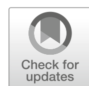

Microplastics and Nanoplastics

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Lucia Pittura, Stefania Gorbi, Carola Mazzoli, Alessandro Nardi, Maura Benedetti, and Francesco Regoli

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Abstract

Microplastics have become a constant and ubiquitous component of the marine environment, being found in water surface, along water column, and in sediments, beaches, and organisms worldwide. Assessing microplastics in the environment is necessary to understand sources, distribution, abundance, and ecological consequences for marine ecosystems, especially considering that microplastic contamination is expected to increase in years to come in view of increasing global annual primary plastic production.

This chapter intends to provide tools to approach the issue of microplastics as consciously as possible, including the importance to consider microfibers as a category per se. Definitions of microplastics and microfibers will be provided along with a brief discussion of elements affecting their distribution in the marine environment and how they can cause biological effects. The focus of the chapter is, however, on the most common and suitable sampling strategies, analytical approaches, and standard requirements, for conducting a reliable assessment of microplastics in marine matrices.

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Microplastics · Nanoplastics · Microfibers · Water · Sediment · Biota · Sampling · Characterization · μ-FTIR · Raman spectroscopy

8.1 Introduction

Microplastics have become a constant and ubiquitous component of the marine environment, being found in water surface, along water column, and in sediments and organisms worldwide (Qu et al. 2018). Microplastic contamination is expected to increase in years to come, especially in view of increasing global annual primary plastic production that would reach 1.1 billion tons in 2050 and with a total cumulative production between 1950 and 2050 of 34 billion tons, none of which would be biodegradable (Geyer 2020). Assessing microplastics in the environment is, thus, necessary to understand sources, distribution, abundance, and ecological consequences for marine ecosystems. This problem is also on the agenda of national and international organizations worldwide, asking experts to use their knowledge to compile recommendations and guidelines for routinely monitoring programs (van Bavel et al. 2020).

This chapter intends to provide tools to approach the issue of microplastics as consciously as possible, firstly, describing what constitute a microplastic, what kind of other categories exist, how microplastics originate, and their possible fate in the field and, secondly, focusing on the most suitable sampling strategies, analytical approaches, and standard requirements for a reliable assessment of microplastics in marine matrices.

International consensus has not yet been reached on a definition and categorization of microplastics, often resulting in an ambiguous comparison of data (Hartmann et al. 2019). To date, it is globally accepted that the lower size limit for microplastics is 1 μm (Frias et al. 2018), while, under this value and down to 1 nm, plastics are defined as nanoplastics (Gigault et al. 2018). The upper size limit of microplastics is, instead, still under debate. Frias and Nash (2019) proposed 5 mm, whereas other authors suggest to restrict the definition of microplastics to particles smaller than 1 mm based on the International System of Units definition for "micro" (Cole et al. 2011).

Microplastics and nanoplastics can have a primary origin, when they are intentionally produced in the micro-nanometer size range to be directly used in a wide range of applications (e.g., personal care products and cosmetics, abrasive powders, powders for injection molds, and 3D printing). Otherwise, secondary microplastics represent results of weathering or fragmentation of larger objects either during use or following loss to the environment (UNEP 2021).

Another criterion for defining microplastics is associated to shape. Also in this case, there is no standardized classification. BASEMAN consortium (whose goal was the validation and harmonization of analytical methods for microplastic analysis in environmental matrices) suggested eight categories based on the most common

microplastic types described in peer-reviewed publications: (1) pellet, (2) fragment, (3) fiber, (4) film, (5) rope and filament, (6) microbead, (7) sponge/foam, and (8) rubber.

Despite that color is not considered to be crucial to define microplastics, recording this characteristic is considered important for studies concerning aquatic organisms, as some species are thought to potentially ingest microplastics based on a color preference behavior (Frias and Nash 2019).

Although textile fibers are broadly classified as a shape of microplastics, they were suggested as a category of their own. Compared to other types of microplastics, they are much more abundant in the environment, and they are different in source (textile vs. common use), polymer typology (often natural vs. synthetic only), and mitigation actions (industry vs. public awareness, Avio et al. 2020). These differences of microplastics and microfibers are highlighted by definitions given by Bessa et al. (2019) and Liu et al. (2019). Microplastics are synthetic solid particles of polymeric matrix, with size ranging from 1 μm to 5 mm, consisting of either items that are manufactured to be of microscopic dimensions (primary) or that are formed from the weathering and fragmentation of larger plastic waste items, which are insoluble in water at 20 °C (Bessa et al. 2019). Microfibers are natural or artificial fibrous materials of threadlike structure with a diameter lower than 50 μm, length ranging from 1 μm to 5 mm, and length to diameter ratio greater than 100. Microfibers are released or shed to the environment from all kinds of fibrous materials, such as clothes; agricultural, industrial, and home textiles; and some textile products, semimanufactured goods, or accessories used in other fields, during production, use, and end-of-life disposal (Liu et al. 2019).

Distribution and accumulation of microplastics in aquatic ecosystems are widely dependent on particle characteristics such as size, shape, density, and chemical composition along with environmental parameters including wind, temperature, and water current velocity (Gola et al. 2021). Hydrodynamic processes, coastal currents, drift, and river outflow act to disperse microplastics from their sources. Additionally, rotational ocean currents transport surface plastics to convergence zones of oceanic gyres, leading to concentrated areas of accumulation (Coyle et al. 2020). Several factors potentially influence the vertical distribution of plastics along the water column, such as wind-induced mixing, incorporation into marine aggregates or fecal matter, biofouling (Cole et al. 2016), size and shape of materials, and relative density that might vary with additives added during production (Reisser et al. 2015, Table 8.1). For plastic denser than seawater, the shape and the near-bottom current velocity magnitude strongly define the settling (Bagaev et al. 2017).

Due to their small size and widespread occurrence, microplastics can be ingested by wide range of marine organisms causing a range of effects like mechanical damages, attachment of polymers to external surfaces, hindering of mobility and clogging of the digestive tract, inflammation, cellular stress, and decreased growth (Setälä et al. 2016). The chemical impact can be related to additives present in the plastic from manufacturing, as well as to the environmental contaminants which are adsorbed by the hydrophobic nature and high surface-to-volume ratio of microplastics. There is, however, an active debate regarding the toxicological

Table 8.1 Buoyancy of most common polymer in seawater (from Bessa et al. 2019)

Abbreviation	Polymer	Density (g cm^{-3})		Buoyancy of polymer in seawater (1.025 g cm^{-3})
PS	Polystyrene	0.01–1.06		Positive
PP	Polypropylene	0.85–0.92		Positive
LDPE	Low-density polyethylene	0.89–0.93		Positive
EVA	Ethylene vinyl acetate	0.93–0.95		Positive
HDPE	High-density polyethylene	0.94–0.98		Negative
PA	Polyamide	1.12–1.15		Negative
PA 66	Nylon 66	1.13–1.15		Negative
PMMA	Polymethyl methacrylate	1.16–1.20		Negative
PC	Polycarbonate	1.20–1.22		Negative
PU	Polyurethane	1.20–1.26		Negative
PET	Polyethylene terephthalate	1.38–1.41		Negative
PVC	Polyvinyl chloride	1.38–1.41		Negative
PTFE	Polytetrafluoroethylene	2.10–2.30		Negative

relevance of adsorbed pollutants on microplastics and their possible transfer to marine organisms due to the variability of experimental results (Benedetti et al. 2022). Despite that a detailed review of fate, distribution, and biological effects of microplastics is outside the aim of this chapter, these issues are fundamental for a comprehensive risk assessment in the marine environment.

8.2 Sampling the Marine Environment for Microplastic Detection

Monitoring microplastics in the environment is based on sequence of steps starting with the collection of appropriate samples, followed by isolation of particles from the matrix and their physical and chemical characterization.

The sampling strategy always depends on the aim of the research, while sampling devices are related to the matrix to be collected, the size limit of microplastics to be targeted, and available equipment for immediate processing.

