2$) dark field optical images obtained by stitching into grey scale images, on which particles are easily detectable using their brightness, counted, classified by size and shape, and their coordinates recorded for further micro-Raman chemical identification. Thus, the mean number of microplastic, pigmented, and additive particles (projected to 1 L sample volume), their size, and type distributions were estimated (see Figure 2, additives not included).[21,22]

*Table 1: Details of the plastic particle standards used in the present study to assess the nanoGPS relocalization and the exposure of human podocytes to plastics (PVC, PA, PP). Adapted with permission from Springer Nature.[22]*

| Material | Type | Manufacturer | Size (µm) |
|---|---|---|---|
| Polyethylene (PE) | Clear microspheres, powder | Cospheric | 1-10 |
| | | | 10-106 |
| Poly (vinyl chloride) (PVC) | Powder | Pyropowders.de | < 50 |
| Polyamide - Nylon 6 (PA) | Powder | GoodFellow | 15-20 (average particle size) |
| Polystyrene (PS) | Polybead Micron Microspheres, 2.5% solids in water | Polysciences Inc. | 1 |
| Polypropylene (PP) | Chromatographic Grade, powder | Polysciences Inc. | 25-85 |

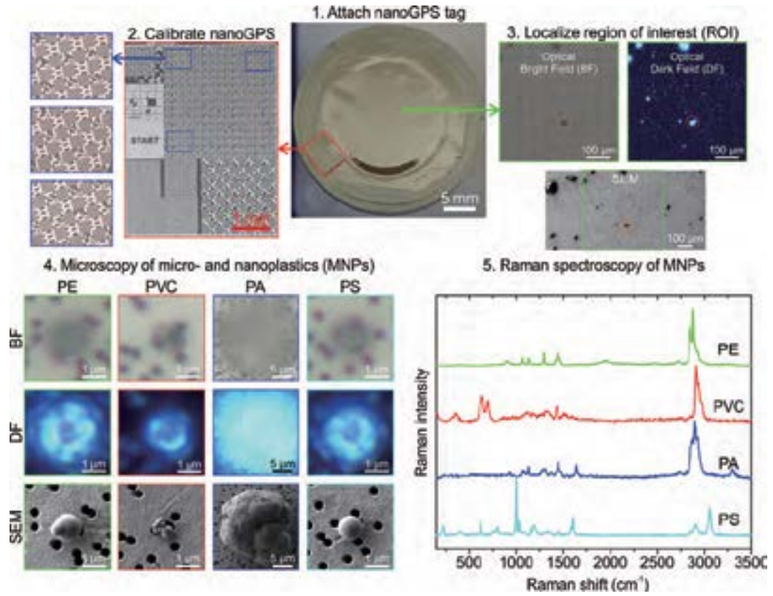


*Figure 1 Correlative microscopy and spectroscopy workflow for micro- and nanoplastics on an Al coated PC membrane used to filter MNPs from water. First, a so-called nanoGPS tag is attached directly to the sample. Second, three images are recorded at random positions on a pattern (different patterns correspond to various instrument magnifications) and fed into a software that calibrates the global, stage coordinates into local, tag (sample) coordinates including sample rotation. This procedure is repeated for each instrument to be used in the workflow. Third, identical ROIs are precisely relocalized in independent instruments, regardless of the sample orientation. Fourth, the same single or agglomerated particles are imaged at optical (BF, DF) and SEM spatial resolutions to assess size, shape, number, and surface morphology of MNPs down to nanoscale. DF imaging is used to clearly distinguish MNPs from the porous structure of large-area filters. Fifth, unambiguously chemical identification by micro-Raman spectroscopy is applied. The Raman spectra are taken with permissionfrom the Society for Applied Spectroscopy.[14]*

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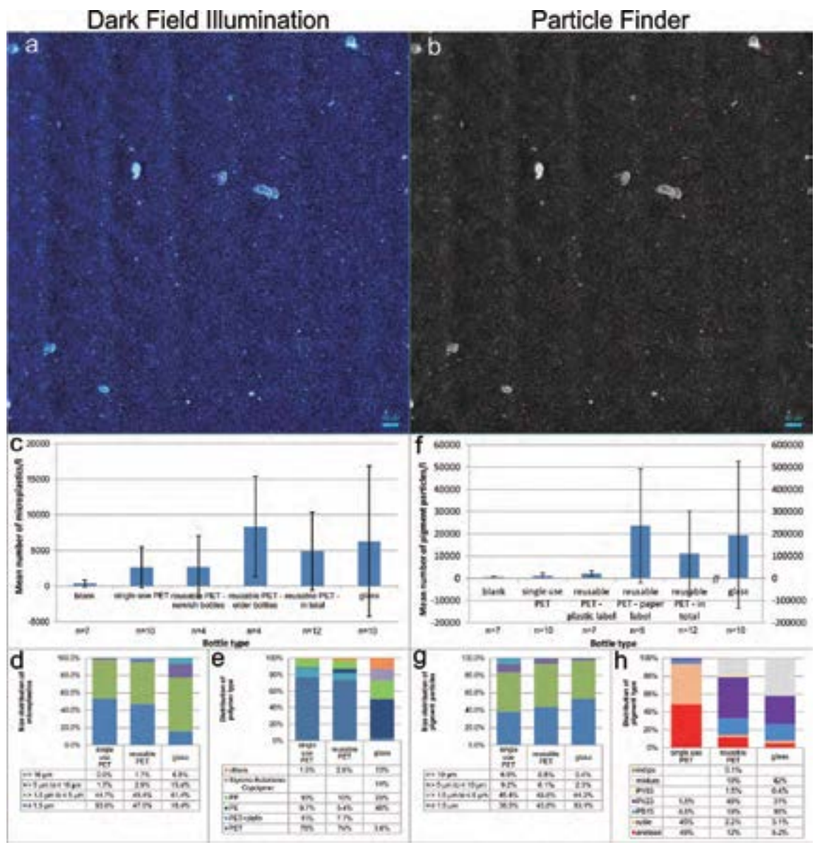


*Figure 2: (a) Example of a dark field montage (1 mm²) obtained by stitching, on which particles from mineral water samples shine brighter than the pores of the Al coated PC membrane filter. (b) ParticleFinder software converts the DF image into a grey scale image used to automatically detect, classify, and measure Raman spectra of individual particles at their center, marked by red points. (c, d, e) Mean number of microplastics ± standard deviation projected to 1 L sample volume, size, and plastic type distributions function of the bottle material. (f, g, h) Mean number of pigments ± standard deviation projected to 1 L sample volume, size, and pigment type distributions function of the bottle material. Adapted with permission from Elsevier.[21]*

### Human podocytes exposure to MNPs

Conditionally immortalized human podocytes that contain a heat sensitive CV40T antigen were cultured as described previously.[23] Podocytes were proliferated under growth permissive conditions at 33°C and further differentiated through the inactivation of SV40 T-antigen at 37°C. After 7 days of differentiation, cells were treated with different concentrations of diluted standard plastic (PVC: 0.5, 1 mg/ml; PA: 0.5, 1 mg/ml; PP: 2.5, 5 mg/ml) and tyre wear (0.125, 0.5 mg/ml) particles for 7 h to evaluate their possible effects on the cells. In order to decrease the aggregation of particles, they were sonicated before the incubation. Following the particle treatment, cells were washed two to three times with phosphate buffered saline (PBS) and fixed for further biological, imaging, and spectroscopy assays. For this study, the podocytes were grown on the surface of silicon wafers previously coated with platinum (Pt thickness 100 nm) that were attached along with nanoGPS tags to SEM holders to avoid relative sample - tag position shifts when moving between instruments.

### Analytical methods

Complementary analytical techniques present on different instruments were used to visualize and detect MNPs on filters and inside cells as well as to determine the changes in cells caused by the contact with MNPs. All measurements have been performed at room temperature. The latter point was first addressed by using a live-dead cell imaging kit based on two-color fluorescence cell viability assay (Thermo Fischer Scientific). Based on this assay, cell-permeable and cell-impermeable dyes were used for staining of live and dead/dying cells, respectively. Following the particle treatment, the live/dead cells were assigned based on the kit instruction. Fluorescent images were collected with the use of an Evos M5000 imaging microscope (Thermo Fischer Scientific) (see Figure 3).

Furthermore, we employed a confocal micro-Raman spectrometer (HORIBA LabRAM HR Evo-Nano or XploRa PLUS), operated by the LabSpec 6 software (with data analysis and ParticleFinder), equipped with bright and dark field illumination (BF, DF) objectives coupled to a camera to image MNPs and cells (~ 1 µm spatial resolution). Three lasers (532, 633, and 785 nm) focused by 50× (NA 0.75) or 100× (NA 0.9) objectives were used for Raman excitation and collection in a backscattering geometry with

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laser powers of 1.2 mW or 3.2 mW (532 nm), 11.2 mW (633 nm), and 5.3 mW (785 nm). Two gratings (300 and 600 grooves/mm) and integration times of 1- 20 s and 2x accumulations were applied. The acquired Raman spectra and maps (step size 1 µm) were analyzed to chemically identify the particles and the structural damage induced by them on the human podocytes. A SEM (Zeiss field emission Auriga, secondary electron detector) was used for a detailed morphological imaging of MNPs and cells (< 10 nm spatial resolution) at a low voltage of 1 kV to avoid modifications caused by electron scanning. The height profiles of the same cells investigated by micro-Raman and SEM were measured by a confocal imaging microscope (Leica DCM 3D), the relocalization of identical cells being realized using the nanoGPS technology (see Figure 4). Moreover, because of the superposition of Raman bands related to the plastic materials and cells, we applied a classical least squares algorithm (CLS) available in LabSpec 6 to highlight the spatial distribution of MNPs on the mapped cells (see Figure 5).

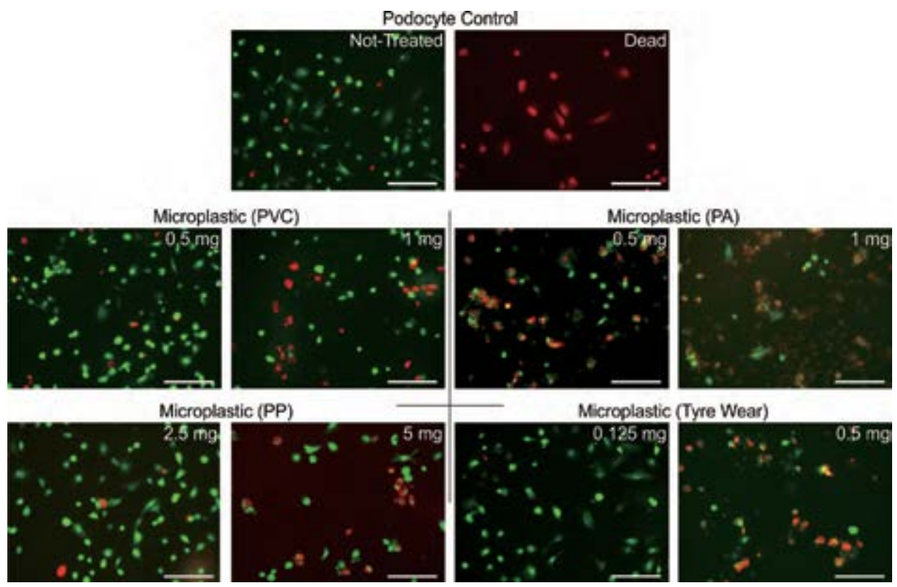


*Figure 3: Fluorescence live - dead cell imaging (green - red) to assay the cytotoxicity of microplastic and tyre wear particles on podocytes, following 7 h particle exposure at relevant concentrations (mg/ml) and washing with PBS. The control cells were non-treated or intentionally killed to check the live - dead cell imaging kit. The concentrations to initiate and induce a notable impact on podocytes depends on the polymer type. During particle incubation the cells are under stress and start to die gradually. Consequently, some of the degraded cells are washed away and not assigned with colors. Some attached particles with intrinsic fluorescence are also visible. The preliminary results of this assay are yet mostly qualitative and show an overall effect of particle treatment on the cell viability. Scale bars are 300 µm.*

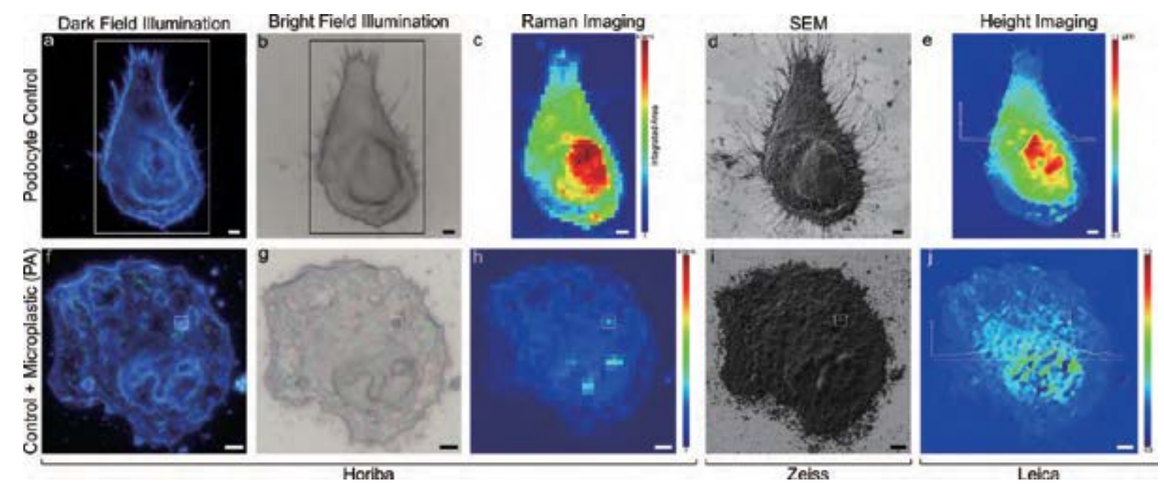


*Figure 4: Correlative microscopy and spectroscopy workflow applied to podocytes untreated, control (first row) and particle treated (second row) with 1 mg/ml PA (Table 1 and Figure 3) using the nanoGPS position encoder tag (Figure 1). Two representative cells were easily relocalized and investigated in three independent instruments from different manufactures (Horiba, Zeiss, Leica) with complementary analytical techniques. First, an integrated optical microscope with dark (a, f) and bright (b, g) field illumination and micro-Raman spectrometer are used for a fast visual inspection of cells, followed by Raman imaging (c, h), showing less Raman signal for treated cells (note the same scale) that is an indication of podocytes damage after exposure to PA. Second, SEM imaging (d, i) reveals detailed surface morphology changes at nanoscale induced by the PA treatment and visualizes a PA nanoparticle (~ 30 nm), as confirmed by micro-Raman spectroscopy, delimitated by the square in the second row. Third, an interferometric profilometer is employed to measure the height profile without (e) and with (j) plastic contamination (note the same scale), PA incubated cells being flatter. Two horizontal profiles are also shown (maximum heights of ~ 1.5 µm and ~ 0.8 µm for the control and treated cell, respectively). Scale bars are 3 µm.*

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### Results and Discussion

#### nanoGPS relocalization

The nanoGPS relocalization technology for correlative microscopy and spectroscopy investigations is illustrated in Figure 1 for standard micro-sized plastic particles (Table 1), with some particles being by chance < 1 µm. First, a nanoGPS tag is rigidly mounted next to the Al coated PC membrane filter, both on a SEM holder that is moved between instruments, such that the tag and sample keep their positions relative to each other. The smaller the distance between tag and sample, the better the relocalization accuracy that can be further influenced by stage and imaging characteristics. Second, the multiscale and multimodal patterns on the tag are employed to calibrate the stage of each instrument, different feature sizes being used for distinct instrument magnifications (see SEM image of the entire tag). Three images are taken at random positions on a chosen pattern and fed along with the global, stage coordinates into the NaviGo software. In this example, images were recorded with the 10× objective of the optical microscope on the micro-Raman spectrometer. The software automatically determines the local, sample coordinates and rotation with respect to the tag. This calibration procedure is repeated for all instruments in the workflow and can be recalled anytime by recording one single image on the same pattern, independent of stage and sample rotation.

In the third step, one or more ROIs are located on the filter and their sample coordinates are saved in one instrument and retrieved in other instruments by converting sample, local into stage, global coordinates. In our case, largearea optical images acquired by stitching under BF and DF illumination are compared to a large field of view SEM image, with the same particle marked on all overview pictures. Next, MNPs can be directly relocalized and imaged at spatial resolutions of optical and electron microscopies (step four) and their spectral fingerprints determined by micro-Raman spectroscopy (step five) (PP is not shown). While on the BF and DF optical images these particles appear to be single, SEM imaging reveals that PE and PVC are cluster particles. When approaching the filter pore size, particles are barely visible in BF, but clearly noticeable in DF because they shine brighter than the pores, as seen for PVC. Moreover, SEM shows smooth surfaces with spherical and fragment-like shapes for the studied polymer particles. It should be noted that BF, DF, and Raman are usually performed before SEM; however, low-voltage

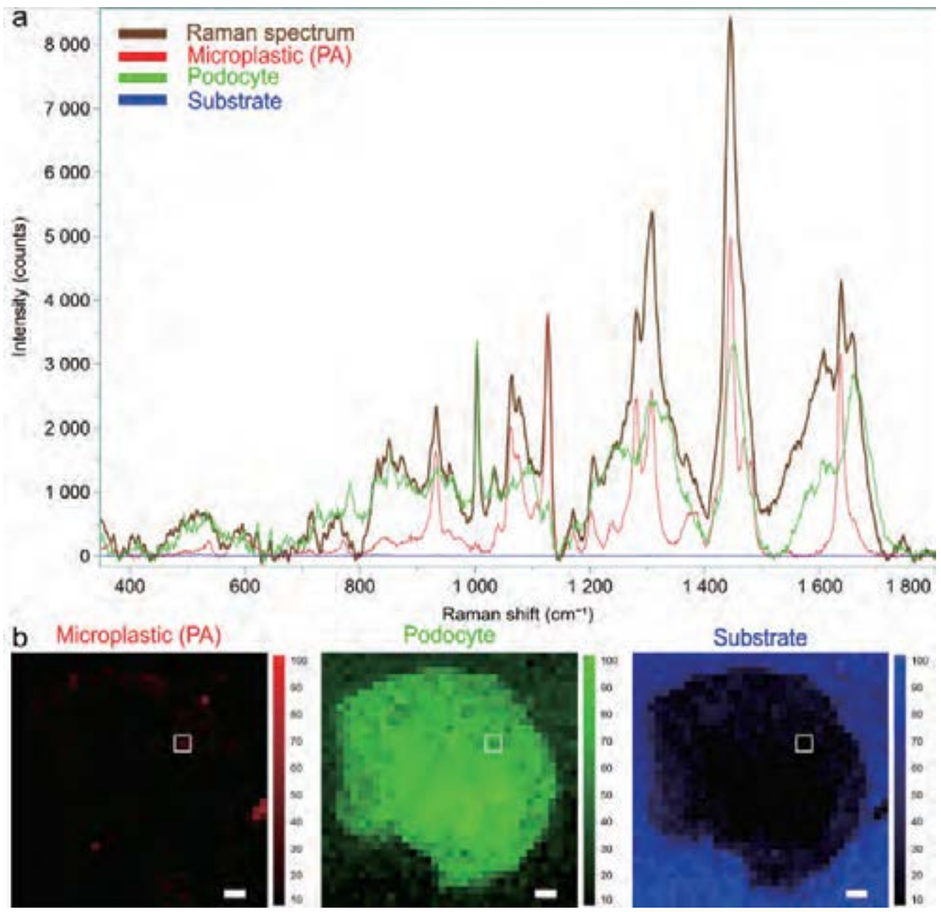


*Figure 5: (a) Classical least squares (CLS) fitting is applied to decompose each measured Raman spectrum into its spectral components based on given reference spectra. (b) Separate score maps are generated for each component as illustrated for the podocyte cell treated with PA shown in Figure 4 (second row). The square indicates the position of a PA particle. Thus, despite the superimposed and complex Raman bands of cells and MNP particles, the spatial distribution of MNPs can be clearly localized. Scale bars are 3 µm.*

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SEM does not damage MNPs, so that Raman after SEM is also possible.[14] All in all, nanoGPS tagging enables sample navigation and observation at different length scales in independent instruments, thus detailed morphological (size, shape, surface, number) and chemical characterization of the same micro- and nanoparticles is achievable.

## ParticleFinder

The ParticleFinder software module combined with DF optical microscopy and micro-Raman spectroscopy represents another example of correlative analysis applied here to study contamination by microplastic, pigment, and additive particles in bottled mineral water. 32 samples from 21 different brands of mineral water were investigated to determine the number, size, and type of particles, the results being summarized in Figure 2.[21] DF imaging is used to scan five large-area image montages (1 $mm^2$) on each sample to warrant significant particle statistics. Such a montage generated by stitching (Figure 2a) is then converted into a grey scale image, on which all particles ≥ 1 µm are automatically detected and individually measured by micro-Raman (Figure 2b).

We identified varying amounts of microplastics in water from all bottle types, partly resulting in large error bars when calculating the mean particle number (Figure 2c); however, some trends are clearly visible. On average, higher number of microplastics were found in water from reusable (PET and glass) compared to single use PET bottles. Interestingly, newish, reusable PET showed less microplastics than older, reusable PET, but similar to single use PET, suggesting that the bottle age can critically affect MP contamination. Regarding the average size distribution, 90.5% of MPs were ≤ 5 µm in all bottles and ~ 50% were ≤ 1.5 µm in PET bottles (Figure 2d), these MP size classes being addressed for the first time in such samples.[21,24] The predominant polymer type detected in PET bottles was PET considered to originate from the bottle material, while some PET particles displayed olefinic or pigment spectral interferences. In glass bottles, we mainly found PE and PS attributed to abrasion of caps on the glass bottleneck as well as PS, styrenebutadiene- copolymer, and PET most likely from the machinery used for the cleaning process (Figure 2e).

In addition to microplastics, pigmented and additive particles were also detected in the analyzed mineral water samples. Large variations in the number of pigmented particles in water from different bottle and label types were observed (Figure 2f). On average, single use PET contained less pigments similar to blank samples, while reusable PET and glass bottles with printed paper labels showed higher amounts of pigments. Alike MPs, older, reusable PET displayed more pigments than newish, reusable PET and most of the pigmented particles belonged to size classes investigated for the first time, 91.5% were ≤ 5 µm and 45.1% were ≤ 1.5 µm (Figure 2g).[21,24] We found that the pigment types mainly correspond to the colors used for printing on the paper labels (Figure 2h). These pigment particles originate from the paper labels and enter into the bottles during the cleaning process.[25] Additive particles were detected in reusable PET bottles and considered to leach from the bottle material (68.6% were ≤ 5 µm and 11.7% were ≤ 1.5 µm). These results demonstrate that ParticleFinder can be used for automatic detection, classification, and Raman measurement of particles < 1.5 µm from real samples, which is very important due to toxicological reasons, since this size class is considered small enough to penetrate deeply into organs.[21,22]

## Effects of MNPs on podocytes

The potential risk of plastic particles on human health is addressed in this study using human podocytes as a highly-specialized kidney cell type. Since kidneys are involved in the filtration process and do not regenerate their cells continuously, they are likely to accumulate MNPs over the lifetime.[26] We performed cell viability tests after incubation of podocytes with four different MNP types (standards PVC, PA, PP, and tyre wear) using a live-dead (green - red) cell fluorescent based kit. Representative results for relevant plastic concentrations after 7 h exposure with respect to control cells are summarized in Figure 3. The cytotoxicity response is found to depend on the polymer type, a higher concentration is needed for PP (5 mg/ml) compared to PVC, PA, and tyre wear (0.5 - 1 mg/ml) to achieve a similar cell mortality rate. Two mechanisms are proposed to explain the damage induced by the plastic particles on podocytes and finally their death. First, particles can attach on the cell surface and limit the nutrient uptake, the degree of attachment depending on particles' adhesion properties and sizes. Some particles still remained attached

after three times washing with PBS following incubation and can be visualized based on their intrinsic fluorescence as shown in Figure 3. Second, smaller size particles can be taken up into the cells by phagocytosis as illustrated in Figure 4 for PA particles.

The correlative microscopy and spectroscopy characterization of identical cells using the nanoGPS relocalization technology is demonstrated in Figure 4, exemplary shown for PA treated cells. Two representative podocytes (control and incubated) are localized in three independent instruments and studied with complementary analytical techniques down to nanoscale resolution. Optical imaging (~ 1 µm spatial resolution) under DF (a, f) and BF (b, g) illumination show the degradation and deformation of cells after particle exposure. The structural damage is further confirmed by micro-Raman mapping (c, h), treated cells display Raman spectra with less intensity (note the same scale for the integrated area maps). High spatial resolution SEM imaging (< 10 nm) is used to assay the integrity of cell features at nanoscale, exposed cells do not regularly show normal biological features like heterogeneous surface, nucleus, and foot processes (d, i). Height profile imaging acquired with an interferometric profilometer quantifies the deformation of incubated cells that flatten with respect to control cells (e, j), with height changes from ~ 0.8 µm to ~ 1.5 µm, respectively (note the same scale). Given the complex peak structure of Raman spectra from cells and plastic particles and the large overlap between peaks, we employed a CLS fitting algorithm that decomposes each measured Raman spectrum into its spectral components and provides score distribution maps for each component as displayed in Figure 5. This enables us to spatially resolved MNPs without underlying podocyte and substrate backgrounds, which are shown separately. Taking advantage of the nanoGPS relocalization capability in a correlative workflow, the same PA particle (outlined by the square in Figure 5b and Figure 4 - second row) was imaged by SEM and found to be a nanoparticle (~ 30 nm) most likely taken up into the cell by phagocytosis (Figure 4i). All in all, these preliminary experiments indicate the negative influence of plastic particles on human podocyte cells; however, more assays are needed to account for other relevant polymers present in the environment and their separate and mixed effects on different human organs, tissues, and cells.

**Conclusion**

The present study introduces an efficient measurement protocol for the assessment of contamination, accumulation, and hazards related to micro- and nanoplastic particles in bottled mineral water and human kidney cells. This protocol combines context microscopy and fingerprinting spectroscopy with automated relocalization (nanoGPS) and detection (ParticleFinder) of the same MNPs and cells in separate instruments from distinct manufactures (HORIBA, Zeiss, Leica). Results on microparticle contamination (average number, size, type) in mineral water and toxicity effects of MNPs (standards PVC, PA, PP, and tyre wear) on podocytes (in-vitro) are reported. It was found that the bottle material (single use, reusable PET and glass), bottle age (older, newish reusable PET), and label print (paper, plastic) affect the distributions of microplastics, pigments, and additives. In contrast to non-treated controls, podocytes incubated with MNPs tend to lack usual cell characteristics such as heterogeneous surface, nucleus, and foot processes, confirming the potential risk of plastic particles on the viability of cells. These findings were revealed by a biological cell test supported by complementary methods involving optical (bright, dark field) and scanning electron microscopy, micro-Raman spectroscopy (with CLS spectra fitting), and height interferometric profilometry. Further work will deal with different plastic types, concentrations, and exposure times.

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* Editorial note: This content is based on HORIBA's investigation at the year of issue unless otherwise stated.

George Sarau and Melina Yarbakht contributed equally to this work.

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# HORIBA Solution

Filtration appartus
Raman microscope
MVAPlus, ViewSharp, Navsharp, VRM
ParticleFInder
Know It All
Reference standard sample

After considering the optimal microplastic analysis workflow and the needs and challenges of the people approaching this topic. HORIBA has developed a full solution to for this application that will help our existing and future customers by providing all the tools needed in a single bundle:

1. This **Booklet**, which will be updated with new releases twice a year to keep you up to date on microplastic (new protocols, new regulations etc.)
2. **Filtration apparatus**
3. **Filters & Filter Holder**
4. **Raman microscope** stand alone (with detector options available) or coupled with a Fluorescence microscope in a single instrument
5. **Video Raman Matching** with GPS like technology to reliably locate particles on the filter
6. **Reference standard sample** to validate your analysis
7. **ParticleFinder™**: Fully automated particles analysis software which allows
   - Viewing and locating the particles from the optical images
   - Characterization by size/shape
   - Analysis using Raman microscopy
   - Chemically identification using dedicated libraries
   - Result reporting with statistics on shape, size etc.
8. **KnowItAll**. Software for spectral identification with a dedicated polymer library built-in that includes over 150 spectra.

The provided KnowItAll HORIBA edition includes advanced capabilities (Multicomponent search etc.) with the additional ability to build your own library.


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# HORIBA Solution



## Filtration apparatus

Included in the starter kit are:

- 100 ml Glass funnel
- Sintered glass support with 13 mm filtration area
- Silicone stopper
- 1L Borosilicate glass flask
- Diaphragm vacuum pump

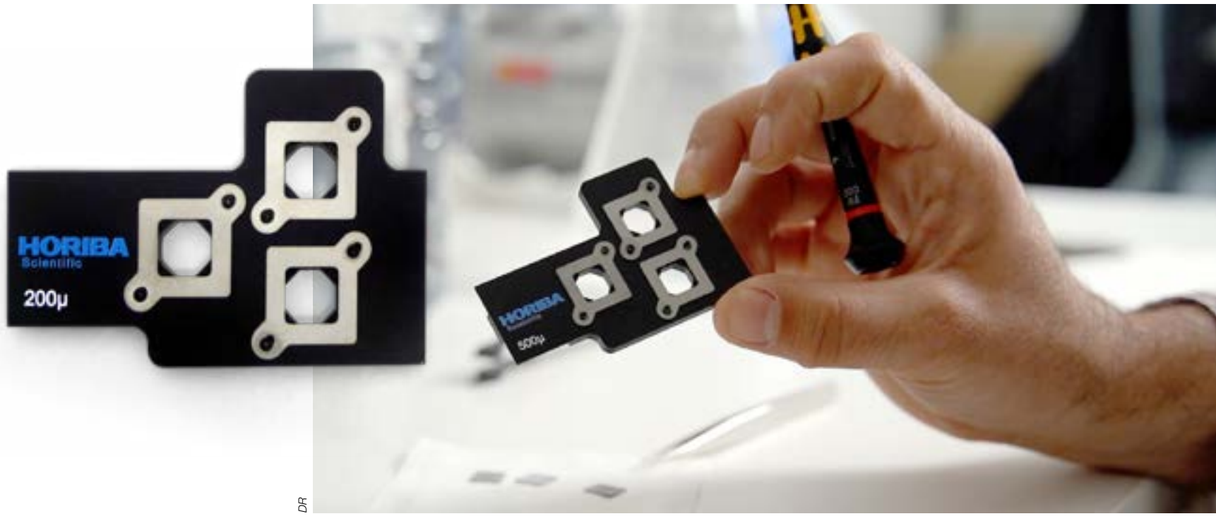
DR

## Filters & Filter holder

The filters we have selected for this microplastic starter kit are square shaped Silicon filters from SmartMembrane (http://www.smartmembranes.de/en/). We are providing 25 filters of 3 different pore size: 1 µm (5 filters), 2.5 µm (10 filters) and 5-6 µm (10 filters). Filters are packed in small boxes with 5 filters in each box.


DR

Together with the filters we provide a holder specifically developed to hold square-shaped filters. The holders are two in order to be optimised according to the thickness of the filters and each holder has places for 3 filters (picture below), allowing efficient use of the system as up to 3 filters can be analyzed without needing the user to be present to change the sample.

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# HORIBA Solution

Filtration appartus
**Raman microscope**
MVAPlus, ViewSharp, NavSharp, VRM
ParticleFInder
Know It All
Reference standard sample

## Raman microscope

Two platforms are available:

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**XploRA™ PLUS**

**LabRAM Soleil™**

DR

Both Raman microscopes can be equipped with a standard detector (CCD - Charge Couple Device) or an Imaging detector (EMCCD - Electron Multiplied Charge Couple Device). For microplastic analysis using our dedicated ParticleFinder™ software our standard detector is the perfect choice, providing excellent performance for this application in a cost-effective package. For multipurpose systems that will acquire large maps, the imaging detector may reduce measurements times for these maps, while not degrading microplastic analysis performance.

All platforms/configurations include 5X, 20X, 50X-LWD (Long Working Distance) and 100X-LWD objectives and two excitation laser lines: 532 nm and 785 nm.

The XploRA™ PLUS is also available with Fluorescence illumination to combine two measurement methodologies widely used for microplastic analysis: Nile Red staining/Fluorescence microscopy and Raman microscopy. Nile Red can be the fast-screening technique for particle counting and shaping and Raman can validate the results by chemically identifying a subset of the particles: We added the 785 nm excitation laser to optimize the collection of Raman spectra on Nile Red stained particles. It is also useful to reduce fluorescence interference from organic contamination.

To ensure full flexibility, Fluorescence illumination is provided with band-pass excitation filter from 460-490 nm and emission in the green (515-555 nm), where plastics fluoresces more selectively (as mentioned in the “Measurement Methodologies” session), and with an excitation filter at 535 nm with emission in the red (610 nm).

To complete the visual configuration both Raman microscopes are equipped with at least a 5 megapixel 5MP CMOS video camera, upgraded to a 6 megapixel version with the Fluorescence option. The LabSpec 6 software platform is common to both systems an provides complete instrument control, data processing and this microplastic package also includes, in addition to ParticleFinder™ and KnowItAll which will be described in detail later, a number additional tools:

# HORIBA Solution

Filtration appartus
Raman microscope
**MVAPlus, ViewSharp, NavSharp, VRM**
ParticleFInder
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Reference standard sample

## MVAPlus

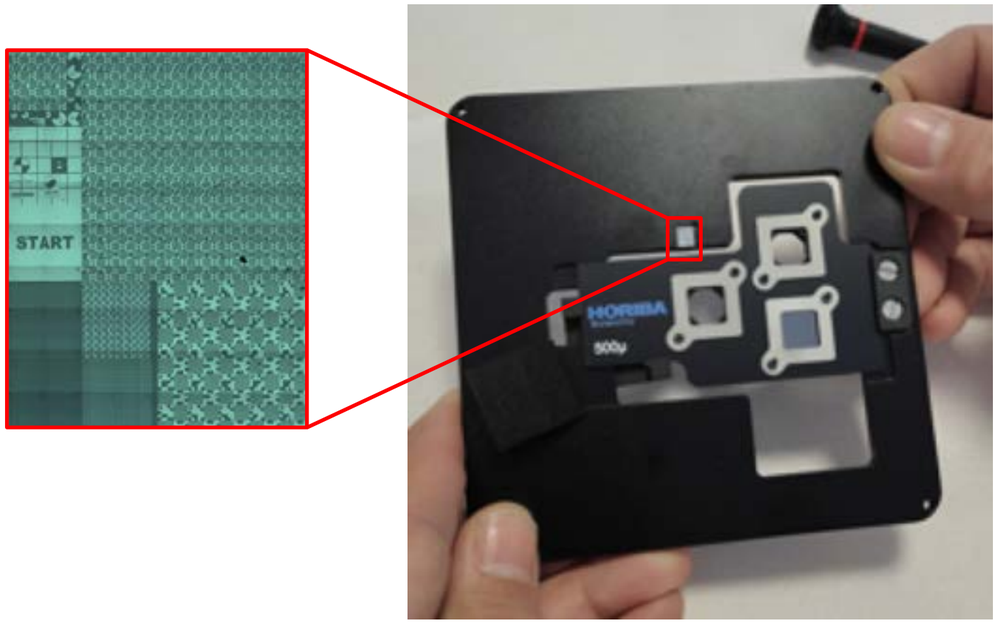
Multivariate Analysis module includes a number of multivariates (chemometric) methods, providing additional tools for data analysis such as PLS (Partial Least Square), CLS (Classical Least Square), PCA (Principal Component Analysis), MCR (Multivariate Curve Resolution) and Cluster Analysis.

## ViewSharp™ and NavSharp™

ViewSharp and NavSharp which provide a clear view of the sample's surface and guarantees the highest focal quality in Raman Images (autofocusing during Raman collection) and particle images, provides a topography image and allows 3D chemical visualization.

## Video Raman Matching (VRM) with nano-GPS Technology

Video Raman Matching is the perfect tool to reliably move to your particles even when transitioning from low to high magnification (do the mosaic with a 10x and measure the particles confidently with higher magnification). It allows also a perfect correlation between the chemical information and the visible image. VRM technology is based on GPS technology, a patented tag allows locations to be identified and to accurately position  the sample and /or particles. This technology is the HORIBA gateway to Correlative Microscopy.




DR

*Fig. 8. Video Raman Matching (VRM)*

# NEW ParticleFinder™

**ParticleFinder™** is the perfect software solution for particle analysis. It provides a step-wise, automated routine to locate, characterize and chemically identify, through Raman analysis, particles. ParticleFinder™ is ideal to analyze microplastics but can also be used for other environmental applications (e.g. Particulate analysis) and also for pharmaceutical applications (powder dispersion) and in any field where chemical and morphological characterization of particles is needed.