Water, sediment, and biota can be collected as bulk or volume-reduced samples (Stock et al. 2019). In the first case, the whole collected volume is taken without any reduction during the sampling process, theoretically, allowing to capture all microplastics regardless of their size and visibility. Volume-reduced samples are subjected, during the sampling, to specific treatments (i.e., filtration, separation, and concentration) to preserve only portions of samples that will be further processed in a laboratory (Wang and Wang 2018). Selective sampling consists of direct collection of microplastics recognizable by the naked eye, and it is applicable only to large microplastics (1–5 mm, e.g., plastic pellet on beaches) (Hidalgo-Ruz et al. 2012).

8.2.1 Sampling of Seawater

Different typologies of devices are used to collect microplastics in seawater, categorized in non-discrete sampling devices for volume-reduced samples and discrete devices for bulk samples (Cutroneo et al. 2020). Depending on the device, it is possible to collect different layers of the water compartment: the upper 30 cm or "surface waters" (Han et al. 2020), including the air-water interface or directly below the interface; the "subsurface layers" still within the upper mix layers affected by winds, surface currents, and vessel movements; and the deeper layers of water column (van Bavel et al. 2020). The choice of device depends on available equipment and characteristics of sampling location, such as turbulence at the surface, hydrodynamic profile, and depth, besides the focus of the research. Irrespective of the sampling method, environmental metadata should always be collected to support data interpretation including bathymetry, water temperature, salinity, water currents, surface wind, and weather conditions (van Bavel et al. 2020).

Nets originally designed for plankton are widely used for collecting microplastics (Fig. 8.1a). They consist of a funnel shape mesh, generally made of nylon and 1.5–4.5 m long, with a circular or a rectangular mouth maintained constantly opened by a rigid frame and connected to a final cylinder called code-end. The main advantage is the possibility to filter large volumes of water concentrating and gathering microplastics in the code-end. There are various mesh sizes for nets possibly ranging from 20 μm to 5000 μm, despite that those of 300 μm are the most commonly used to facilitate comparison among studies. However, 300-μm mesh fails to depict the overall pattern of microplastic pollution, since it does not retain microfibers and smaller microplastics (Tamminga et al. 2019), which are both of great biological relevance. However, nets with lower mesh sizes can easily get clogged up, compromising the sampling process (Löder and Gerdts 2015).

Plankton nets are used for both vertical and horizontal sampling of the water column, allowing the collection of replicate samples in the bongo conformation, with two plankton nets connected through a couple of circular aluminum frames

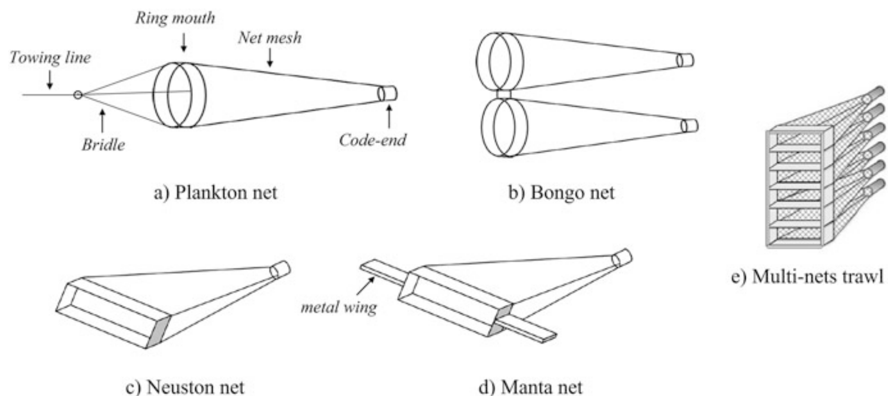


Fig. 8.1 Most common typologies of net devices for sampling microplastics in seawater column (**a**, **b,** and **e**) and on surface and subsurface waters (**c** and **d,** modified from van Bavel et al. 2020)

(Fig. 8.1b). In horizontal sampling, a weight (about 10–20 kg) is fixed to the net, then slowly dropped to the maximum depth (avoiding contact with the bottom), and trawled obliquely at a speed of less than 2 knots to let the water pass through the net with a steady flow. In vertical sampling, the net is lifted toward the surface from a specific depth, thus sampling the entire water column (Campanale et al. 2020).

Manta and neuston nets have frames of rectangular shape (Fig. 8.1c–d) and are the most suitable for surface and subsurface water sampling: nets are deployed from the ship and towed horizontally along horizontal transect for specific periods, varying from a minimum of 10 min (Setälä et al. 2016) to a maximum of 240 min (Pan et al. 2019), at a known speed (between 1 and 5 knots, Cutroneo et al. 2020). Manta nets have two buoys or, more typically, two metal wings equipped on the sides of the frame which give the appearance of a manta ray, to ensure stability and keep the net floating on the surface. Manta nets can thus maintain a constant immersion depth under the sea surface, and the filtered water volume can be estimated accurately. Neuston nets are kept at the surface by floats or suspended beneath the water's surface, filtering the surface layer even in the presence of waves, despite that the volume is difficult to be estimated accurately because the net's immersion depth changes constantly (van Bavel et al. 2020). Neuston nets, mounted one above the other as multi-net trawl, also provide the possibility to synchronously sample various layers of the water column (Fig. 8.1e).

After the sampling, nets must be carefully rinsed from the exterior to assure that plastic debris are washed into the collector and to clean the net before the next sampling. The material within the collector is finally transferred to a sample container for subsequent processing in laboratory (Fig. 8.2): samples can be fixed with plastic-friendly fixatives (e.g., formalin) to preserve the biological component or stored frozen if microplastics are the only target parameters of the study.

During the trawling, it is fundamental to measure the volume of the filtered water and to normalize the concentration of microplastics per volume unit. For this reason, a flow meter is often present on the net opening (Stock et al. 2019); as an alternative, the amount of filtered water can be calculated by the net opening size and trawl distance, the latter easily obtained using smartphone applications for GPS tracking during the sampling.

In addition to nets, pumping systems represent non-discrete sampling devices allowing to filtrate in situ large volumes of water, from surface up to a depth of 100 m (Cutroneo et al. 2020). Pumping systems are equipped with a flow meter to determine the volume of filtered water. Microplastics are collected through a series of filters/sieves, available in different mesh sizes to separate microplastics into different dimensional classes at the time of sampling. Filters can be recovered in petri dishes and preserved until laboratory analysis, whereas sieves need to be rinsed with decontaminated water (e.g., microfiltered water) and microplastics collected and preserved in a glass jar. Some sieves are designed with the possibility to disassemble the frame and mount a clean filter mesh. The first filter/sieve of the filtration unit is often of 5-mm mesh size used to remove larger plastic particles, and a 300-μm mesh filter/sieve is typically included in the battery for comparing results with those obtained with nets (Setälä et al. 2016; Tamminga et al. 2019; Rist et al.

Fig. 8.2 Net-based method for sampling microplastics in surface waters. Activities carried out by the Polytechnic University of Marche (Italy) in the Adriatic Sea using a manta net during activities of RESPONSE project funded by JPI Oceans, 2020–2023 (photo credit, L. Pittura and C. Mazzoli)

2020; Karlsson et al. 2020; Schönlau et al. 2020). Such comparisons revealed that in situ pump filtration methods are more accurate in volume measurement and versatile for point sampling and filter size choice, enabling standardization of sampling (Razeghi et al. 2021); in addition, pump filtration allows to collect smaller microplastics and more significant sampling of microfibers compared to nets (Campanale et al. 2020). On the other side, pump systems are more expensive, and trawling methods can cover larger areas, thus better overcoming some of the problems related to patchiness (Karlsson et al. 2020). Net trawling and pump sampling methods can be considered complementary techniques providing a more comprehensive approach for monitoring microplastics in water compartment (Tamminga et al. 2019; Razeghi et al. 2021).