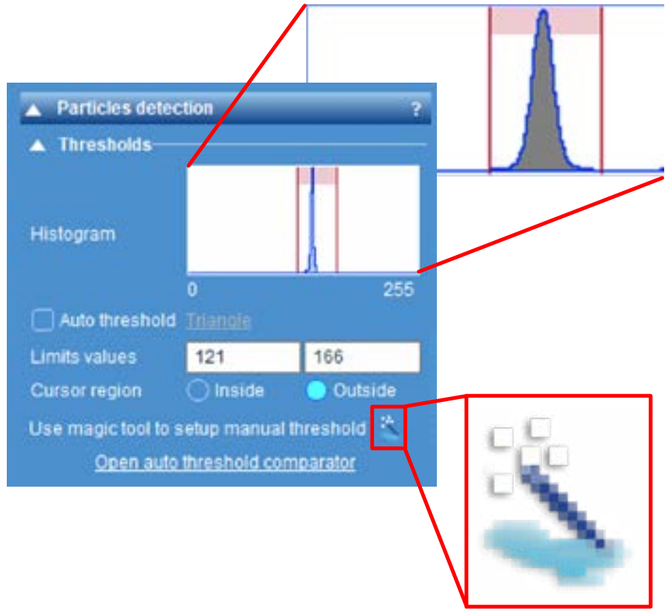
ParticleFinder™ routine has 6 consecutive steps:

**Image acquisition** (Single field of view) or Mosaic

**Image acquisition 1**
Single image, or montaged or mosaic wide field of view image

**Threshold** Two main threshold options are available: Auto-thresholding and Manual-thresholding. Auto-thresholding with dark or light backgrounds and manual-thresholding by selecting on the histogram the desired grey scale intensity distribution through the active video image.

**Threshold 2**
Manual and Auto-thresholding, Magic wand selection and ease visual comparison




Manual thresholding is also available with a simplified routine by using the Magic Wand: A single click on the particle with the magic wand allows to automatically adjust the grey scale intensity distribution according to the grey scale of the particle selected.

# HORIBA Solution

Filtration appartus
Raman microscope
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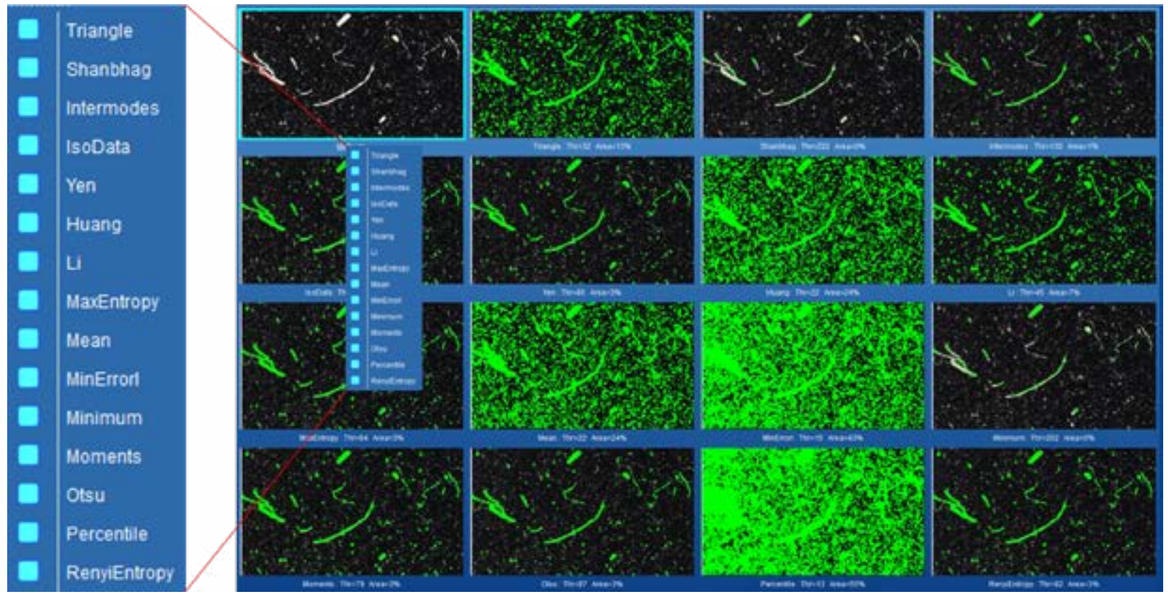
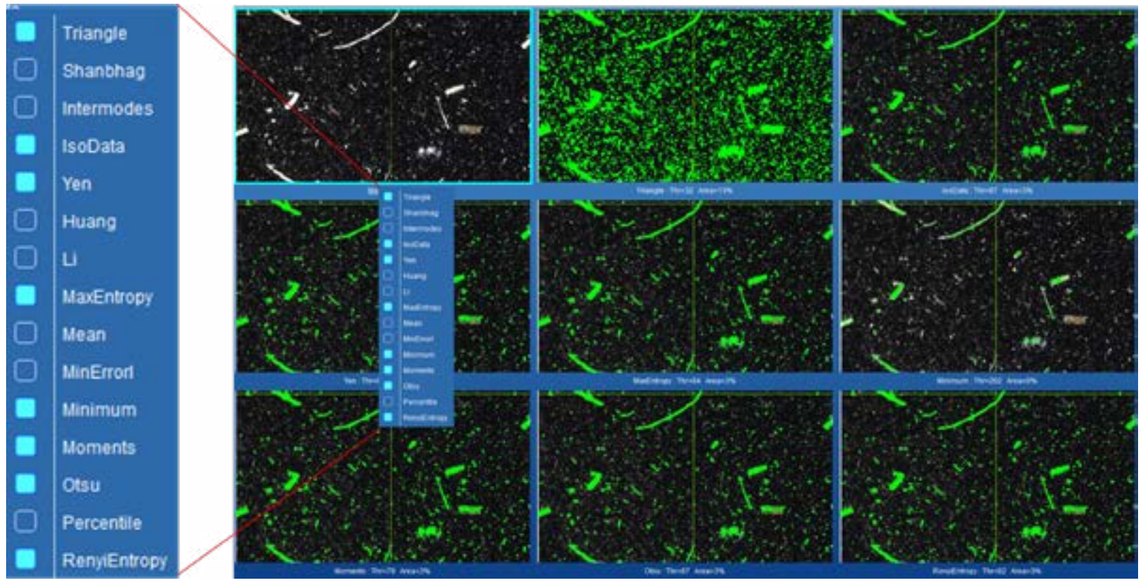
**ParticleFInder**
Know It All
Reference standard sample

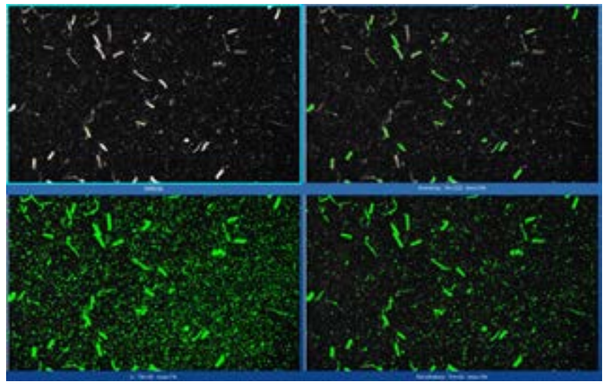
The Auto-thresholding provides unbeatable flexibility; indeed, it allows to select between several algorithms
➡ Up to sixteen are available.
Each of these algorithms will automatically set the range of the grey scale intensity distribution on the histogram based on the video image and the algorithm sensitivity.


The choice of the best algorithm does not require any expertise since a visual comparison allows to easily identify the one most suitable for the selection of the particles and/or the fibers of interest.




The number of algorithms to be visually compared is not limited: all 16 or 8 or only 3 can be displayed.



# HORIBA Solution

Filtration appartus
Raman microscope
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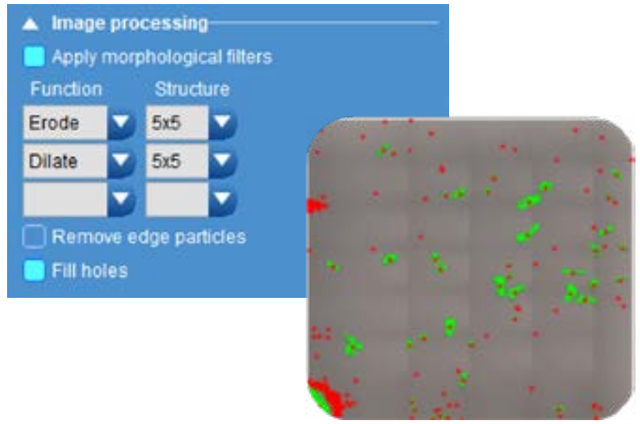
**ParticleFInder**
Know It All
Reference standard sample

**Process** Additional image processing option such as erode/dilate/open/close/majority filters and removing touching particles, filling holes, etc,… are also available.

**Process 3**

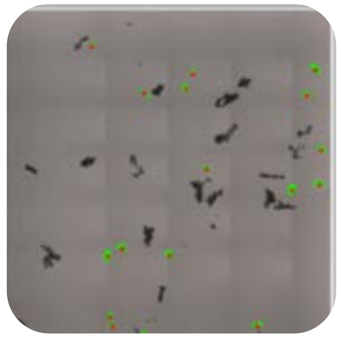
Erode/dilate/open/close/majority, fill holes, remove edge particles




**Select** Define the particles to be chemically analyzed using any of the available morphological information. Selection can also be done first, before image acquisition.

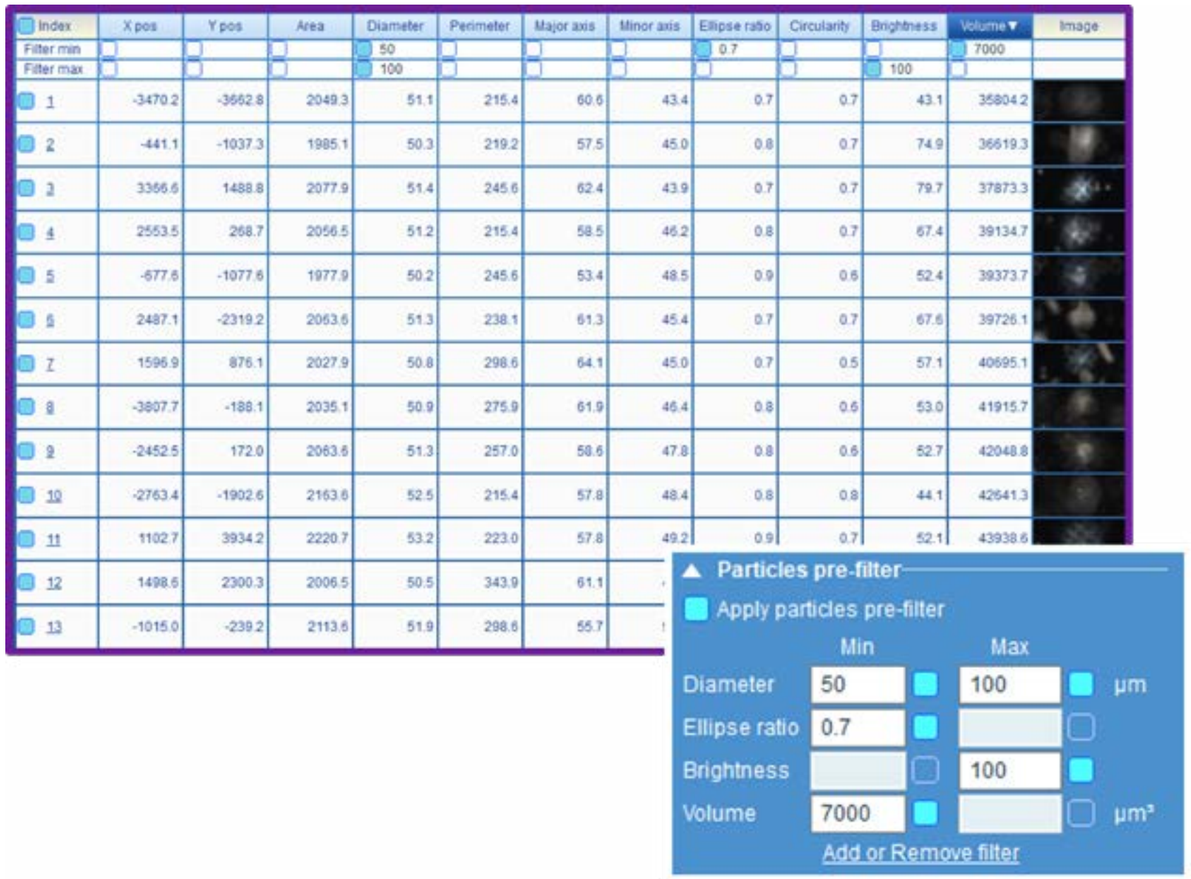
**Select 4**

Screen particles based on any morphological parameters



Selection and pre-filtering can be done using any morphological features, in the example below we select particles and/or fibers with a diameter between 50mm to 100mm, an ellipse ratio higher than 0.7 a brightness below 100 and a volume above 7000 mm².

This versatility and customization ability allows the measurement to be fine-tuned by optimising the number of particles to be analized and hence the time of analysis.



| Index | X pos | Y pos | Area | Diameter | Perimeter | Major axis | Minor axis | Ellipse ratio | Circularity | Brightness | Volume | Image |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Filter min | | | | 50 | | | | 0.7 | | | 7000 | |
| Filter max | | | | 100 | | | | | | 100 | | |
| 1 | -3470.2 | -3662.8 | 2049.3 | 51.1 | 215.4 | 60.6 | 43.4 | 0.7 | 0.7 | 43.1 | 35804.2 | |
| 2 | -441.1 | -1037.3 | 1985.1 | 50.3 | 219.2 | 57.5 | 45.0 | 0.8 | 0.7 | 74.9 | 36619.3 | |
| 3 | 3366.6 | 1488.8 | 2077.9 | 51.4 | 245.6 | 62.4 | 43.9 | 0.7 | 0.7 | 79.7 | 37873.3 | |
| 4 | 2553.5 | 268.7 | 2056.5 | 51.2 | 215.4 | 58.5 | 46.2 | 0.8 | 0.7 | 67.4 | 39134.7 | |
| 5 | -677.6 | -1077.6 | 1977.9 | 50.2 | 245.6 | 53.4 | 48.5 | 0.9 | 0.6 | 52.4 | 39373.7 | |
| 6 | 2487.1 | -2319.2 | 2063.6 | 51.3 | 238.1 | 61.3 | 45.4 | 0.7 | 0.7 | 67.6 | 39726.1 | |
| 7 | 1596.9 | 876.1 | 2027.9 | 50.8 | 298.6 | 64.1 | 45.0 | 0.7 | 0.5 | 57.1 | 40695.1 | |
| 8 | -3807.7 | -188.1 | 2035.1 | 50.9 | 275.9 | 61.9 | 46.4 | 0.8 | 0.6 | 53.0 | 41915.7 | |
| 9 | -2452.5 | 172.0 | 2063.6 | 51.3 | 257.0 | 58.6 | 47.8 | 0.8 | 0.6 | 52.7 | 42048.8 | |
| 10 | -2763.4 | -1902.6 | 2163.6 | 52.5 | 215.4 | 57.8 | 48.4 | 0.8 | 0.8 | 44.1 | 42641.3 | |
| 11 | 1102.7 | 3934.2 | 2220.7 | 53.2 | 223.0 | 57.8 | 49.2 | 0.9 | 0.7 | 52.1 | 43938.6 | |
| 12 | 1498.6 | 2300.3 | 2006.5 | 50.5 | 343.9 | 61.1 | | | | | | |
| 13 | -1015.0 | -239.2 | 2113.6 | 51.9 | 298.6 | 55.7 | | | | | | |



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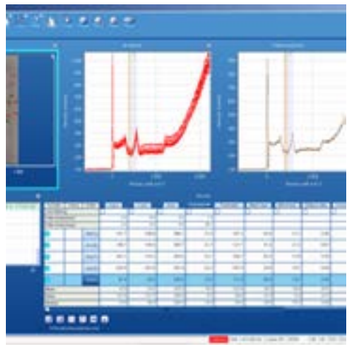
# HORIBA Solution

Filtration appartus
Raman microscope
MVAPlus, ViewSharp, NavSharp, VRM

**ParticleFInder**
Know It All
Reference standard sample

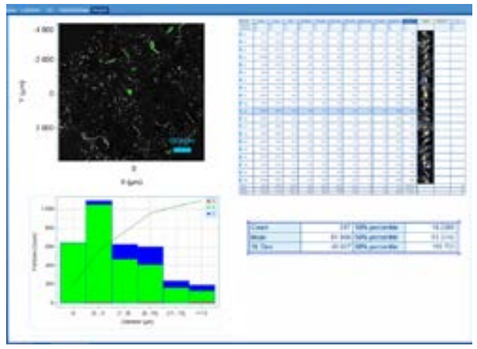
**Acquire Raman** Collect a Raman spectrum for each particle. It is possible to: a) Do a single spectrum; b) Do multiple spectra and averaging them to get one; c) Do small maps to get full picture of big particles.

**5**
**Acquire Raman**
Automatically analyze/identify each particle

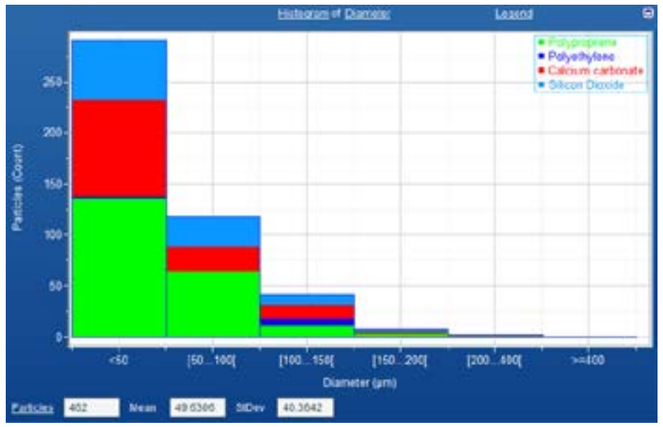


**Report and Chemically Identify** Several reporting options are available along with chemical identification by means of KnowItAll™ and the HORIBA Microplastics library.

**6**
**Identify and Report**
Chemically identify each particle by dedicated libraries



The new reporting capability can create any type of histogram and display the different morphological parameters (diameter, area, aspect/ratio, volume, perimeter, etc…) according to the chemistry of the particles. Below, we created a histogram showing the number of particles according to the diameter distribution and the chemistry, the following ranges (ranges which are fully customizable) have been selected for this example: 50 mm, 50-100 mm, 100-150 mm etc…




The same data can be also shown in a table instead of histograms

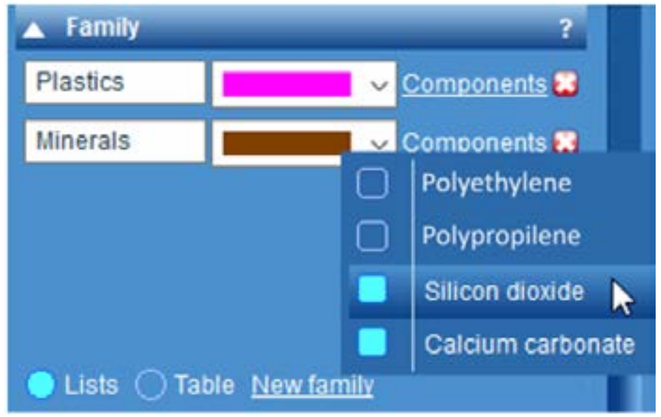
| ID | <50 µm | [50...100[ µm | [100...150[ µm | [150...200[ µm | [200...400[ µm | >=400 µm |
|---|---|---|---|---|---|---|
| Polypropilene | 135 | 64 | 11 | 3 | 1 | 0 |
| Polyethylene | 3 | 0 | 6 | 0 | 0 | 0 |
| Calcium carbonate | 94 | 24 | 14 | 2 | 1 | 0 |
| Silicon Dioxide | 59 | 30 | 11 | 3 | 1 | 0 |
| | 291 | 118 | 42 | 8 | 3 | 0 |

# HORIBA Solution

Filtration appartus
Raman microscope
MVAPlus, ViewSharp, NavSharp, VRM

**ParticleFInder**
Know It All
Reference standard sample

Additionally, it is possible to create families in order to merge different categories and simplify the displaying of the data. In this example we merged all the plastic materials (polyethylene and polypropylene) and the inorganic material (silicon dioxide and calcium carbonate) respectively in Plastics and Minerals.




**ParticleFinder™** can be used with 2 different approaches to maximize performance in any laboratory condition - Static mode or Dynamic mode.

In static mode an image is acquired, which can be a single Field Of View FOV (the size of the FOV depends on the magnification of the objective) or a mosaic (where several tiles, i.e. single images, are stitched together to create a wider image of your sample), after imaging these are processed to characterize the particles' shape/size/location, as described above, and analyzed to chemically identify the particles. Summarizing the Static Mode works by following the 6 steps in order: 1 ➡ 2 ➡ 3 ➡ 4 ➡ 5 ➡ 6 or, if selection is done first, 4 ➡ 1 ➡ 2 ➡ 3 ➡ 5 ➡ 6.

# HORIBA Solution

Filtration appartus
Raman microscope
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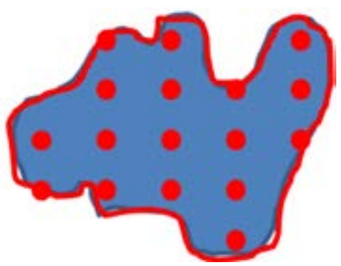
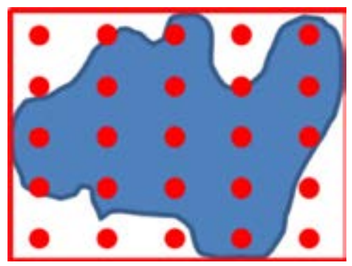
**ParticleFInder**
Know It All
Reference standard sample

*In Step 5, Raman Acquisition, we would like to add some additional information on the acquisition options available for each particle to allow you to appreciate the extreme flexibility of ParticleFinder™.*

Each particle can be identified by collecting a single spectrum, several spectra (a matrix of points 2X2, 3X3 etc. can be defined) and averaging them, a small map which can be “Particle only” or “Minimum rectangle” as depicted in the picture below.



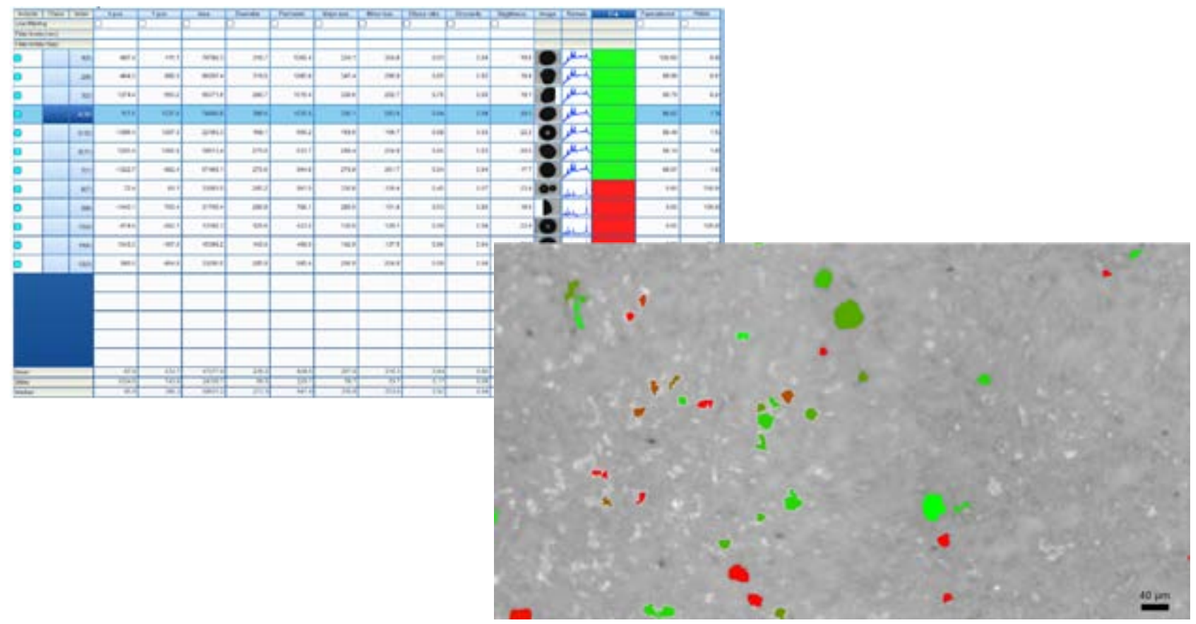
*Particle Only*



*Minimum Rectangle*

In Dynamic mode, to reliably analyze your particles even in tough conditions (instability due to the bench, laboratory environment etc.), for mosaics (multiple tiles) the software does not acquire the full image but completes the full routine on each tile. So firstly we select the particles of interest (step 4) for example all particles in the range 1 to 20 µm) and the required thresholding/processing (for steps 2/3) are setup just once (it does not need to be done for each tile individually). Then the first tile (single image) is acquired (step 1), it is then processed (step 2), the particles characterized (step 3) and have Raman spectra/maps taken of them (step 5). The software then moves to the next tile and the process (steps 1 ➡ 2 ➡ 3 ➡ 5) is automatically repeated. The particles are then characterized and the data reported (step 6).

Chemical identification of the particles can be also visually represented by false coloring the results table and map by plastic type: Each color is assigned to a different material (PE, PP, PET, etc.)



*False colors identify different types of Plastic*

# HORIBA Solution

Filtration appartus
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**ParticleFInder**
Know It All
Reference standard sample

**ParticleFinder™**

The best solution for **Microplastic identification** with HORIBA instruments (XploRA™ PLUS and LabRAM Soleil™)

- *Automatic Workflow*
- *Intuitive Interface*
- *Maximum Flexibility*


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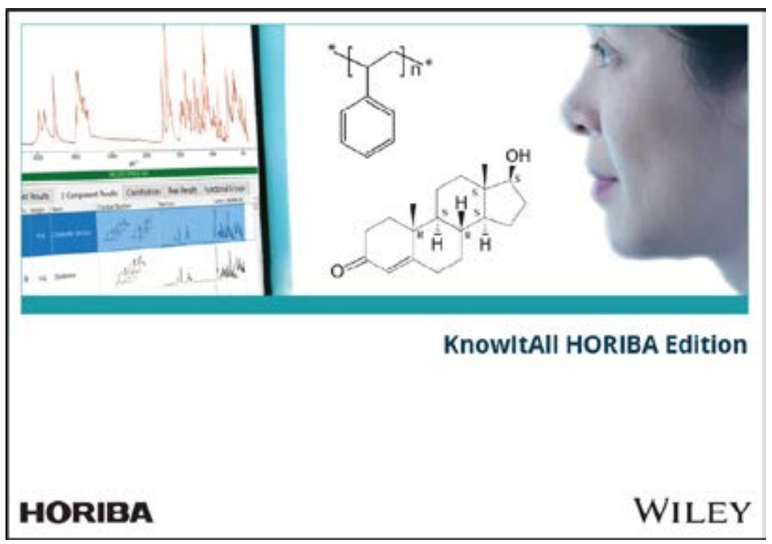
# HORIBA Solution



KnowItAll HORIBA Edition integrates spectral data and tools into a single interface, so chemists can perform multiple tasks in relation to that data and ultimately extract greater knowledge from it. Easily transfer information from one tool to another and move from one task to the next, without having to leave the main interface or open another program. **KnowItAll HORIBA Edition offers solutions to identify, analyze, and manage Raman spectral data and supports multiple file formats**.

*Table 8. KnowItAll Solutions to identify, analyze, and manage Raman spectral data and supports multiple file formats.*

| Data Toolbox | |
|---|---|
| **ID Expert™** | One-click spectral identification tool: to perform applicable basic analyses (single and multi-component search, peak search, and functional group analysis) on an unknown spectrum |
| **SearchIt™** | Advanced database searching including mixture analysis, pure compound search, simultaneous multi-technique search |
| **MineIt™** | Multi-technique spectral database building and data mining. Includes patented Overlap Density Heatmap technology to visualize similarities and dissimilarities in datasets. |
| **QC Expert™** | Perform a QC comparison of a sample Raman or IR spectrum against a reference spectrum |




**KIA/MineIt** is the tool which allows you to create your own databases. Users can:

- Build databases with one or more analytical techniques
- Build databases with multiple spectral scans in the same record
- Import analytical data from different instruments in the laboratory
- One-click import of common native instrument file formats and *.csv format (spreadsheet)
- Enhance each record with peak information, structures, and properties, such as sample source, boiling point, etc.
- Import multiple structure formats (with stereochemical bonds and identifiers)
- Use “Batch Import and Export” for efficient handling of spectra, structures, and property files

*Researchers can build searchable databases that include one or more analytical techniques (Raman, IR, MS, Near IR, NMR, UV-Vis), chemical structures, and other metadata by using the Minelt tool.*

# HORIBA Solution



- Supports unrestricted spectral range and resolution - Store spectra at the extract range and resolution at which each spectrum was measured rather than having to use a fixed range and resolution
- “Auto-Property” computes values such as formula, molecular weight, etc. for entire datasets
- Make databases more powerful by attaching spreadsheets, MSDS, and other documents or adding hyperlinks to web pages
- Create cross-references to data from other techniques; i.e., a Raman spectrum can be linked to an IR spectrum
- Quickly add properties and structures from PubChem to your database

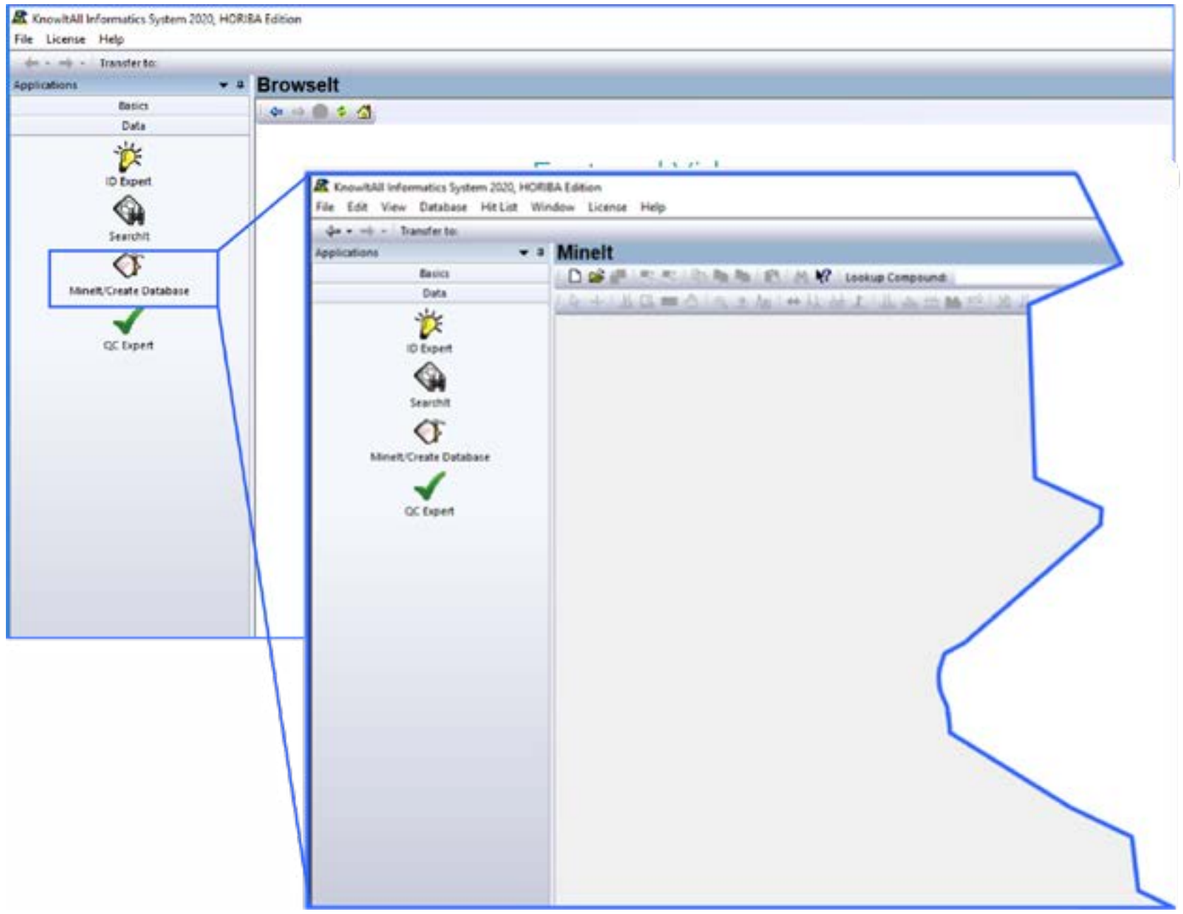
Moreover, databases can be customized:

- Databases can be customized to meet laboratory specifications
- Users can create custom fields to store associated metadata relevant to their work
- Choose from three types of property fields: text, numeric, hyperlink
- Generate “preferred property” forms so users enter properties consistently
- Set spectral parameters such as x- and y-resolution

The next section provides a visual guide of how to build your own database with KIA/MineIt.

## Step 1 - Database selection

Select the MineIt/Create database option from the menu on the left side on the main page of KnowItAll

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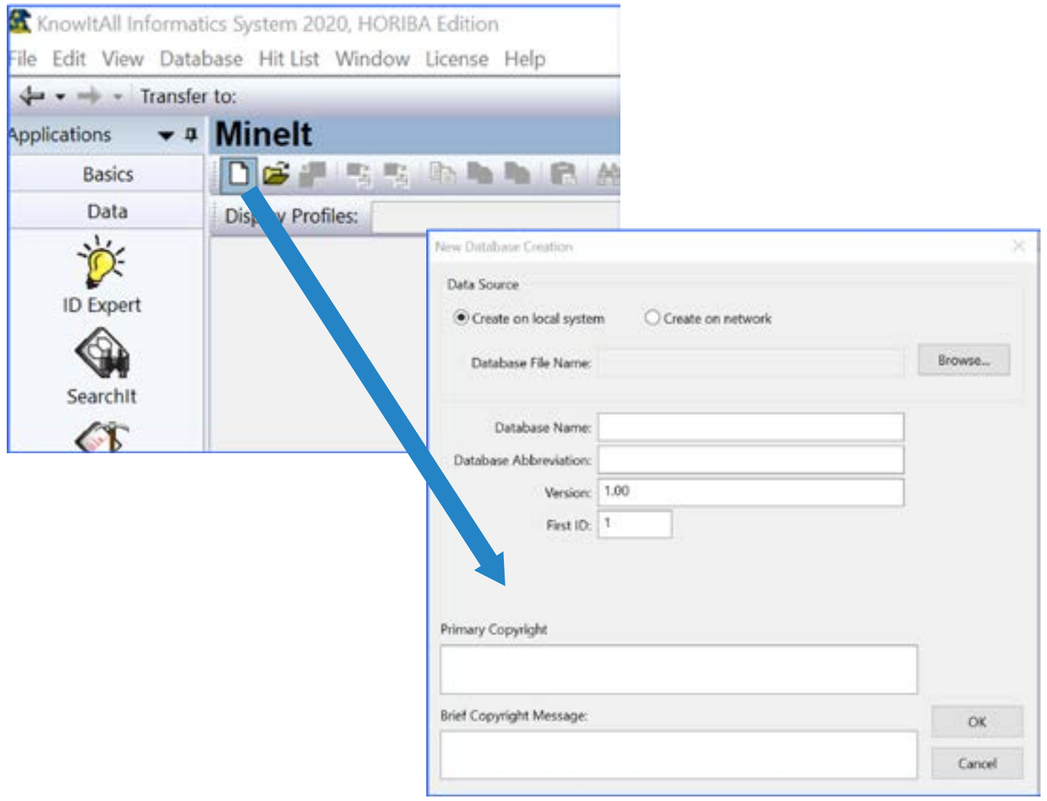
# HORIBA Solution



### Step 2 - Database creation

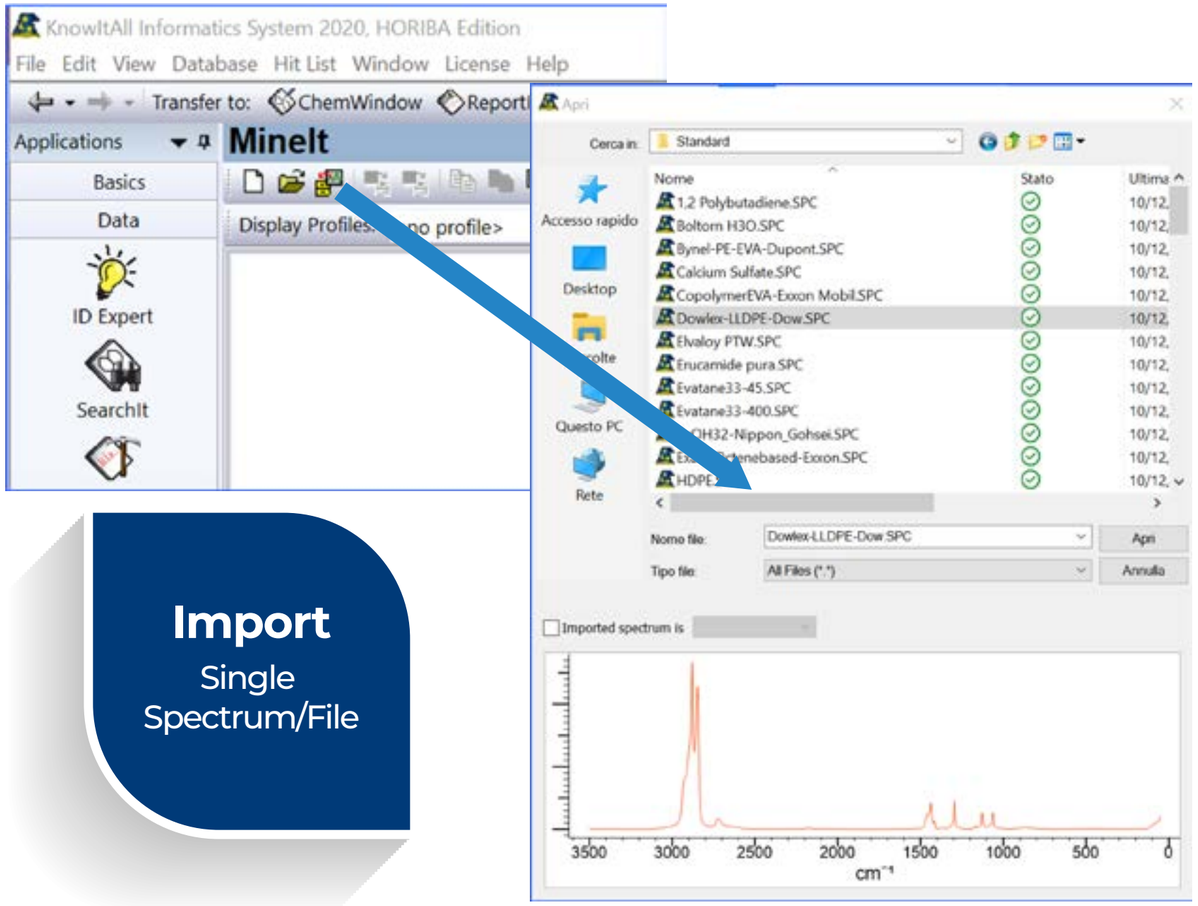
Select create new user database




Browse to the location where you want to store the database, on your local computer or on a network location. Define a name and an abbreviation for the database to allow easy identification.