Diverse pumping systems are commercially available or custom made. They can pump water from a specific depth that is directed to the filtering system outside the water or can work directly submersed. Submersible pumps can be lowered from the vessel using a winch sideways or toward the stern of the ship (Cutroneo et al. 2020). Depending on the technical specification of pumps, different volumes can be sampled, diverse depths can be covered, and filtration time can vary from several hours to a few minutes. For example, a new custom-made plastic-free pump-filter system (UFO system—Universal Filtering Objects system) was applied for

collecting microplastics down to 10 μm in the Arctic, filtering approximately 1 m^3 of water from 5-m depth (Rist et al. 2020). The device is composed of a metal hose deployed in the water, a pump controlled by an inverter, and a modular filtering device. The mouth of the hose is equipped with a stainless-steel metal cage of 5-mm mesh to protect the system against large debris. The filtering unit consists of three parts: the water first passes through a filter of 300-μm mesh to retain larger items with the purpose of protecting the finer filtering mesh from clogging. The water is then divided onto two parallel units with filters of 10 μm. Outlets were recombined and connected to a mechanical flowmeter to quantify the filtered volume (Rist et al. 2020, Fig. 8.3). Preston-Whyte et al. (2021) used a Micro Plastic Particle Pump developed by KC Denmark for sampling near surface water (0.5-m depth) in a port environment. Using a crane, the particle pump was deployed from the quayside and held submerged. During deployment, 2000 L of pumped water was directed through stacked sieves (5 mm, 500 μm, 300 μm, and 200 μm). On recovery, sieves were individually removed, and all particulate matter on the sieve was transferred to a 1-L glass jar (Fig. 8.4a). Actually, the pump system of KC Denmark enables to sample down to 40 m, and a flexible range of filter sizes is available (ranging from 1000 μm down to 20 μm). The same model of pump system used by Preston-Whyte et al. (2021) was, in fact, used by the Polytechnic University of Marche (Italy) to sample microplastics in water column up to 30 m along the Adriatic coast (Mediterranean Sea), within activities of the RESPONSE JPI Oceans project: sieves were individually removed and stored in glass petri dishes for subsequent processing in the laboratory (Fig. 8.4b).

Discrete sampling devices can be used to collect defined volumes of water from specific depths. The main advantage of collecting bulk samples is that, theoretically, all present microplastics can be sampled without any size limitation, preventing any loss possibly occurring in volume-reduced samples. This sampling is rapid and reduces the risk of contamination, due to the short handling and sample exposure to the surrounding environment. The main disadvantage consists of the limited amount of samples that can be collected, stored, and processed (Campanale et al. 2020).

Discrete sampling devices can include a plexiglass water sampler, a rosette sampler system (CTD [conductivity-temperature-depth] sampler), and the lander system (Fig. 8.5). The plexiglass water sampler is particularly useful and convenient for microplastic collection in shallow waters with weak currents, while it would not be applicable to deeper aquatic environments because it is usually made of acrylic materials and can be fragile when it is subjected to pressure. The rosette sampler system is typically comprised of a set of Niskin bottles (8–12 L) equipped with CTD sensors, and it can be adopted to collect water samples at various depths in marginal seas and pelagic zones up to 6800 m in depth. Niskin bottles can also be fitted on a lander system to collect bottom water near the seafloor (Liu et al. 2020).

Additional methods can be as simple as a glass bottle of 1 liter, used during various citizen science-driven projects to collect surface waters (van Bavel et al. 2020). Samples obtained with discrete devices are transferred to a jar, and the inside of the device is rinsed with decontaminated water to collect plastic particles that

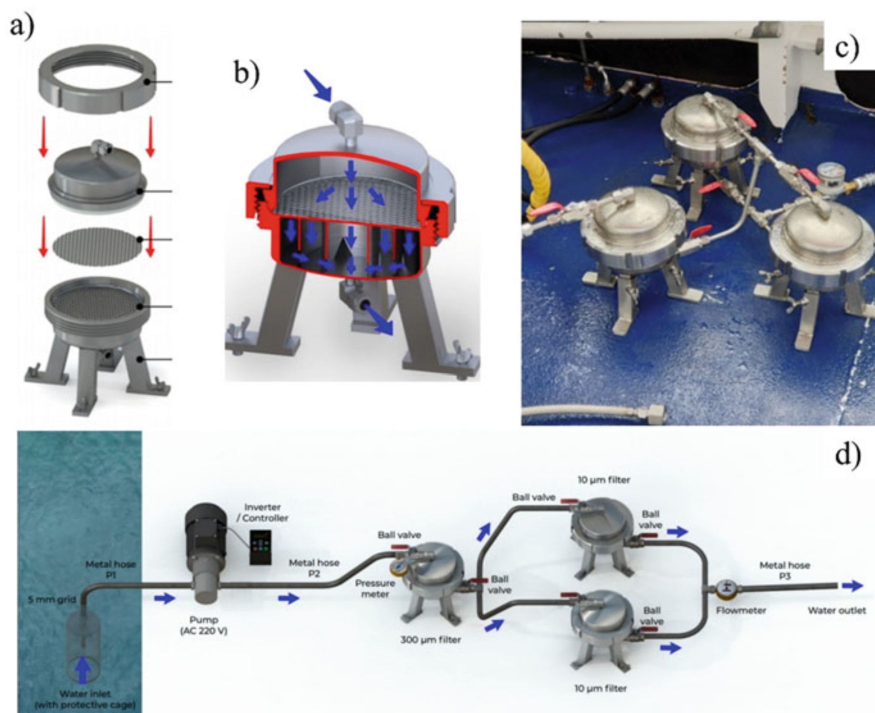


Fig. 8.3 UFO system as an example of custom-made pump-filter device for sampling microplastics in seawater: (**a**) mounting schematic of a single UFO unit, (**b**) cross section of a single UFO (arrows illustrate the water flow), (**c**) picture of the real setup operating on board during the survey, and (**d**) overall schematics of UFO setup (modified from Rist et al. 2020)

remain attached. The sample is preserved until laboratory analysis or filtered or sieved directly on board (Cutroneo et al. 2020).

8.2.2 Sampling of Sediments

Microplastics can be investigated in various typologies of sediments including those of intertidal beaches and subtidal seabed sediments. Different sampling approaches are obviously required, although it is always recommended to collect data associated to the sampling site, such as date of sampling, coordinates of location, morphological and hydrographic characteristics of the area, type of sediment, presence of macroplastics, local point sources (e.g., proximity to urban and/or industrial areas, to river streams and/or estuaries, and to wastewater treatment plants), or any other relevant information for interpretation of results. Exhaustive examples of datasheets to collect data while sampling intertidal and subtidal sediments are provided in the

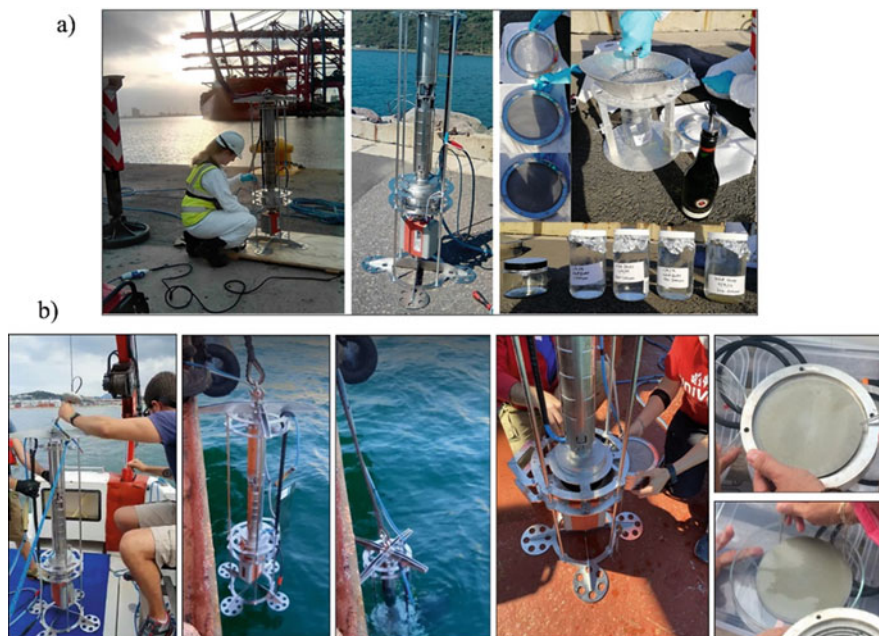


Fig. 8.4 Micro Plastic Particle Pump (KC Denmark) as an example of submersible device for sampling microplastics in seawater. (**a**) Operation of the pump in a port environment and sample collection (modified from Preston-Whyte et al. 2021). (**b**) Operation of the pump and sample collection carried out by the Polytechnic University of Marche (Italy) along the coasts of Adriatic Sea during activities of RESPONSE project funded by JPI Oceans, 2020–2023 (photo credit, S. Gorbi and F. Regoli)