### Step 3 - Import spectra

Spectra can be imported one at a time (Import) and/or all together by using the batch import option




**Import**
Single
Spectrum/File

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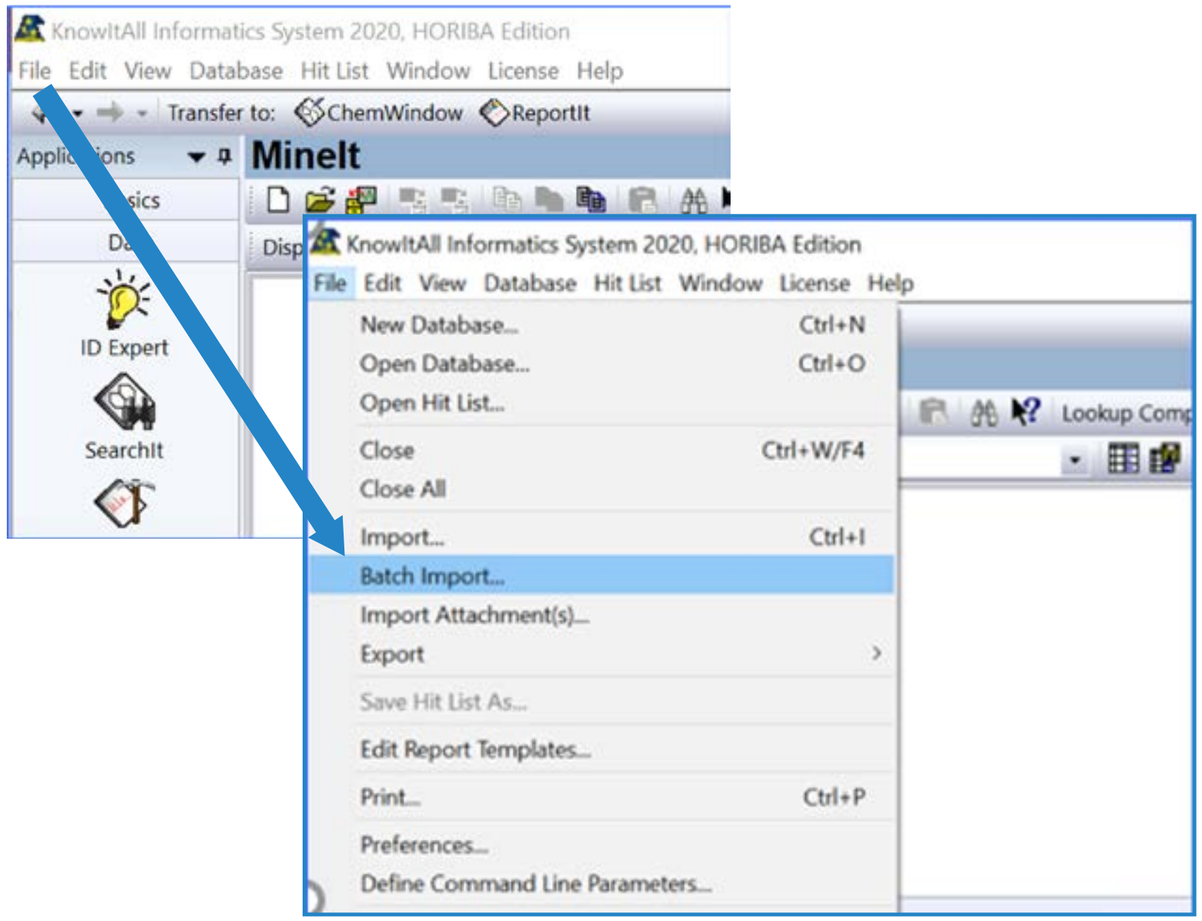
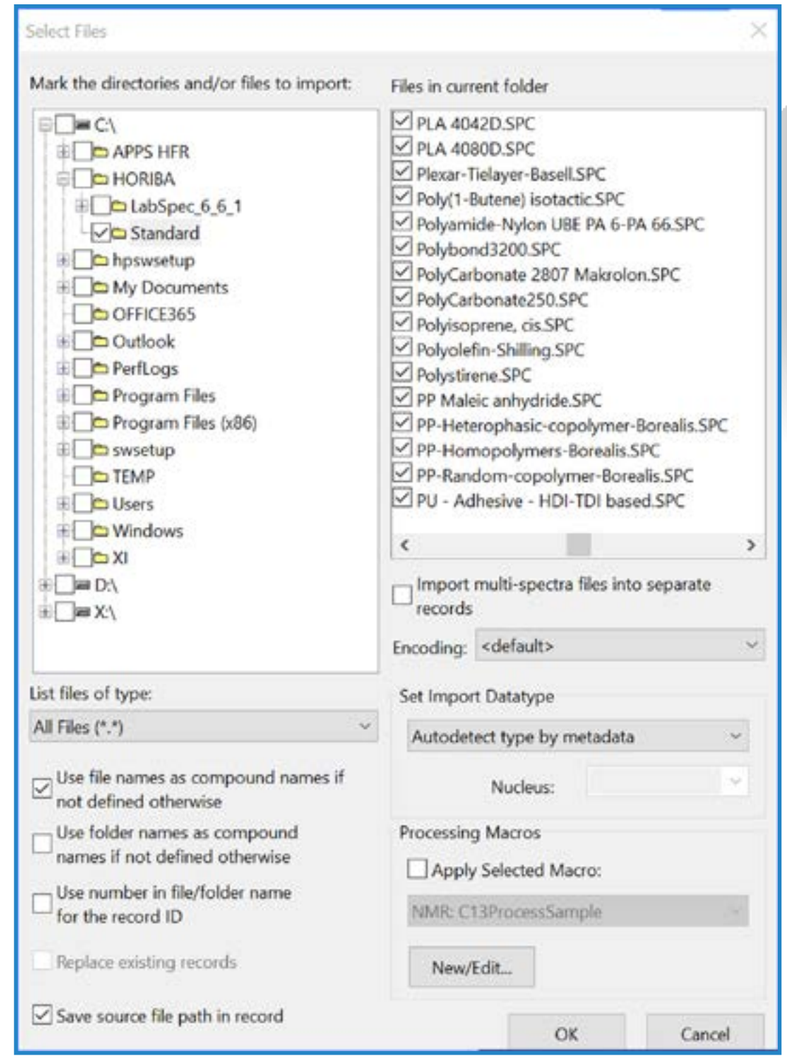
# HORIBA Solution

Filtration appartus
Raman microscope
MVAPlus, ViewSharp, NavSharp, VRM

ParticleFInder
**Know It All**
Reference standard sample




**Batch Import**
Multiple Spectrum/File

The batch import option allows multiple spectra to be selected and metadata types are automatically recognized.

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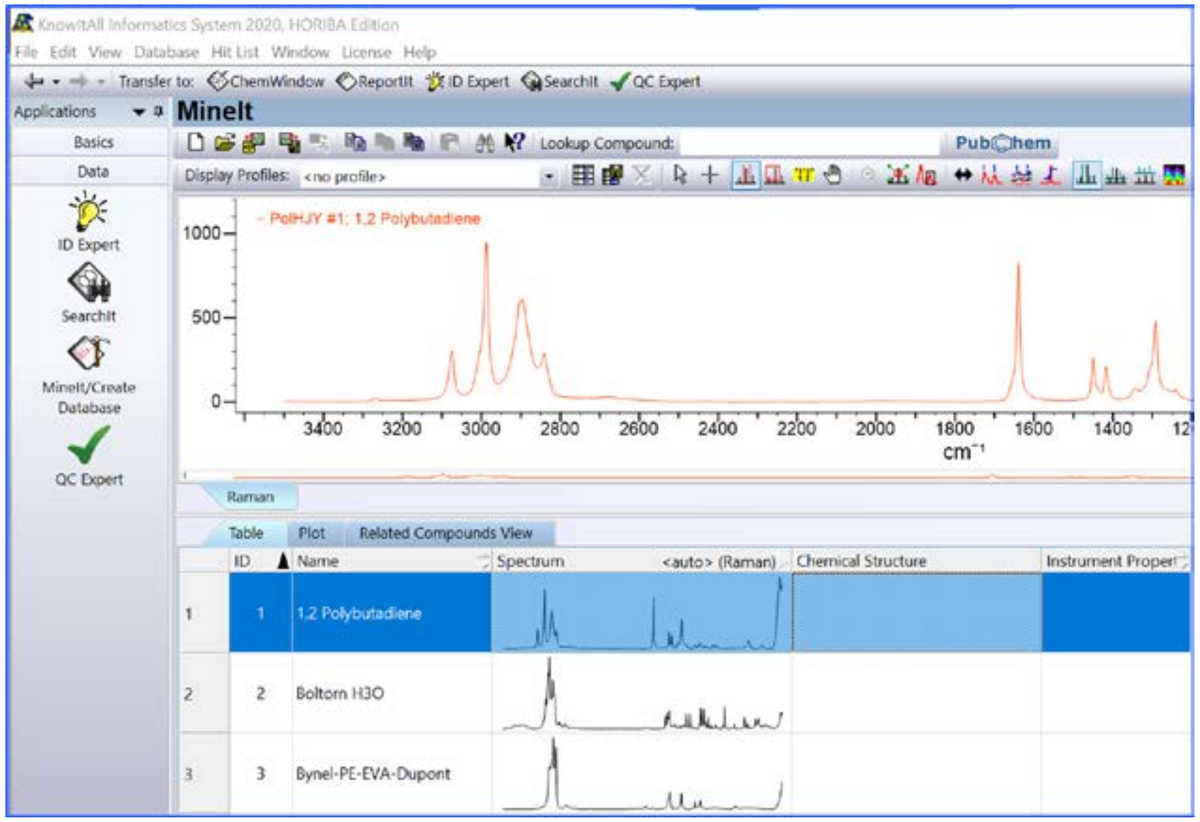
# HORIBA Solution



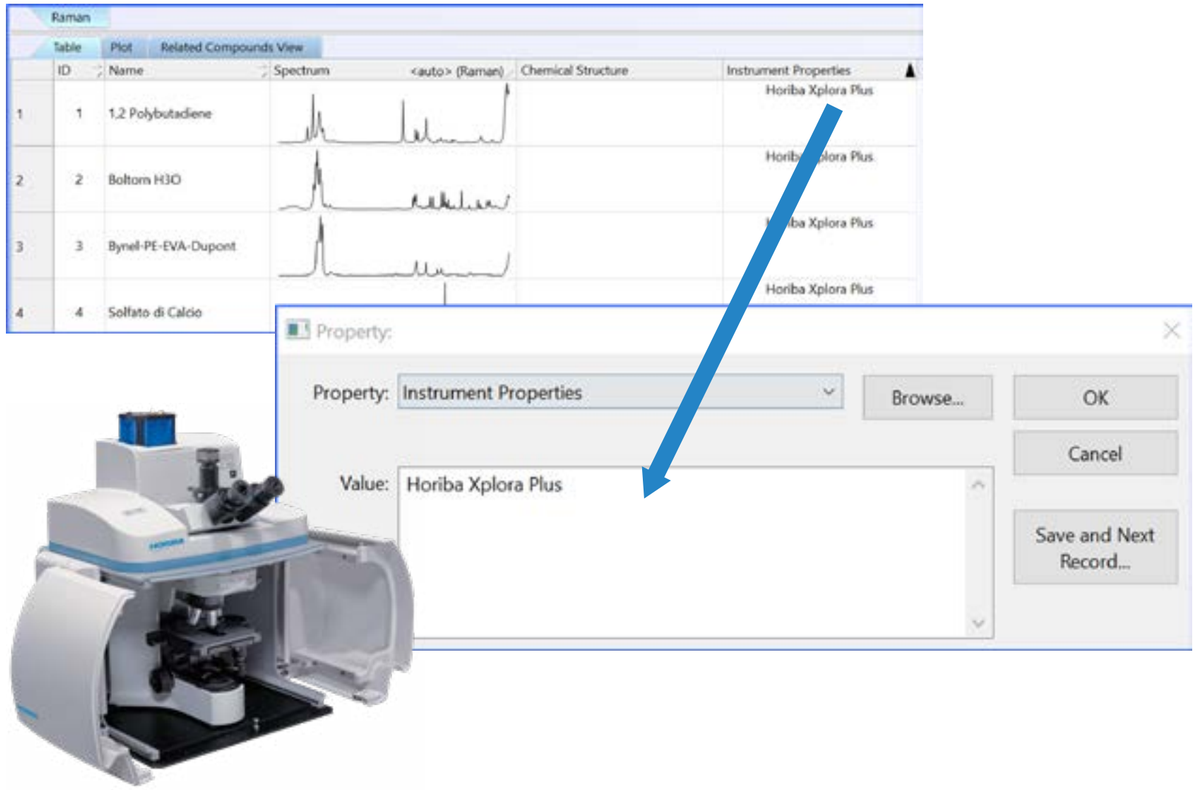
### Step 4 - Addition to the library

Once the spectrum/spectra are imported the library is ready to be used. However, users can also customize the library by adding other information (such as physical, chemical and instrumentation parameters, flags, names, etc.) by adding as many columns as needed.




### Step 5 – Library customization

The existing information on each column (for example “Instrument Property” in the picture shown below) can be modified for each spectrum by double clicking inside the column; the modification can also be assigned to all spectra and not just to a single one. Here we specified that the instrument used for data collection was the HORIBA XploRA PLUS.

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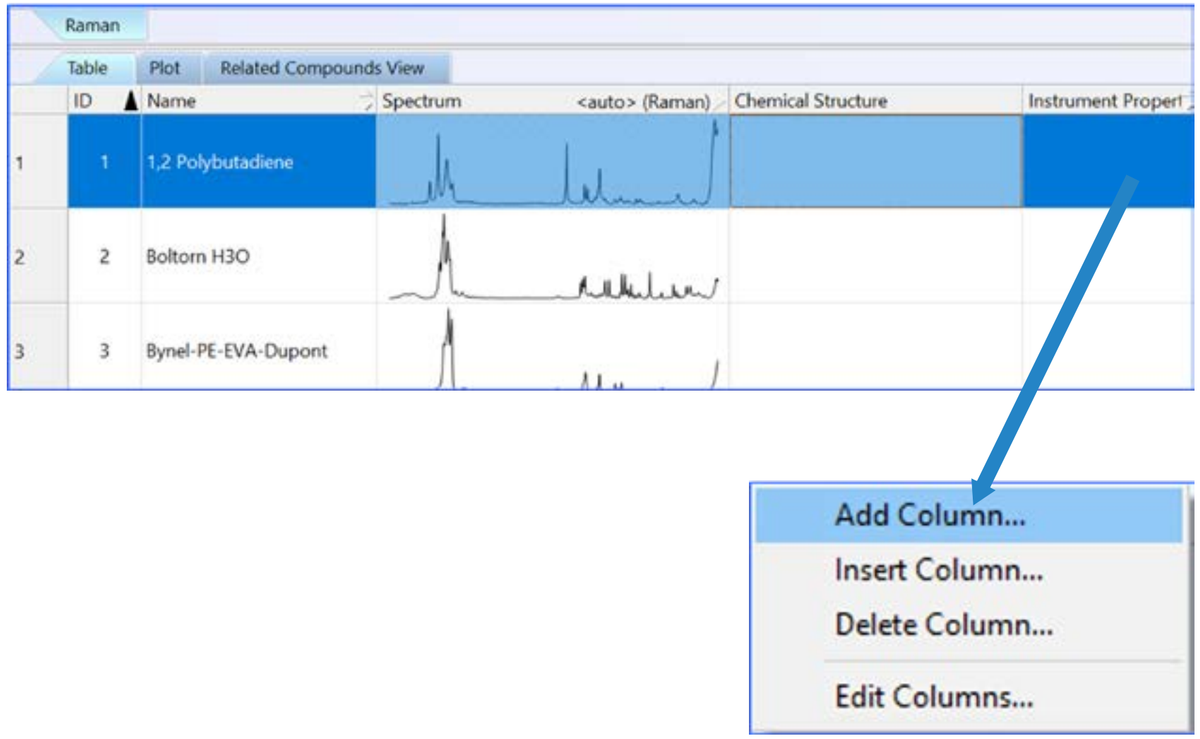
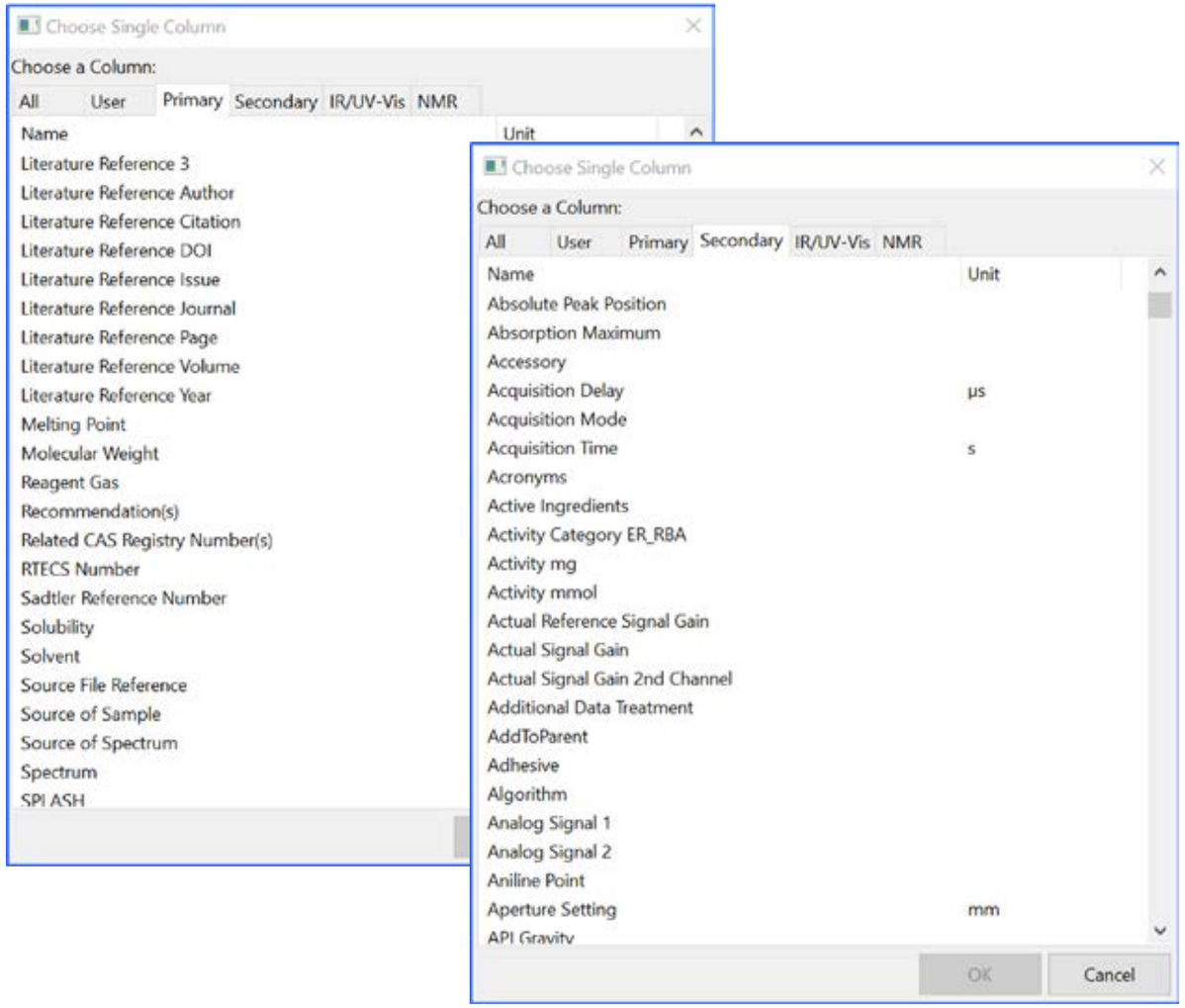
# HORIBA Solution



Adding a column to see additional information is also straightforward: right click on the table and select “add column”.




A submenu will open-up, allowing a new column to be select from an extensive list (see the example on the right)



## Reference standard sample

As a part of samples of microplastics in order to confidently approach the microplastic analysis and to validate your workflow and your lab environment. The standards are delivered in tablet form with a detail receipt and instruction on handling and usage.

The standards are prepared by the Norwegian Institute for Water Research (NIVA); NIVA is the Norway's leading institute for fundamental and applied research on marine and freshwaters and was founded back in 1958. NIVA has an extensive experience in the microplastic monitoring and evaluation.

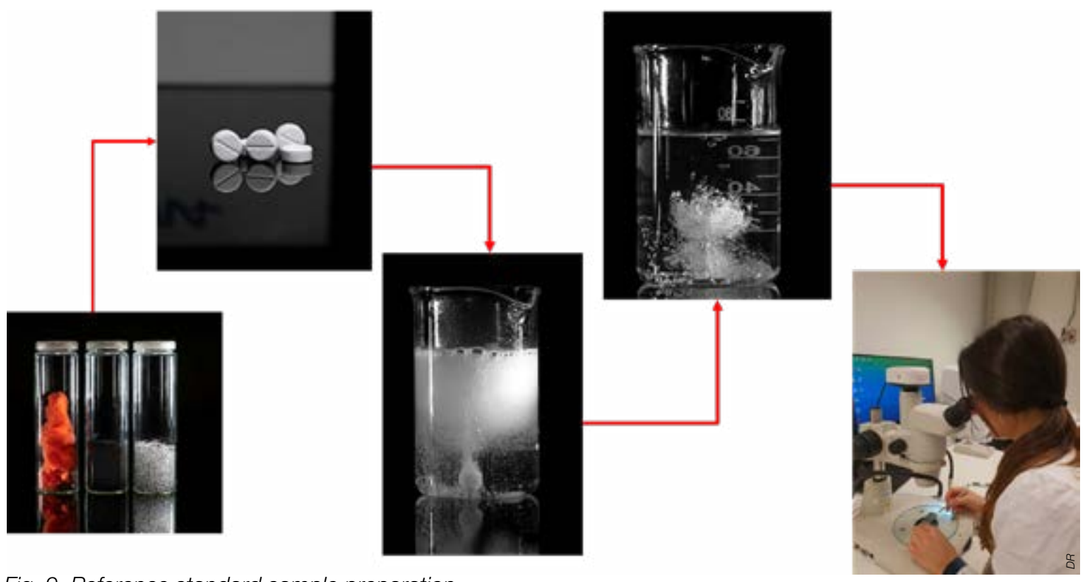



*Fig. 9. Reference standard sample preparation*

The tablets contain a well defined individual microplastic polymers or a polymer mixture (Polyethylene, Polystyrene, Polyvinyl Chloride and Polyethylene Terephthalate) ranging from 50 to 355 µm. HORIBA's full solution will provided also reference standards in micron size.

# Applications

## Marine Water

As application example to show the full workflow of ParticleFinder™ software we used a marine water sample. All the data are collected by using the LabRAM Soleil™.

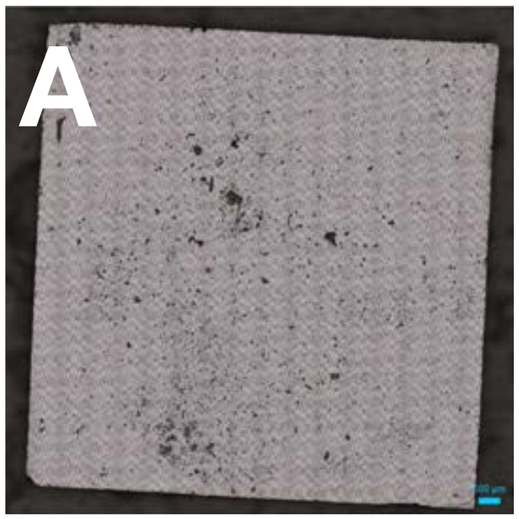

DR

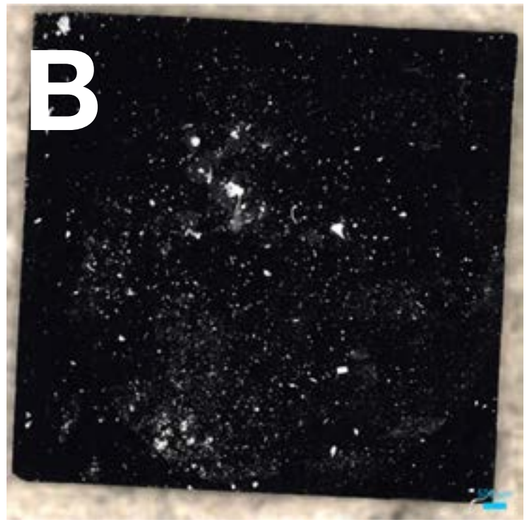
### Image acquisition

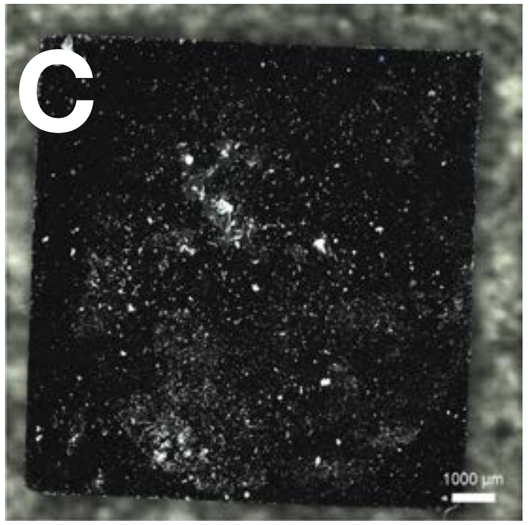
The mosaic image is acquired with a 10x objective by using the flatfield correction in order to avoid the stitching effect that could affect the particles selection. The Raman spectra are acquired with a 50x long working distance objective.

Our VRM (Video Raman Matching) coupled with nanoGPS technology allow us a fast and precise calibration enabling multi-magnification Raman Imaging to optimize your analysis time.

*The sample was pre-treated with KOH to remove the organic residues and a silicon filter with pore size of 1 micron was used for the filtration step.*



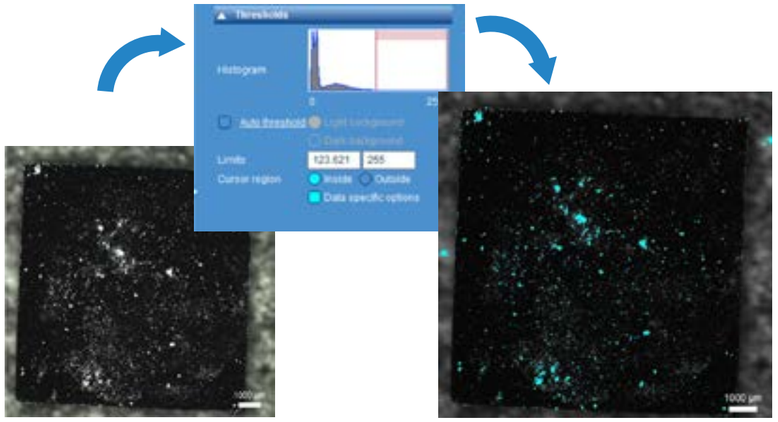




In picture A,B and C we collected the same optical image in three different modes: A. Brightfield mode; B, Brightfield mode with visible polarizer in cross-polarization; C, Darkfield mode.

The flexibility of our platforms (XploRA™ PLUS and LabRAM Soleil™) in term of optical imaging to visually identify Microplastic and thus correctly measure them is a key aspect. For this sample we selected the Darkfield image mode (highlighted in yellow).

### Threshold and Process

Threshold & Process step allow the visual selection of Microplastic. The selected Microplastic particles are highlighted in the picture on the right.



# Applications

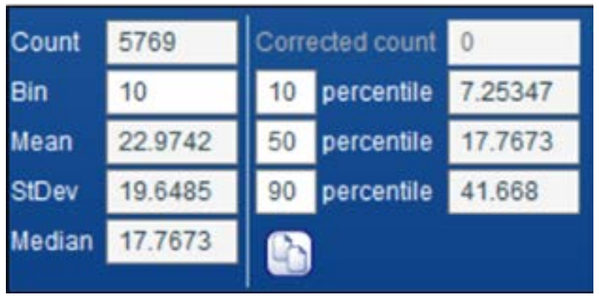
## Characterize and Locate

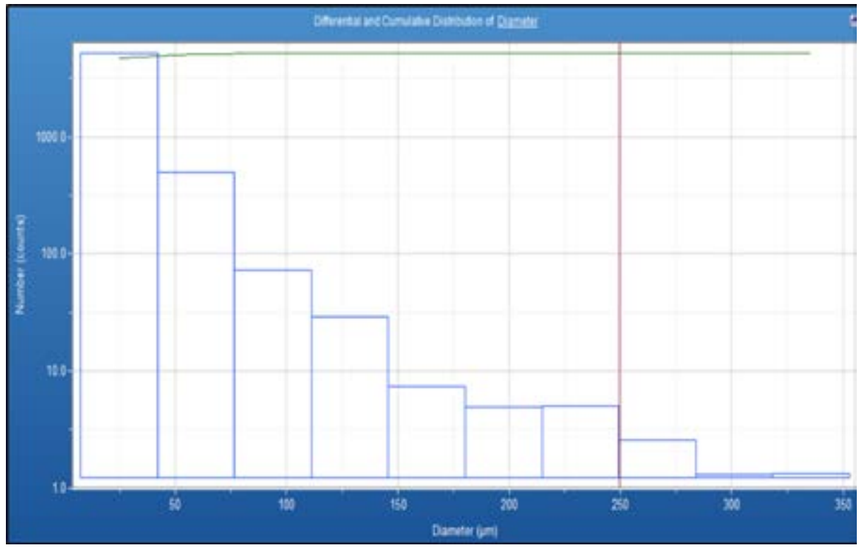
A table is generated after the particles selection where particles and their morphological characteristics (such as area, circularity, diameter etc…) are listed.

| Include | Class | Index | X pos | Y pos | Area | Diameter | Perimeter | Major axis | Minor Axis | Ellipse ratio | Circularity | Image |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Use filtering | | | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ | |
| Filter limits(min) | | | 0.0 | -4401.6 | 7.0 | 8.0 | | | | | | |
| Filter limits(max) | | | 0.0 | 5939.3 | 8.0 | 10.0 | 0.0 | | | | | |
| ☐ | | 1527(5403) | -1462.0 | 5149.1 | 237.6 | 17.4 | 64.7 | 19.7 | 15.4 | 0.78 | 0.84 | |
| ☐ | | 1528(3590) | -3126.6 | 2951.8 | 237.6 | 17.4 | 60.2 | 21.6 | 14.0 | 0.65 | 0.91 | |
| ☐ | | 1529(1511) | 3222.1 | -75.5 | 309.9 | 19.9 | 64.7 | 21.0 | 18.7 | 0.89 | 0.96 | |
| ☐ | | 1530(2079) | -210.6 | 879.6 | 309.9 | 19.9 | 67.4 | 22.0 | 18.3 | 0.83 | 0.93 | |
| ☐ | | 1531(4037) | 138.5 | 3549.0 | 144.6 | 13.6 | 55.7 | 16.7 | 11.0 | 0.66 | 0.77 | |
| ☐ | | 1532(114) | 2906.4 | -4052.0 | 712.8 | 30.1 | 113.2 | 44.5 | 20.6 | 0.46 | 0.84 | |
| ☐ | | 1533(5088) | -1653.6 | 4805.4 | 1952.5 | 49.9 | 200.6 | 55.8 | 48.7 | 0.87 | 0.78 | |
| ☐ | | 1534(543) | -3989.8 | -2465.9 | 124.0 | 12.6 | 51.1 | 14.2 | 10.8 | 0.76 | 0.77 | |
| ☐ | | 1535(961) | -2788.5 | -1367.6 | 454.5 | 24.1 | 85.9 | 33.0 | 17.7 | 0.54 | 0.88 | |
| ☐ | | 1536(1400) | -2766.0 | -276.0 | 836.8 | 32.6 | 137.0 | 55.1 | 22.3 | 0.40 | 0.75 | |
| ☐ | | 1537(1062) | -2438.0 | -1011.4 | 433.9 | 23.5 | 82.1 | 29.3 | 18.9 | 0.65 | 0.90 | |
| ☐ | | 1538(5744) | 2870.7 | 5708.9 | 464.9 | 24.3 | 82.1 | 29.2 | 20.2 | 0.69 | 0.93 | |
| Mean | | | -1606.4 | 1642.7 | 717.6 | 23.0 | 92.4 | 29.7 | 19.3 | 0.76 | 0.84 | |
| Stdev | | | 2528.1 | 2638.1 | 2682.6 | 19.6 | 116.7 | 32.2 | 16.6 | 0.19 | 0.11 | |
| Median | | | -1902.8 | 1928.2 | 247.9 | 17.8 | 64.7 | 20.5 | 15.5 | 0.81 | 0.87 | |

The majority of Microplastic particles are in the range between 0 to 40 microns underlying even more the importance of a tool like Raman Microscopy which allows to analyse particles down to the sub-micron range.

This picture shows the overall number of particles counted by Particle Finder™ for this marine sample: 5769.




*The histogram shows the size distribution of the 5769*

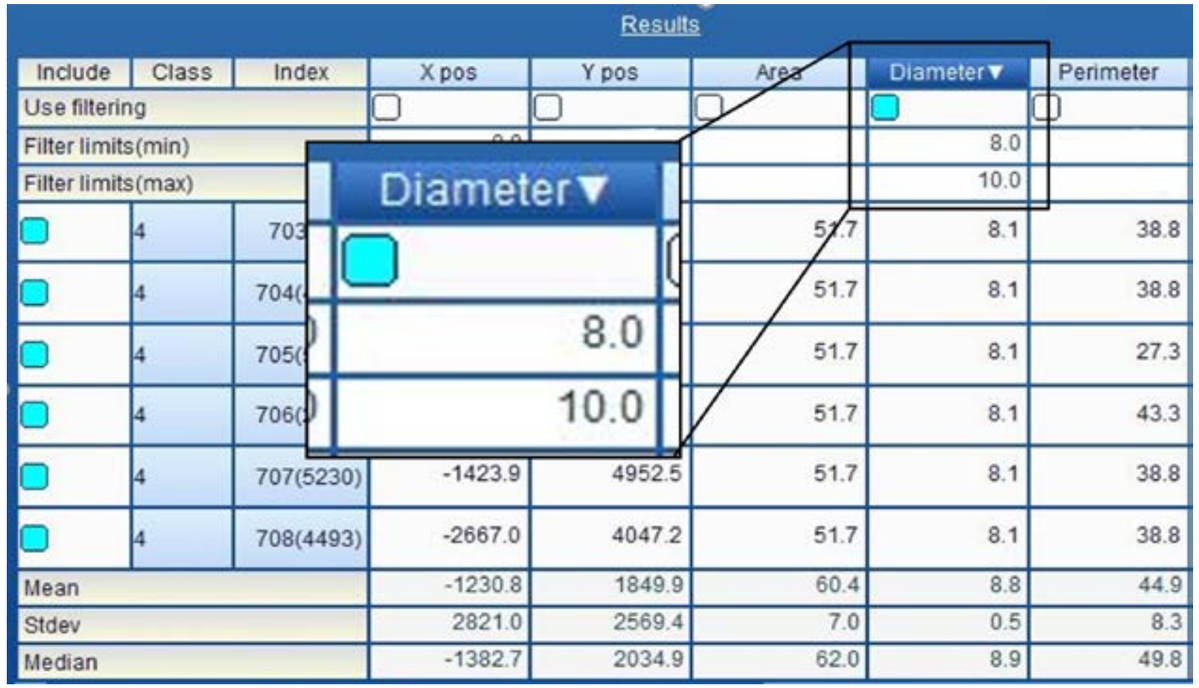
# Applications

## Select

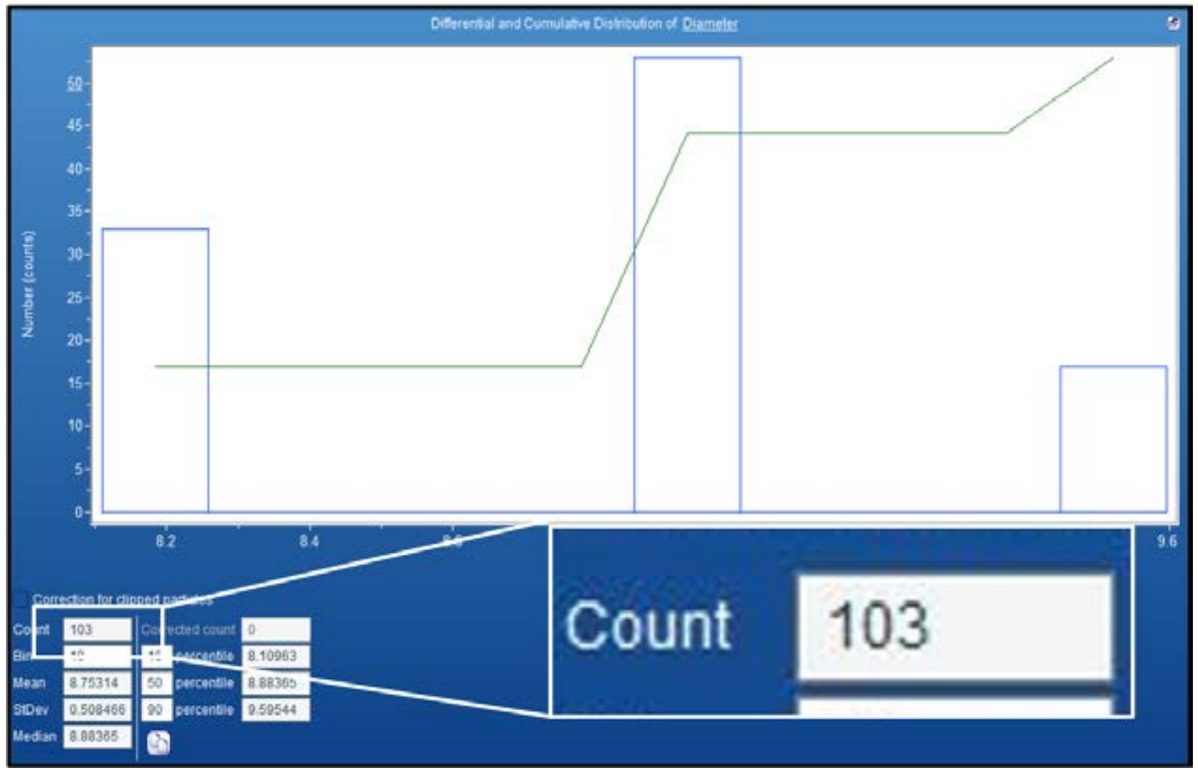
To show the high flexibility of ParticleFinder™ we select two particles size range to be chemically identified with Raman for this sample :

- Particles in the size range 8 to 10 microns;
- Particles in the size range 7 to 15 microns.