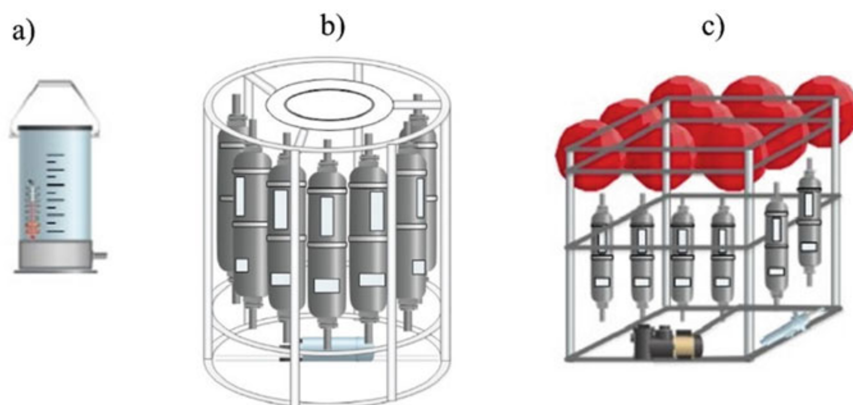


Fig. 8.5 Most common types of discrete sampling devices for sampling microplastics in seawater: (**a**) plexiglass water sampler, (**b**) CTD sampler, and (**c**) lander system (modified from Liu et al. 2020)

protocol for monitoring microplastics in sediments produced by Frias et al. (2018) within the BASEMAN JPI Oceans project.

8.2.2.1 Intertidal Sediments (Beaches)

Sampling sediments on beaches is relatively easy from a technical point of view, and it can be carried out by trained nonscientist operators with unsophisticated equipment: a nonplastic sampling tool (tablespoon, trowel, or small shovel and corers), a frame to define the sampling unit, a 5-mm metal sieve to exclude macro-debris directly at the beach, and a nonplastic container to store the sample (Fig. 8.6). The not trivial aspect is the monitoring design, the correct identification of sampling area (e.g., shoreline and above the strandline), the depth of sediments to be collected, and the frequency of surveys (Löder and Gerdts 2015). Beaches are dynamic environments, and distribution of microplastics can rapidly change according to the depositional regime and environmental characteristics like tides, currents, and winds (Hanke et al. 2013). Other critical issues are the number of replicates and the quantity of sediments to be collected (weight or volume), to ensure a representative sampling and accurate estimation of microplastic concentration (Prata et al. 2019). Sampling strategies for microplastics on beaches are summarized in guidelines of the UNEP (Cheshire et al. 2009), the OSPAR (Wenneker et al. 2010), the NOAA (Lippiatt et al. 2013), and the MSFD Technical Subgroup on Marine Litter (TSGML, Hanke et al. 2013).

Sediment collection is recommended to be performed on the strandline (top of the shore), where litter is more likely to accumulate, defining a transect of 100 m parallel to the water edge (Fig. 8.7); for heavily littered beaches, the transect can be reduced to 50 m. If the survey is extended to the whole beach, at least two transects shall be identified between water edges and above the strandline (back of the beach) with a minimum distance of 50 m. Along transects, sampling is performed within conventional areas of 30×30 cm or 50×50 cm marked through the use of a quadrat (sampling units): collection of the top 5 cm of sediment (total volume of

Fig. 8.6 Tools for sampling sediments on beaches for microplastics and sampling activities by high school students coordinated by researchers of the Polytechnic University of Marche, Italy (photo credit, L. Pittura)

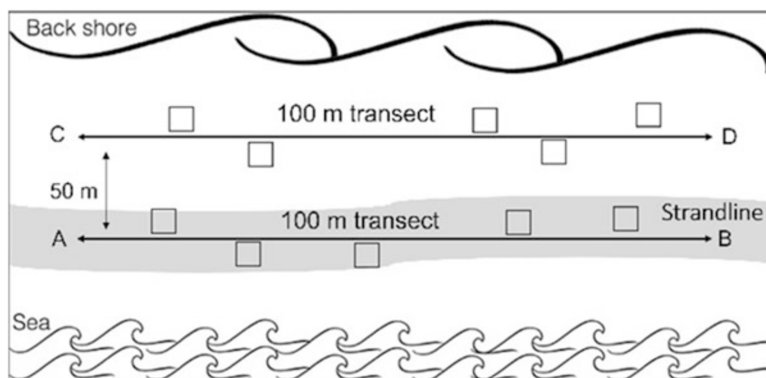


Fig. 8.7 Schematic representation of sampling strategy for microplastics on beach with transects and quadrants (modified from Frias et al. 2018)

approximately 4500 cm^3 = 4.5 L) is a common approach, and a minimum of five replicates, separated by at least 5 m, is recommended. Sampling units should be chosen in a random way to be representative of the sampling area (Fig. 8.7). Four surveys per year in spring (April), summer (mid-June to mid-July), autumn (mid-September to mid-October), and winter (mid-December to mid-January) are suggested, but a higher frequency may be initially necessary to identify significant seasonal patterns.

8.2.2.2 Subtidal Sediments (Seabed Sediments)

The most common devices to collect submerged sediments for microplastic analysis are grabs and corers (Fig. 8.8a–e), the choice of which depends on purpose of the survey, sediment typology, and various environmental and logistical constraints, such as water depth and vessel characteristics.

Grabs are easy to use and allow to collect samples in a relatively short time. The most common are van Veen, Ekman-Birge, and Shipek grabs. The van Veen grab is constituted by two jaws which close when these arrive at the bottom holding the sediment inside. Some models have upper doors to collect the upper centimeters of sediment directly inside the device. The Ekman-Birge grab has a box shape with upper windows and lower jaws; the smaller type can be manually operated by a rod that allows the insertion into the sediment. The Shipek grab has a bucket that rotates into the sediment when it reaches the sea bottom (Romano et al. 2018), facilitating the collection of difficult substrates (gravel or compacted sands).

Compared to grabs which retrieve disturbed sediments, core samplers collect cylindrical sections maintaining the sediment integrity from the surface to the deeper layers. This allows to determine historical profiles and inputs of microplastics as indicators of anthropogenic activity. As advantages, corers produce small volumes of sample and potentially enable to sample at a depth of more than 5000 m (Löder and Gerdts 2015). Among these devices, box corer (e.g., Reineck box corer) consists of a stainless-steel square box of variable size and a support frame that stabilizes the

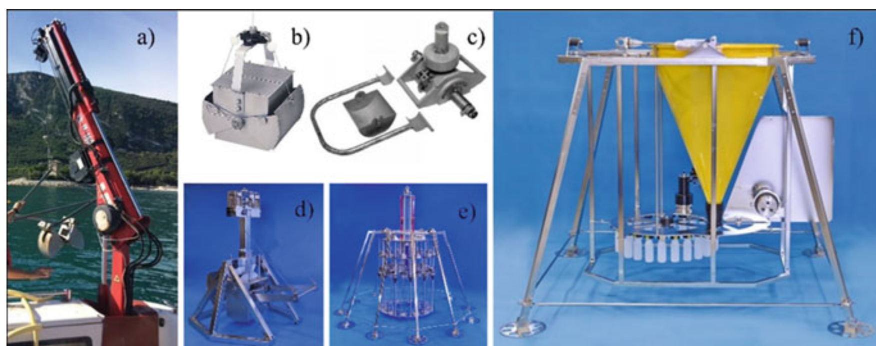


Fig. 8.8 Devices for sampling interdital sediments for microplastics: (**a**) van Veen grab (photo credit, S. Gorbi), (**b**) Ekman-Birge grab, (**c**) Shipek grab, (**d**) box corer, (**e**) multiple corer, and (**f**) sediment trap for microplastic vertical fluxes

sampler, ensuring the vertical penetration in the sea bottom. The box allows the recovery of nearly undisturbed samples of around 30 cm thick, and samples can be collected in a single device or a multiple corer constituted by 4 up to 12 core barrels (Romano et al. 2018).