The overall number of particles are 103 and 1807 respectively for the different size ranges.




| Include | Class | Index | X pos | Y pos | Area | Diameter | Perimeter |
|---|---|---|---|---|---|---|---|
| Use filtering | | | | | | | |
| Filter limits(min) | | | | | | 8.0 | |
| Filter limits(max) | | | | | | 10.0 | |
| | 4 | 703 | | | 51.7 | 8.1 | 38.8 |
| | 4 | 704( | | | 51.7 | 8.1 | 38.8 |
| | 4 | 705( | | | 51.7 | 8.1 | 27.3 |
| | 4 | 706( | | | 51.7 | 8.1 | 43.3 |
| | 4 | 707(5230) | -1423.9 | 4952.5 | 51.7 | 8.1 | 38.8 |
| | 4 | 708(4493) | -2667.0 | 4047.2 | 51.7 | 8.1 | 38.8 |
| Mean | | | -1230.8 | 1849.9 | 60.4 | 8.8 | 44.9 |
| Stdev | | | 2821.0 | 2569.4 | 7.0 | 0.5 | 8.3 |
| Median | | | -1382.7 | 2034.9 | 62.0 | 8.9 | 49.8 |




The particle size range can be easily selected by typing the desired number in the table (picture on the left for 8 to 10 micron).
The histogram below is providing the number of particles according to their size distribution.

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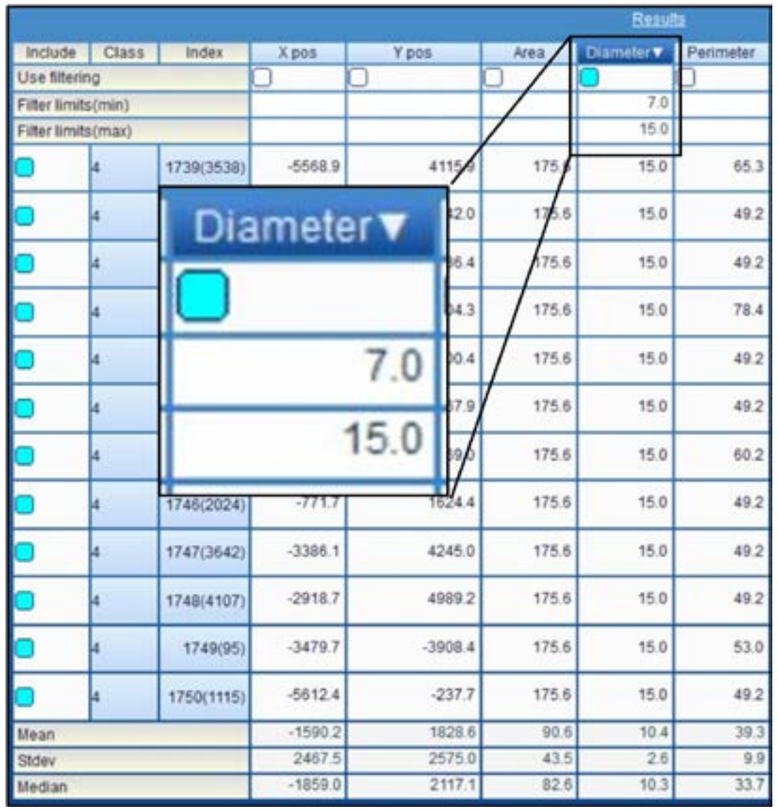
# Applications

On the next two pictures we are showing the particles selection for the range between 7 to 15 micron and the histogram with the particles size distribution for the 1807 particles.



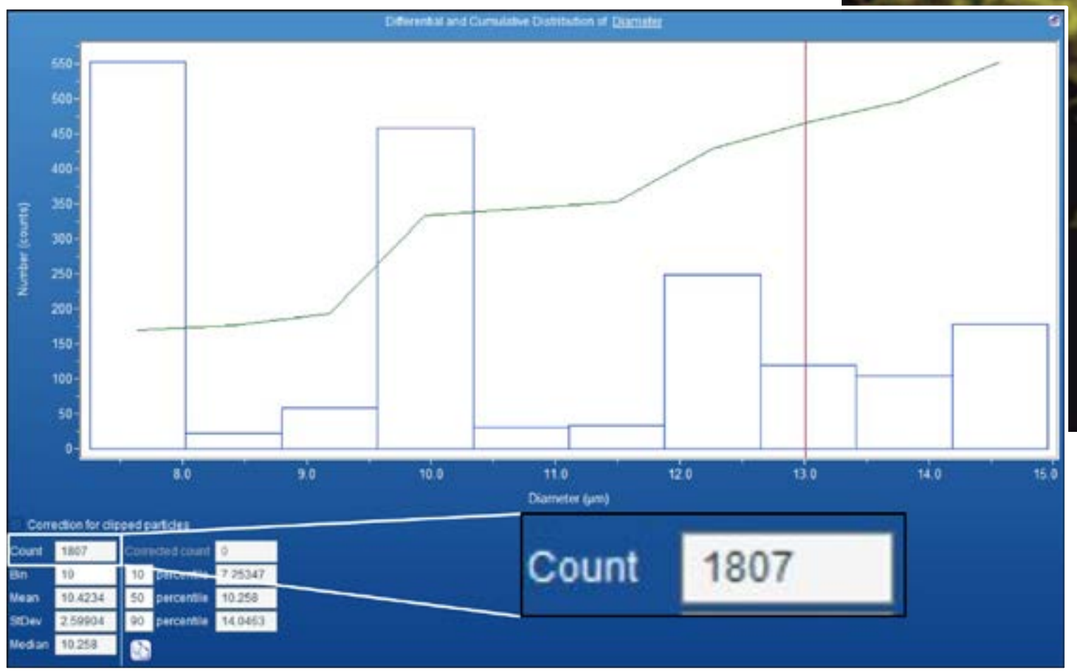
Results

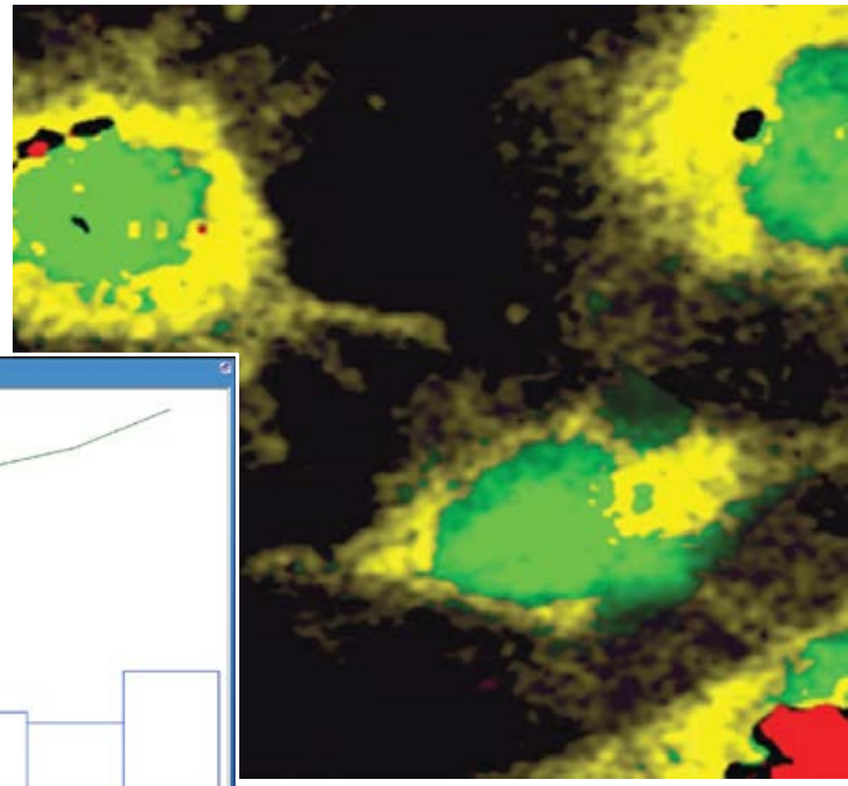
| Include | Class | Index | X pos | Y pos | Area | Diameter▼ | Perimeter |
|---|---|---|---|---|---|---|---|
| Use filtering | | | | | | | |
| Filter limits(min) | | | | | | 7.0 | |
| Filter limits(max) | | | | | | 15.0 | |
| | 4 | 1739(3538) | -5568.9 | 4115.9 | 175.6 | 15.0 | 65.3 |
| | 4 | | | 42.0 | 175.6 | 15.0 | 49.2 |
| | 4 | | | 36.4 | 175.6 | 15.0 | 49.2 |
| | 4 | | | 34.3 | 175.6 | 15.0 | 78.4 |
| | 4 | | | 30.4 | 175.6 | 15.0 | 49.2 |
| | 4 | | | 27.9 | 175.6 | 15.0 | 49.2 |
| | 4 | | | 39.0 | 175.6 | 15.0 | 60.2 |
| | 4 | 1746(2024) | -771.7 | 1624.4 | 175.6 | 15.0 | 49.2 |
| | 4 | 1747(3642) | -3386.1 | 4245.0 | 175.6 | 15.0 | 49.2 |
| | 4 | 1748(4107) | -2918.7 | 4989.2 | 175.6 | 15.0 | 49.2 |
| | 4 | 1749(95) | -3479.7 | -3908.4 | 175.6 | 15.0 | 53.0 |
| | 4 | 1750(1115) | -5612.4 | -237.7 | 175.6 | 15.0 | 49.2 |
| Mean | | | -1590.2 | 1828.6 | 90.6 | 10.4 | 39.3 |
| Stdev | | | 2467.5 | 2575.0 | 43.5 | 2.6 | 9.9 |
| Median | | | -1859.0 | 2117.1 | 82.6 | 10.3 | 33.7 |

Diameter▼
7.0
15.0

## Raman acquisition

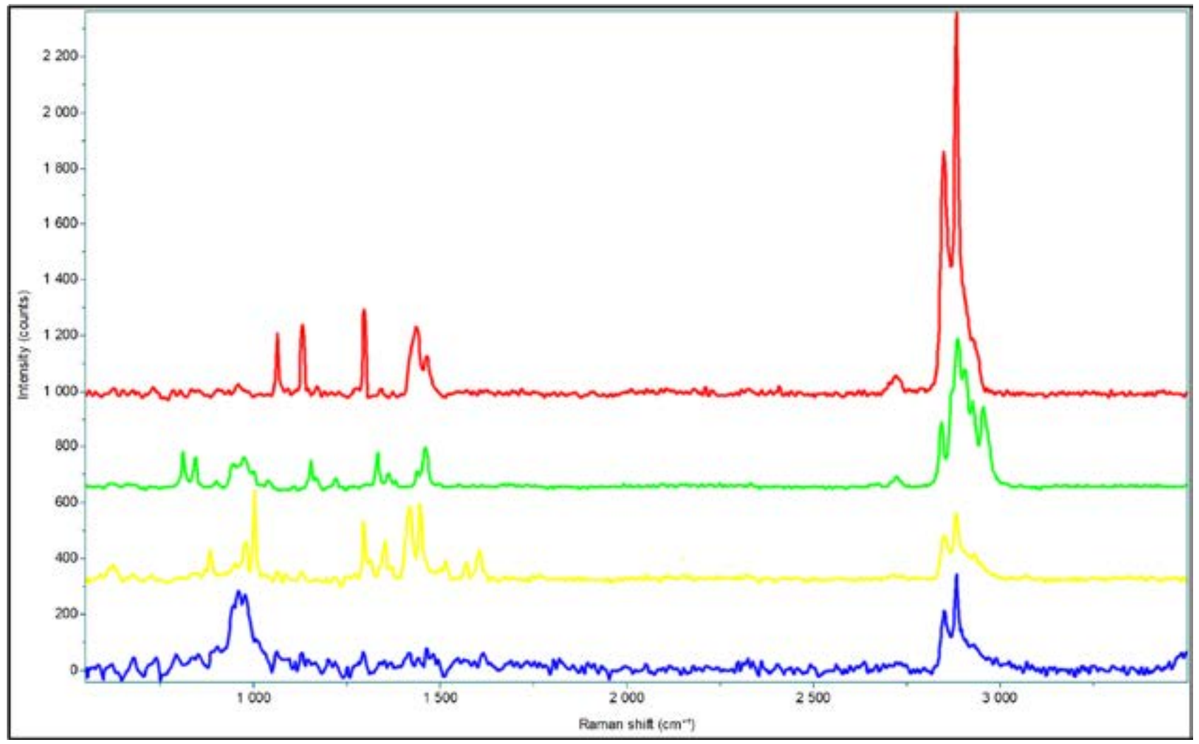
After the particles selection Raman spectra are collected for each particle. In this case we used a 532 nm laser, a 50x long working distance objective and an acquisition time of 7 seconds per spectrum. Spectra are collected in the range from 50 to 3500 $cm^{-1}$.



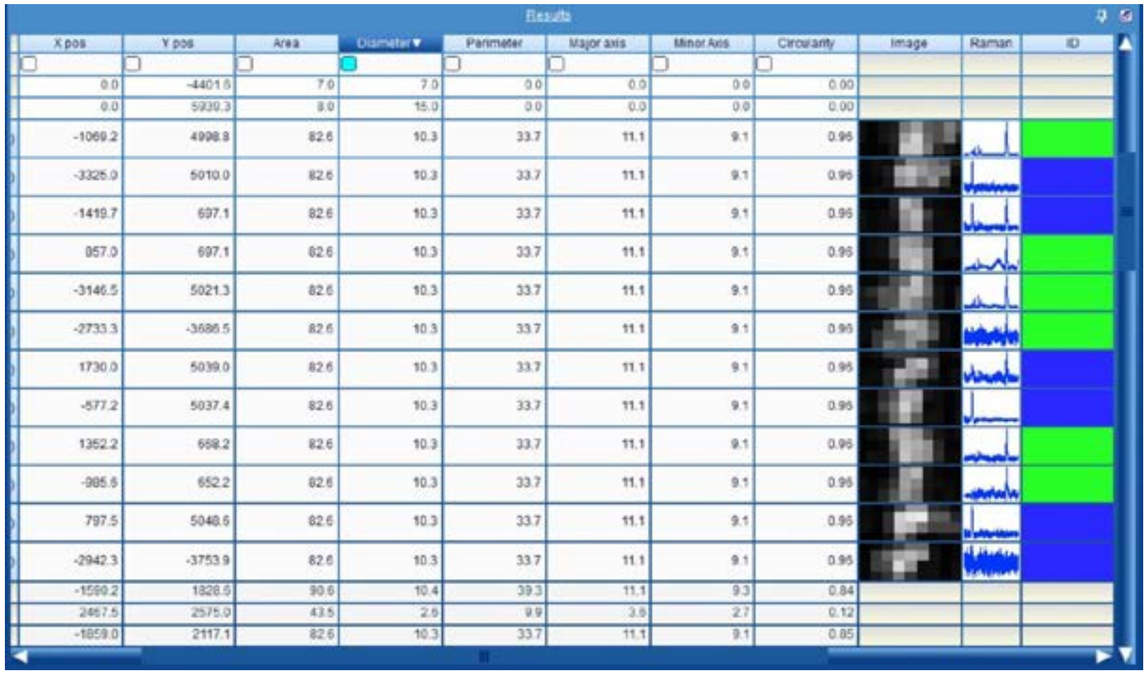



## Chemical identification and reporting

The chemical identification by exploiting the HORIBA polymer library identify four main polymer types which spectra are displayed below: Polypropylene [PP], High Density Polyethylene [HDPE], Polyethylene (low density) [PE] and Polytetrafluoroethylene [PET].




The table generated at the end of the acquisition reports two additional columns respect to the one obtain in the Characterise & Locate step:
Raman spectrum (Raman column) and chemical identification (ID column). Chemical identification is colour coded in the table to quickly visually/identify Particle vs. Polymer Type.