Independently on the device, subsamples of ideally 250 ml of sediment should be collected to be representative of sampling location (Hanke et al. 2013). Samples are then placed into containers (preferably of nonplastic material) and stored frozen at −20 °C in the dark if these are not immediately analyzed (Frias et al. 2018).

While analysis of microplastics in sediments can answer questions related to their presence, accumulation, and characteristics, the sinking process of microplastics along the water column can be monitored through the deployment of sediment traps (Saarni et al. 2021; Fig. 8.8f). These instruments, commonly used in oceanography to measure settling of organic and inorganic particles, consist of an upward-facing funnel that directs sinking particles toward a collection vessel. Traps typically operate over an extended period of time (weeks to months), and their series can be cycled to follow temporal changes in sinking flux with time, for instance, across a seasonal cycle.

8.2.3 Sampling of Biota

The selection of appropriate species is crucial to assess the ingestion of microplastics and their potential biological effects. A sampling design that includes multispecies, covering different trophic positions, habitat, and feeding strategies is preferable to a single-species approach. This strategy provides a more ecologically relevant assessment of microplastics in the marine environment facilitating comparisons among areas with different species but with similar trophic web structures (Avio et al. 2020). In general, the selected species should meet as many as possible of the following criteria:

(i) They occur naturally with high abundance and wide geographic distribution.
(ii) The biology and ecology are well known.
(iii) They are easy to sample and to process in laboratory.
(iv) They are already used as bioindicators in monitoring programs for other contaminants.
(v) The ingestion of microplastics is documented.
(vi) They have ecological relevance (i.e., key species in maintaining ecosystem functions) and commercial value (Fossi et al. 2018; Bray et al. 2019).

Bivalves fulfill most of such criteria and are abundant in intertidal and coastal locations worldwide. Adults are sessile organisms relatively easy to collect and process in laboratory conditions, having been used as bioindicators to monitor contaminants and marine environmental status for several years (e.g., Mussel Watch Programme): mussels have already been suggested as suitable sentinel species for microplastic pollution (Bessa et al. 2019). Also fish were highlighted as valuable indicators of the occurrence of microplastics in the marine environment, since they exploit almost every kind of habitat, occupy many ecological niches, and are an important food source for human populations worldwide (Sbrana et al. 2020). Other candidate species include benthic sediment-dwelling organisms (including marine worms) that are abundant and are widely used for biomonitoring contaminants in aquatic systems (Bessa et al. 2019). Based on such considerations, Fossi and collaborators (2018) proposed some species for monitoring different habitats (from coastal areas to offshore and from benthic environments to pelagic waters) at different spatial scales in the Mediterranean Sea. Mussels (*Mytilus galloprovincialis*), polychaetes (*Arenicola marina*), and crabs (*Carcinus* spp.) were highlighted as small-scale bioindicators of microplastics along the coastline, while red mullet (*Mullus barbatus*), sole (*Solea* spp.), European hake (*Merluccius merluccius*), and catshark (*Galeus melastomus*) as small-scale sea-bottom indicators being demersal fish living in close connection with sediments and depending on benthic prey for feeding. The bogue *Boops boops* and the pompano *Trachinotus ovatus* can be sentinel species of microplastics in coastal waters, while, for monitoring open waters at small scale, mesopelagic and pelagic fish (the European anchovy *Engraulis encrasicolus* and the European pilchard *Sardina pilchardus*) were suggested. Large pelagic predators *Thunnus alalunga* and *Coryphaena hippurus* were proposed as medium-scale bioindicators of microplastics in open waters, while, for basin-scale studies, large filter feeding whales (e.g., *Balaenoptera physalus*) and sharks (e.g., *Cetorhinus maximus*) were proposed as the most suitable for their migratory behavior.

Despite that the MSFD-TSGML recommends at least 50 individuals per species as a suitable sample size, the majority of published studies differs considerably (Wesch et al. 2016). Sample size could be lowered in those species or population showing high frequency of microplastic ingestion. Even though larger sample size provides more reliable results (Hermsen et al. 2018), as a practical suggestion, the number of individuals analyzed per single species could be reduced in favor of more species to be included in the study (Avio et al. 2020).

Due to the diversity of habitats and species, a large variety of techniques have been used for sampling biota. Organisms can be collected in grasps, traps, creels, or bottom trawling (benthic species), by manta or bongo nets (planktonic and nektonic invertebrates), by hand (e.g., bivalves or crustaceans), or by electrofishing (Stock et al. 2019). In addition to scientific sampling campaign, sources of samples for microplastic monitoring may include sportfishing events, farmed organisms, or animals bought at fish markets. All ethical requirements must be followed, i.e., avoiding protected/endangered species or invasive sampling methods (Bessa et al. 2019). The analysis of dead animals is particularly useful for obtaining data on microplastic ingestion by large marine vertebrates (e.g., seabird, turtles, and cetaceans) without killing individuals for scientific purposes (Hanke et al. 2013; Wesch et al. 2016).

Target tissues for analyses are mainly those of the digestive tracts (the esophagus, the stomach, and the gut) for larger biota, while whole specimens are usually analyzed for smaller species. Depending on the research question, additional tissues can be selected, for example, muscle (fillet) of commercial fish to evaluate the potential exposure to microplastics for humans. After collection, samples (whole organisms or dissected tissues) can be stored at −20 °C until further processing in laboratory: fixatives, like formalin, ethanol, or formaldehyde, have also been used to preserve microplastics. The time between organism collection and their dissection/storage should be as short as possible to avoid gut clearance (Lusher et al. 2017). During sampling/dissection of biota, it is advisable to record the following information: date and time of activity; type of equipment used for sampling; sampling site or location data (GPS coordinates, site name, depth, and environmental conditions); number and name of species collected from the same site; number of individuals for each species; length, weight, and sex of individuals; and additional observations (Bessa et al. 2019).

8.3 Sample Processing for Microplastic Isolation

In following sample collection, microplastics must be isolated from water, sediment, suspended matter, and organic material (Stock et al. 2019). Isolating microplastics in an appropriate manner is fundamental to achieve high extraction efficiencies, preservation of particles, and accurate data generation (Lusher et al. 2020). Sample processing includes three main steps: (1) organic matter digestion, (2) density separation, and (3) filtration. There are several methods to perform each of these steps that vary in complexity, time, and cost of materials, and the more appropriate choice often depends on the typology of samples.

8.3.1 Organic Matter Digestion

This digestion step is crucial for facilitating the extraction of microplastics from biological samples, and it is often applied to sediments and water samples as

pre-treatment before density separation and/or filtration (Frias et al. 2018; Gago et al. 2018). The removal of organic matter must be performed without altering microplastics in terms of number, shape, polymer characteristics, and color (Bessa et al. 2019). Thus, the overall recommendation is the use of a previously tested digestion protocol, and the choice should be driven considering digestion efficiency, polymer resistance, dangerousness of reagents, and costs (Campanale et al. 2020). There are different types of digestion, including acid, alkaline, oxidative, and enzymatic, and all these methods have advantages and limitations (Lusher et al. 2020).

Nitric acid (HNO_3) is widely used in acid treatments: it is very effective in digesting the organic material present in the sample with >98 % weight loss for biological tissue. However, it causes degradation of polystyrene, polyamide, and polyethylene or change of color (polyvinyl chloride), especially if it is used in high concentrations and at high temperatures. Hydrochloric acid (HCl) has also been suggested, but it seems to be inefficient in treating large quantities of biologic material, and it causes alteration of some polymers. Therefore, acid digestion may be avoided or used with caution since it may lead to underestimation of microplastics in environmental samples (Lusher et al. 2020).

Alkaline digestion is an alternative with great potential, but it may also damage or discolor plastics, leave oily residues and bone fragments, or redeposit tissue debris on plastic surfaces, complicating the subsequent characterization (Prata et al. 2019). Nevertheless, digestion with 10 % potassium hydroxide (KOH) at a maximum temperature of 40 °C was suggested as a cost-effective method for processing biota samples (Bessa et al. 2019). It was recommended to not exceed the temperature of 40 °C since higher temperatures could degrade and reduce the recovery rate of some polymers. The limitation of proposed approach is that it is not particularly suitable for tissues with a considerable amount of fat, as the digestion may take several days and can partially damage some polymers. Combining KOH with a detergent (e.g., Tween 20) may accelerate the degradation process (Bessa et al. 2019). Digestion of 10 % KOH at 40 °C was also proposed as pre-treatment for seawater samples with medium and high organic matter content (water with eggs and larvae, plus zoo- and phytoplankton). In following this step, an additional step was recommended using hydrogen peroxide (H_2O_2) if all organic matter was not digested: H_2O_2 (15 % solution) at 1:1 volume sample/solution ratio is added to oxidize and digest the biological material (Gago et al. 2018).

H_2O_2 is indeed a popular oxidizing agent to efficiently remove organic matter with little effect on microplastic integrity if it is used at less than 20 %: polymeric changes have been identified in terms of transparency and shrinking in size when a 30–35 % solution is applied (Prata et al. 2019).

In contrast to the chemical digestion, the use of enzymes (e.g., proteinase K, trypsin, collagenase, papain, and cellulase) does not affect the polymer's structure and has an excellent removal efficiency of the organic fraction, but it takes more time and higher costs, especially for samples that contain a considerable amount of organic matter to digest and thus require a significant quantity of enzymes

(Campanale et al. 2020): in this respect, it is less affordable during extracting microplastics from large tissues or organisms.

8.3.2 Density Separation

Differences in density can be used to separate plastics from inorganic solid materials (e.g., sand, shells and carapace of invertebrates, and frustule of algae). The density separation step is necessary for sediments to discriminate microplastics from other particles, but it can be applied also to biota samples after digestion and to water samples for minimizing the filtration time and facilitating sorting and characterization steps (Bessa et al. 2019; Gago et al. 2018). The process is based on floating properties of microplastics in denser salt solution. In simple terms, a saturated salt solution with a known density can be carefully mixed with the sample containing microplastics and left to settle: plastic particles will float to the surface, and the supernatant is then collected and filtered for further investigation, while nonplastic debris heavier than microplastics are deposited on the bottom (Ribeiro-Claro et al. 2017; Fig. 8.9). The duration of mixing, as well as the floating time, can vary considerably depending on the sample volume and type, ranging from minutes to several hours or days. For example, coarse sediments settle out relatively quickly, while samples with fine particulate matter require a longer period. The recovery of supernatant can be carried out using tubes, volumetric flasks, separating funnels, or specific devices such as the Sediment-Microplastic Isolation unit (Lusher et al. 2020).

A range of salts differing for their density can be used to separate and extract microplastics: salts also vary in cost and toxicity (Bessa et al. 2019). Sodium chloride (NaCl) and sodium tungstate dihydrate (STD) are both cost-effective and nontoxic salts: for these characteristics, NaCl is recommended by the MSFD-TSML, the NOAA, and the BASEMAN consortium for monitoring programs (Lusher et al. 2020). However, these salts are not effective for the density separation of heavier polymers. Sodium iodide (NaI) is a higher-density salt that allows the separation of most polymers, but it is quite expensive. Zinc chloride ($ZnCl_2$), despite being considered the most effective and least expensive method, is a highly dangerous and corrosive substance: consequently, careful handling, disposal, and recycling of this reagent are required. Sodium polytungstate (SPT) and its derivatives are extremely expensive (although these are recyclable), can be hazardous, and, therefore, are not a first choice for routine monitoring. Furthermore, despite that salts like $ZnCl_2$, NaI, and $ZnBr_2$ allow the density separation of heavier polymers, they are highly soluble in water, and therefore, larger quantities are required in respect to NaCl, SPT, or NaBr (Campanale et al. 2020).

Since some polymers may be lost in separation more frequently than others depending on the salt solution applied (Table 8.2), it is necessary to consider this limitation when the density separation step is included in the processing of sample for microplastic isolation. The way to perform the procedure (e.g., time of mixing and settling and the way to recover the supernatant) can also influence extraction

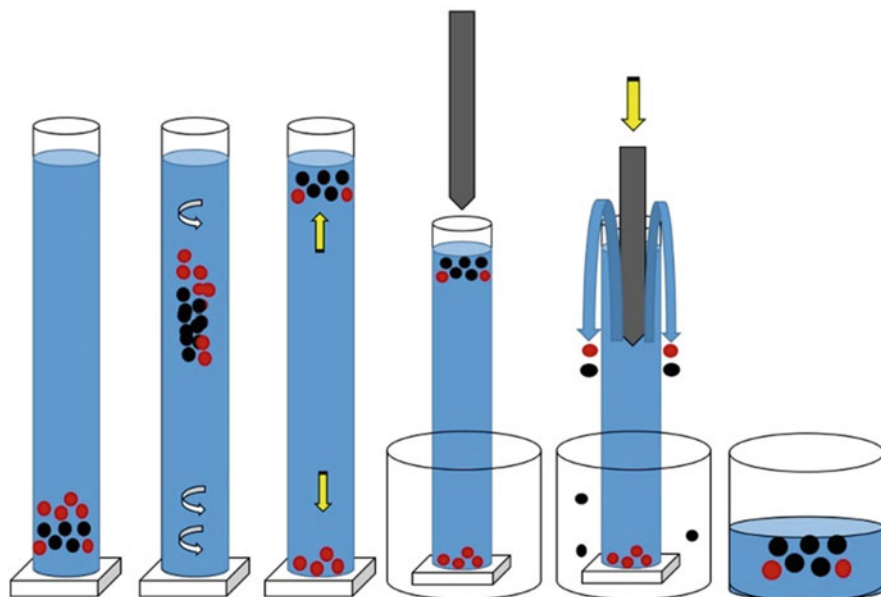


Fig. 8.9 Representation of a density separation for isolating microplastics from digested samples, including mixing, settling, and recovery of supernatant containing microplastics for subsequent filtration. Dark dots, microplastics, and red dots, inorganic or biological material (credit, CG Avio)

results. It is, thus, advisable to evaluate the extraction yield of the density separation method, especially if it was never experienced on the matrix to be processed. A general recommendation is to perform sequential extractions to increase the efficiency of procedure: on average only, the 30.2 % of microplastics were recovered after the first extraction, while reaching between 88.7 % and 100 % following sequential extractions (Lusher et al. 2020).

The effectiveness of a procedure to isolate microplastics from a matrix can be tested using recovery experiments with spiked microplastics (Brander et al. 2020). These tests consist of the addition to the sample of a known number of microplastics, for which also size, shape, color, and polymer are known. The extraction yield is then measured calculating the percentage of spiked microplastics recovered at the end of sample processing, also verifying if changes in physical and chemical characteristics of spiked microplastics have occurred. It is suggested to prepare a heterogeneous mixture of microplastics for these experiments, since the extraction efficiency depends on the method used but also on the characteristics of particles (Löder and Gerdts 2015): commercial microplastics or handmade particles obtained from plastic objects can be used. Quinn et al. (2017) prepared microparticles of eight typologies of plastic polymers and colors from 11 different post-consumer products, cutting them by various physical methods including a coffee grinder, food processor, and liquid nitrogen. These handmade particles were used along with commercial microspheres, to test the effectiveness of NaCl, NaBr, NaI, and ZnBr2 salts in the

Table 8.2 Separation abilities of different density solutions compared to some of common polymers. +, separation; ±, possible separation; and −, not separated (modified from Lusher et al. 2020)

Polymer (abbr.)	Buoyancy of polymers in density solution (g cm^{-3})						
	NaCl (1.0–1.2)	STD (1.4)	NaBr (1.37–1.4)	NaI (1.8)	$ZnCl_2$ (1.6–1.8)	$ZnBr_2$ (1.71)	SPT (2.94–3.1)
PS	+	+	+	+	+	+	+
PP	+	+	+	+	+	+	+
LDPE	+	+	+	+	+	+	+
EVA	+	+	+	+	+	+	+
HDPE	+	+	+	+	+	+	+
PA	+	+	+	+	+	+	+
PA 66	+	+	+	+	+	+	+
PMMA	+	+	+	+	+	+	+
PC	+−	+	+	+	+	+	+
PU	+−	+	+	+	+	+	+
PET	−	+−	+	+	+	+	+
PVC	−	+−	+−	+	+	+	+
PTFE	−	−	−	−	−	−	+

density separation of microplastics from sediments. Miller et al. (2021) produced, instead, high-density polyethylene microparticles of irregular shapes and of various sizes from yellow lids sourced from single-use sterile containers milled in a blender: recovery rates and effects on particles were evaluated after testing four isolation methods from seawater samples. A recovery experiment was performed by Avio et al. (2017) to compare the efficiency of six methods for the extraction of microplastics from fish tissues, including five already published and a new one developed by authors. Four different size classes of polyethylene and polystyrene particles, obtained by sieving a commercial stock powder, were spiked in the gastrointestinal tracts dissected from mullets. The new protocol was validated, showing an extraction yield of 95 % with a density separation step carried out twice, and applied to extract microplastics from fish exposed under laboratory conditions (Avio et al. 2017).