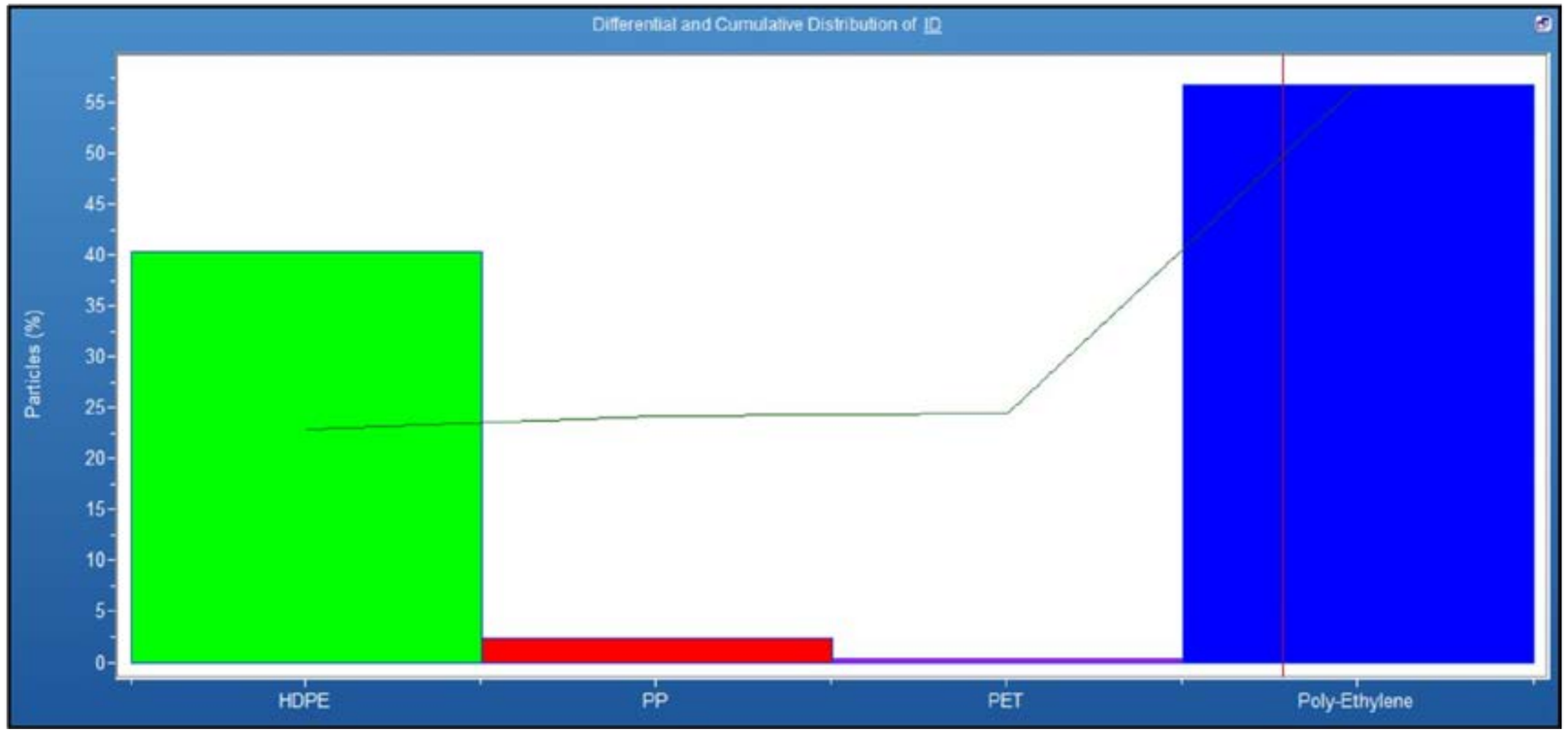
Results

| X pos | Y pos | Area | Diameter | Perimeter | Major axis | Minor Axis | Circularity | Image | Raman | ID |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.0 | -4401.6 | 7.0 | 7.0 | 0.0 | 0.0 | 0.0 | 0.00 | | | |
| 0.0 | 5939.3 | 8.0 | 15.0 | 0.0 | 0.0 | 0.0 | 0.00 | | | |
| -1069.2 | 4998.8 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -3325.0 | 5010.0 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -1419.7 | 697.1 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| 957.0 | 697.1 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -3146.5 | 5021.3 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -2733.3 | -3686.5 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| 1730.0 | 5039.0 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -577.2 | 5037.4 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| 1352.2 | 659.2 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -985.6 | 652.2 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| 797.5 | 5048.6 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -2942.3 | -3753.9 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.96 | | | |
| -1590.2 | 1828.5 | 90.6 | 10.4 | 39.3 | 11.1 | 9.3 | 0.84 | | | |
| 2467.5 | 2575.0 | 43.5 | 2.6 | 9.9 | 3.6 | 2.7 | 0.12 | | | |
| -1859.0 | 2117.1 | 82.6 | 10.3 | 33.7 | 11.1 | 9.1 | 0.85 | | | |

The images of the particle look pixelate because the mosaic is collected with a low magnification objective to speed up the mosaic collection time (better image can be easily obtained by using a 50x objective)

# Applications

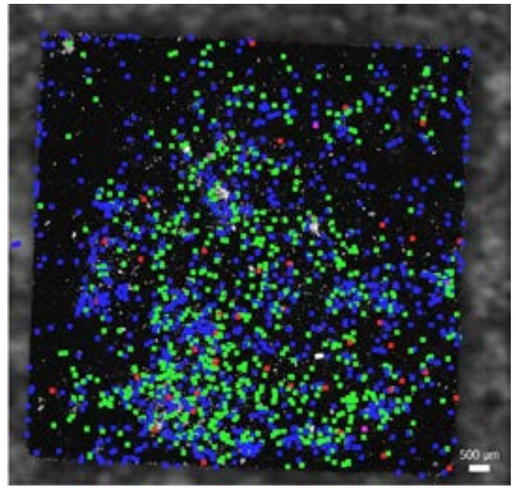
Generated the table and finalized the chemical identification several data treatments and data visualization are available to display the information of the particles. We report below just two of the option available for the size range ranging from 7 to 15 microns.




*HDPE 38,74% (700 particles) in green;*
*PP 2,43% (44 particles) in red;*
*PE 58,55% (1058 particles) in blue;*
*PET 0,58% (5 particles) purple.*

The histogram above shows the percentage of particles by polymers type (a colour is assigned to the different polymers):

At the same time it's possible to displays the particles on the optical image by using the colour code of the histogram.




**We are working on other matrices (sediment, bottled water, salt etc..) and more application will be added on the next release of the Booklet.**

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# Applications

## Analysis of microplastics in hand sanitizers using ParticleFinder™

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### Abstract

Due to the corona crisis, hand sanitizer has become part of our daily routine. However, their use is not without potential risks because of their microplastics content. HORIBA offers all the tools necessary to analyze and characterize the presence of microplastics in hand sanitizers: High-performance Raman microscopes, filtration kit, and especially the powerful particle detection software ParticleFinder™. We analyzed 3 hand sanitizer samples, from different countries, and we were able to identify the different plastic content of each.

### Introduction

At a time of global pandemics, the use and production of hand sanitizers and disinfectants has grown profusely. According to Statista Research Department(1), sales of hand sanitizers in multi-outlet and convenience stores grew by about 58% in 2020 compared to the previous year. Hand sanitizers became an essential item that people carried with them as soon as they stepped outside their homes. Yet, these products are not as harmless as one may think. Similarly, to any over-the-counter pharmaceutical product, serious attention must be paid to verify their inoffensiveness and wholesomeness to ensure safe, worry-free use.

Hand sanitizer consists of roughly 60% alcohol along with other ingredients such as emulsifiers, scent, dyes and what a lot of people are not aware of: Microplastics. According to the 2-minute team(2), 99% of the gel-based products are likely to contain plastics or ingredients that harm the environment.

It is not a secret to anyone that plastics represent a big threat for the environment because of their very slow degradation process. On top of that, microplastics are becoming a concern for the human health as well, since they could contain substances recognized as toxic such as: • Persistent Organic Pollutants (POPs), Polychlorinated Biphenyls (PCBs), Polycyclic Aromatic Hydrocarbons (PAHs), Phthalates etc…(3)

Microplastics can absorb and be an aggregation center for these substances particularly all when dissolved in water, due to their higher chemical affinity with respect to water increasing their load and potential toxicity. Moreover, some studies(4) suggest that the particle uptake by the human body is strongly linked to the size of the particles, as smaller particles may penetrate organs easier.

Considering this, it is crucial to focus on the analytical techniques which allow the identification and characterization of the smallest particles, such as Raman Microscopy.

# Applications

Raman microscopy is a non-destructive, non-contact technique that provides:

- Full morphological information for each particle through the analysis of the optical image (diameter, ellipse ratio, area…);
- Quantitative analysis (number of particles);
- Chemical identification of each particle (by exploiting a dedicated microplastic database library).

Hand sanitizers are usually presented as a gel. Therefore, a sample filtration is required. For this reason, HORIBA developed an easy-to-use filtration kit specifically for this application. Allying this with the powerful particle analysis tool ParticleFinder™, and with the comprehensive spectral identification library KnowItAll®, we get a complete and simple procedure to study and diffrentiate the components of hand sanitizers.

## Instrument and methods

### Samples preparation

We selected 3 hand sanitizer samples coming from different countries. We have called them Sample 1, 2 and 3. They were diluted in Ethanol, then filtered using HORIBA's filtration kit. We used Silicon filters of various pore sizes as they are the most suitable for the Raman analysis of microplastics.

*Figure 1: Filtration apparatus: glass funnel, glass support base, silicone stopper, glass flask, and vacuum pump.*

*Table 1: Filtration conditions for each sample.*

| Sample | 1 | 2 | 3 |
|---|---|---|---|
| Volume of sample in mL | 50 | 25 | 50 |
| Volume of Ethanol in mL | 50 | 75 | 50 |
| Filter pore diameter in µm | 5 | 10 | 10 |

### Raman platform

The Raman acquisition was made using LabRAM Soleil. It is the latest model of the LabRAM microscope series, specifically designed for wide spectral range UV-VIS-NIR confocal imaging, thanks to its ultimate optical design. Its multiple innovative hardware and software features enable us to obtain precise optical images and high-resolution spectra, with advanced automation and at an unbelievable pace.

*Figure 2: LabRAM Soleil Raman microscope*

LabRAM Soleil offers a great variety of optical viewing modes: reflected or transmitted illumination, bright-field/dark-field, phase contrast, etc. We used cross-polarization illumination to reduce the brightness of the Silicon filter and to facilitate the location of particles with ParticleFinder™.

### ParticleFinder™

The ParticleFinder™ application for LabSpec6™ allows automated location of particles, analysis of key particle parameters, such as size and shape, and subsequent chemical characterization with Raman. It is especially beneficial in cases where the number of particles is large, like microplastics, where manually locating and marking each particle would be time-consuming, outweighing the benefits that Raman can offer.

ParticleFinder™ is an integrated part of the LabSpec6™ software, linked to related modules for data acquisition, processing, analysis and display. Its workflow is simple and intuitive.

First, we start with the image acquisition. We used the 50x objective to have an appropriate spatial resolution considering the size of particles (diameter < 80 µm). To analyze the entire 1 cm x 1 cm filter by first imaging it all by montaging together multiple image tiles and then returning to each particle requires an exceptionally high positional accuracy sample stage. However, ParticleFinder™ overcomes this unnecessary constraint using its Dynamic mode. This acquires each image tile in turn but before moving to the next it is analyzed and each particle is analyzed spectrally. This enhances positional accuracy (as XY travel distances remain small) and also minimizes the time between particle location and identification, reducing the chance of the analysis being impacted by a particle moving due to environmental or thermal drift. Since this mode requires no additional user interaction it is highly recommended for ultimate performance and precision for the characterization of both large samples and small size particles.

Then, we can select the particles. An automatic threshold and multiple morphological filters are used to accurately identify the particles. Using pre-filters allows some particles to be excluded from the analysis based on parameters like area, brightness, circularity, etc. This is very useful not only to select particles of interest only, but also to exclude background features like the regular square holes in the silicon filters. We selected only particles whose diameters were between 20 µm and 80 µm to focus on the smallest particles, and whose ellipse ratio exceeded 0.3 in order to avoid the detection of the fibers contained in the gels.

Finally, after the Raman acquisition, the data was analyzed. Besides a baseline correction, no processing was done to the spectra. We identified the different components of our samples using the KnowItAll® spectral library. We then used families to segregate the particles into groups allowing statistics and interpretation of the results based on a higher-level segmentation.

### Results

**The first type of comparison is the image of each filter after the filtration:**

- In the table below, the coloured particles are the ones that were selected for the analysis. They are the smallest particles (between 20 µm and 80 µm diameter) since this is the focus of our study.
- The composition of the samples is different from one another. While the filter corresponding to sample 1 is packed with particles, sample 2 contains many fibers, and the sample 3 one is the least dense.
- Particles in sample 1 are brighter and more opaque than the other samples where the particles are quasi-transparent. Indeed, the mean brightness (in a grey scale of 0 to 255) of the detected particles in each sample is: 108.599 for sample 1, 60.7182 for sample 2, and 43.4436 for sample 3. The opacity of the particles has an influence on the Raman signal, as we see globally better spectra in sample 1.

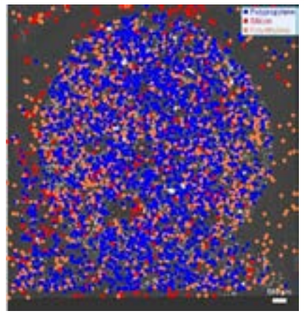
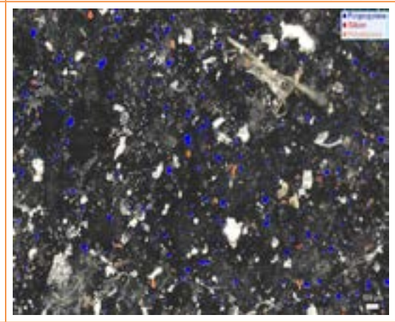
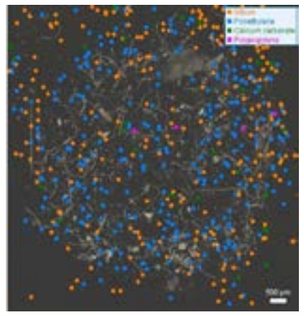
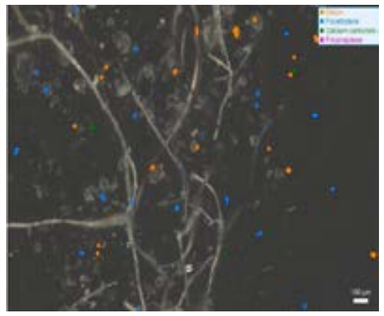
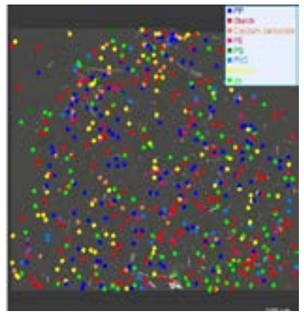
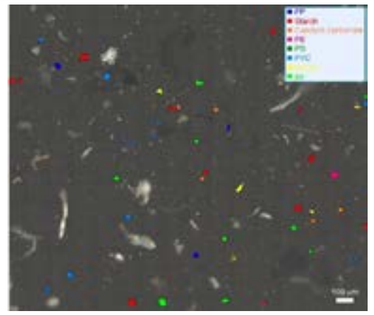
| Sample | Full filter image (scale 500µm) | Zoom on a small area (scale 100µm) |
|---|---|---|
| 1 |  |  |
| 2 |  |  |
| 3 |  |  |

*Table 2: Table showing the images of the full filter (left), and a zoom on a small area of the filter (right). The coloured surfaces correspond to the detected particles*

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**The second type of comparison is the total number of particles and the number of plastic particles in each sample.**

To examine the results, we chose to compare the number of plastic particles per milliliter.

Sample 1 has the biggest number of plastic particles per milliliter with 3733 particles for 50 milliliters. Sample 2 comes in second place with 207 particles for 25 milliliters, and finally Sample 3 with 353 particles for 50 milliliters. Figure 3 illustrates this result.

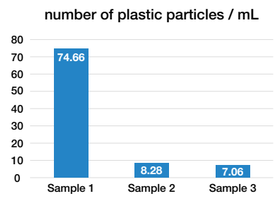



*Figure 3: Bar diagram presenting the number of plastic particles per milliliter for each sample.*

**The third and final type of comparison is the chemical composition of the samples**. We found many components in each sample. The most prominent ones are plastics, but we also found other elements like: Calcium carbonate, which is usually used as a filler in plastics(5), and Starch, which is a biopolymer(6).

To sum up, the three samples differ by the morphological properties of their particles, the number of plastic particles per milliliter (ranging from 7,06 to 74,66), and the types of plastic they contain (Polypropylene and Polyethylene being the most common ones).

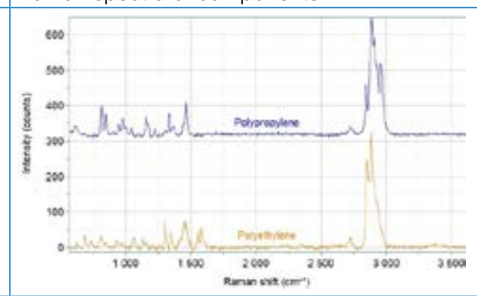
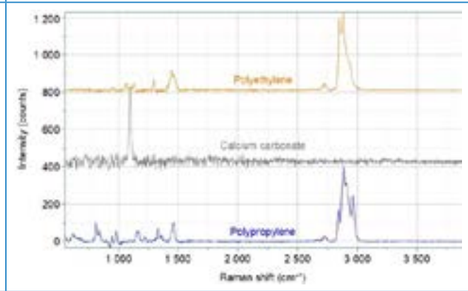
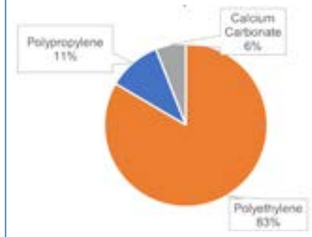
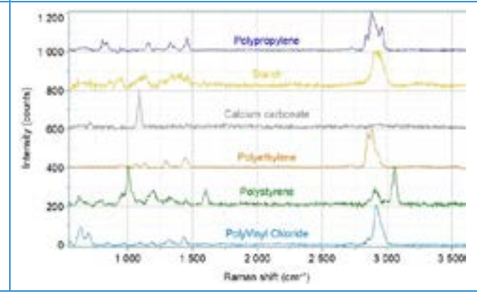
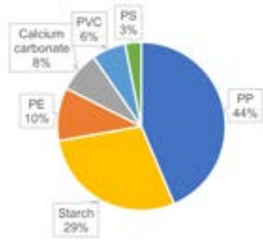
| Sample | Raman spectra of components | Distribution of components |
|---|---|---|
| 1 |   |   |
| 2 |   |   |
| 3 |   |   |

*Table 3: Table presenting the composition of each sample (right) with the corresponding spectra (left).*

## Conclusion

The filn this application note, we described the intuitive and automated method of ParticleFinder™, allying the robustness of Raman microscopy with the sophistication of HORIBA's software to allow the screening of a large quantity of microplastics in a precise way. Thanks to ParticleFinder™, we demonstrated the presence of microplastics in three samples of hand sanitizers, we were able to distinguish the different types of plastics each sample contains, and study their statistical distribution.

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