8.3.3 Filtration

Direct filtration of seawater with low organic matter content (clear water sample), or filtration of supernatants obtained by density separation, is the most frequent method to isolate microplastics from environmental samples. Among different filtering systems used, the vacuum filtration is by far the most common (Lusher et al. 2020; Fig. 8.10a). Types of filters include polytetrafluoroethylene, polycarbonate, nylon, glass fiber, cellulose (nitrocellulose, cellulose acetate, or mixed cellulose), stainless steel, silicon, and Anodisc filters (aluminum oxide). The type of filter

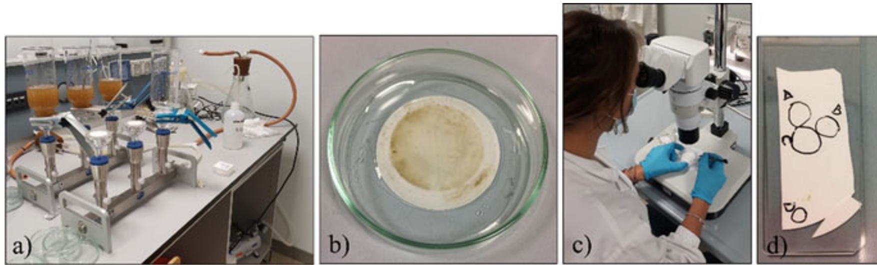


Fig. 8.10 (**a**) Example of a vacuum filtration apparatus used for isolate microplastics from pre-treated biota samples, (**b**) example of a cellulose filter resulting from the filtration step, (**c**) visual examination of the filter under a stereomicroscope and sorting of particles using tweezers, and (**d**) example of a clean support, on which microparticles are transferred for a single-point analysis for chemical identification by micro Fourier Transform Infrared (μ-FTIR) spectroscopy (photo credit, L Pittura)

should be chosen based on availability, porosity, and structure and on suitability for analytical techniques used for the subsequent characterization of retained particles (Martellone et al. 2021).

Pore size will have a significant effect on the overall number of particles collected as they determine the lower size of microplastics detected ranging from 0.3 μm to 200 μm. Larger pore size facilitates rapid filtration but will result in the loss of smaller plastics that are those preferentially ingested by marine organisms (Avio et al. 2020), while small pore or mesh size may result in quick obstruction by organic and mineral matter in the absence of adequate pre-treatment of sample (Prata et al. 2019). Structure of filter will have, instead, a direct effect on the dominant shape of retained microplastics. For example, nylon and cellulose filters can retain more fibers than polycarbonate filters. In fact, nylon and cellulose filters have deep and curvy pore canals organized in a lattice and in a multilayer, respectively, in which fibers are more likely to get stuck; pores on polycarbonate filters are, instead, circular; and canals are shallow and straight allowing fibers to go through more easily (Cai et al. 2020). A standardized pore size, as well as type of filters, should be defined to allow comparison between different studies.

Once filtration of samples is completed, filters are preserved in cleaned glass petri dishes, and remaining solutions can be removed by an oven or a drier at room temperature (Cutroneo et al. 2020). Dried filters are subsequently visually examined through microscopy techniques for the physical characterization of retained particles, which must be followed by the chemical identification of polymers. If the chemical characterization cannot be carried out directly on filters through an automated analysis with spectroscopy techniques (i.e., micro-FTIR and micro-Raman), a manual sorting is previously necessary, consisting of isolation of single particles using tweezers and their transfer onto a clean support (Fig. 8.10b–d).

Polytetrafluoroethylene filters have proved to be a better choice compared to aluminum oxide, glass fiber, and polycarbonate filters, when physical characterization is performed through scanning electron microscopy coupled with energy dispersion spectrometry (SEM-EDX), because they do not pose any interference to the

analysis (Pivokonsky et al. 2018). Glass fiber, cellulose, and stainless-steel filters are instead the most used for filtration and subsequent examination of particles under optical microscopy; however, glass fiber filters, in particular, are not suitable for spectroscopic analysis because they absorb in the infrared area (Martellone et al. 2021).

Aluminum oxide filters are widely used for spectroscopic techniques: they are suitable for analyses by Infrared (IR) spectroscopy in transmittance mode but not in reflectance being transparent to infrared light; they can also be successfully used for Raman spectroscopy. However, Anodisc filters have a self-absorption in the mid-infrared fingerprint range (1400–600 cm^{-1}), hampering a distinct identification of potential microplastic particles and an accurate classification of the polymer type. In addition, aluminum oxide filters are also among the most fragile, because they are thin although they are being rigid.

Silicon filters can represent an alternative, since they guarantee sufficient transparency for the broad mid-infrared region of 4000–600 cm^{-1} and offer good mechanical stability during analysis of microplastic samples using both transmission FTIR microscopy and FTIR imaging as well as Raman microscopy (Käppler et al. 2015). Gold-coated polycarbonate filters are also used for analysis of particles using micro-FTIR spectroscopy in reflectance mode (Martellone et al. 2021).

8.3.4 Quality Assurance and Quality Control (QA/QC) of Analysis

Contamination issues are a challenge in quality assurance and quality control during microplastic studies: the risk of external contamination is extremely important, particularly for microfibers (Prata et al. 2021). QA includes a series of systematic steps or activities to ensure that generated data are accurate and reliable. QC is the process of verifying and checking all data, results, and reported methods to ensure their validity and prevent erroneous conclusions (Brander et al. 2020). QA/QC practices should be considered in advance and carefully followed throughout the whole study process, including sampling and collection, extraction, and analysis.

Regardless of the environmental matrix to collect, the use of plastic devices and materials for sampling and store samples should be eliminated, replacing plastics with glass or metal. If plastic materials cannot be avoided, as the case of nylon nets for seawater or biota sampling, they must be characterized and compared with microplastics extracted from samples: if they match, they must be removed from results. Similarly, certain sampling characteristics intrinsically contain multiple potential sources of microplastics that sometimes cannot be removed. For example, sampling activities carried out on a vessel might expose to potential external contamination derived from hull paints, life vests, ropes, and sails, all materials that should be characterized (Brander et al. 2020). Controls for air contamination should also be performed: leaving a wet filter paper or an open container with filtered water during sample collection is possible to register the deposition of microfibers or microplastics from the surrounding environment.

Another important measure is to control the release of fibers from clothes. Providing protection to operators, including adequate cotton lab coats, can prevent the release of synthetic textile fibers from clothes to some extent. Nonetheless, natural textiles can also release fibers, while cellulosic fibers, such as cotton, can be abundant in indoor air. In this respect, the use of coats, gloves, and paper towels of recognizable colors may help to identify accidental contaminations, thus improving control and prevention procedures (Prata et al. 2021): this recommendation should be applied also during the processing of samples in laboratory.

The processing of samples in laboratory is suggested to be carried out under fume hoods or laminar flow hood. However, fume hoods are poorly efficient in controlling for air contamination since unfiltered air from the laboratory is drawn inside the hood and then expelled outside or filtered. On the other hand, laminar flow hoods draw air in through HEPA filters and create a laminar flow toward the front, preventing the entrance of uncontrolled air: the use of laminar flow hoods is thus preferred. Alternatives include the use of rooms with controlled air flow and access; minimal personnel circulation is always recommended. Metal or glass materials and aluminum foils are also required for all laboratory activities, as plastic objects can release particles and should be fully avoided. All materials must be cleaned with pure water or ethanol, and solutions (especially salt solutions) should always be filtered (0.22- or 0.45-μm pore size) to avoid external contamination with microplastics. During laboratory preparative steps, air deposition controls and running procedural blanks allow to check the background contamination along with the influence of sample processing. Procedural blanks typically include pure reagents (or water) treated with the same procedures as samples.

Results on analysis of both air deposition controls and blank samples should be reported and considered for the final quantification of microplastics in analyzed samples.

8.3.5 Operative Protocol for Isolation of Microplastics from the Gastrointestinal Tract of Marine Species

This paragraph provides a step-by-step description of an operative protocol, including necessary materials, reagents, and equipment, that students can easily perform to extract microplastics from tissues of marine organisms. The protocol is based on methods tested, validated, and directly experienced on a range of marine species by authors of the present chapter and intercalibrated in a joint exercise within several partners of three JPI Oceans projects (EPHEMARE, BASEMAN, and RESPONSE; Vital et al. 2021; Avio et al. 2015, 2017, 2020; Cau et al. 2019, 2020; Bour et al. 2018; Bessa et al. 2019).

The gastrointestinal tract of the red mullet will be the target of the procedure as a practical example, for assessing the ingestion of microplastics by marine biota. However, the same protocol and recommendations can be applied to other species and tissues and to the whole specimens for smaller species (e.g., mussels).

Materials

- Forceps
- Metal tweezer
- Metal spatula
- Glass petri dishes
- Glass flask (250 ml)
- Glass beakers (5 L, 1 L, and 250 ml)
- Glass cylinders (100 ml and 250 ml)
- Glass tube
- Mortar and pestle
- Nitrate cellulose filters (8-μm pore size)
- Acetate cellulose membrane (0.45-μm pore size)
- Magnetic stirrer plate and cylindrical stirrer
- Graduated cylinders (100 ml and 250 ml)
- Stirrer plate and cylindrical stirrer

Reagents

- 10 % KOH solution
- 15 % H2O2 solution
- Saturated solution of NaCl salt (1.2 g/cm3)
- Ultrapure/distilled water

Equipment

- Oven (work temperature 40–50 °C).
- Hood.
- Vacuum filtration system schematically represents in Fig. 8.11 the following:
  - 1 = filtration ramp (Speedflow)
  - 2 = glass filter holder (diam 47 mm) + max volume 500 ml
  - 3 = vacuum tube HW/55 diam mm 8 × 15
  - 4 = non-return PP valve
  - 5 = vacuum trap 2 L
  - 6 = vacuum tube HW/55 diam mm 8 × 15
  - 7 = second vacuum trap 2 L (to protect the pump)
  - 8 = additional filter to protect the pump (optional)
  - 9 = vacuum generator RCK400 34 L/min

Preparation of Solution

- 10 % KOH solution. Dissolve 10 g of KOH for each 100 ml of ultrapure/distilled water.
- 15 % H2O2 solution. Dilute 30 % of hydrogen peroxide in ultrapure/distilled water (1:1, volume/volume).
- Saturated solution of NaCl salt (1.2 g/cm3). Dissolve 10 g of NaCl in 100 ml of ultrapure/distilled water reaching a density of 1.2 g/cm3. To check the density, it is possible to weight 1 ml of solution: if 1 ml of solution weights at least 1.2 g, the

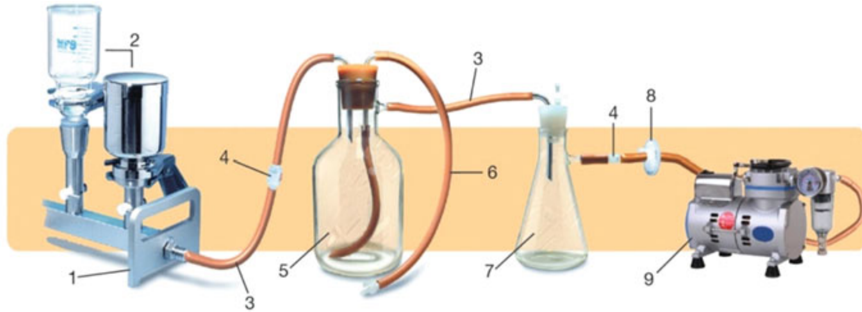


Fig. 8.11 Schematic representation of vacuum filtration apparatus

right density of 1.2 g/cm3 has been obtained. If NaCl salt of pure grade is not available, the commercial cooking salt can be used.

Sample Dissection

Organisms are dissected to obtain the gastrointestinal tract: open the fish from the anus to the mouth using forceps and isolate the gastrointestinal tract including the esophagus, the stomach, and the intestine, with the help of forceps and tweezer (Fig. 8.12). Register the main morphological parameters of dissected specimens: total body weight, total length (from the mouth to the caudal fin), and weight of the gastrointestinal tract.

Digestion of the Gastrointestinal Tract

Put the gastrointestinal tract in a 250-ml glass flask or in a glass beaker and add 10 % KOH solution until the sample is covered or according to the minimum ratio of 5:1, volume/gastrointestinal tract weight. Leave the sample in oven at a maximum temperature of 50 °C until the digestion process is completed (Fig. 8.13).

Density Separation of Digested Sample

Put the digested sample in a graduated glass cylinder (100 ml or 250 ml depending on the volume of digested sample) and add the saturated NaCl salt solution and a cylindrical stirrer. Leave to mix the solution on a magnetic stirrer plate for 10 min and leave to settle for additional 10 min. After this step, a first aliquot of supernatant is collected from the glass tube into a graduated glass beaker (1 L or 5 L depending on processed volumes). The density separation step is carried out twice for a better extraction performance: rinse and refill the cylinder with saturated NaCl salt solution, before repeating the mixing, settling, and recovery of supernatant. All materials used for the collection of supernatant need to be washed with ultrapure/distilled water to collect particles potentially attached to the wall of glass tube and cylinder. The supernatant, obtained from sequential extractions, is ready to be filtered (Fig. 8.14).

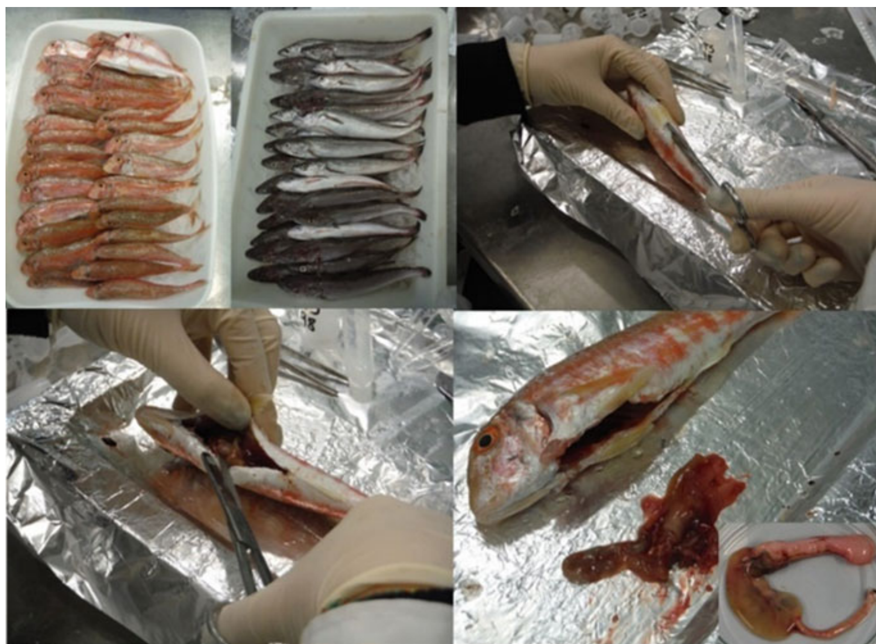


Fig. 8.12 Red mullets and European hakes and dissection of the gastrointestinal tract from the red mullet

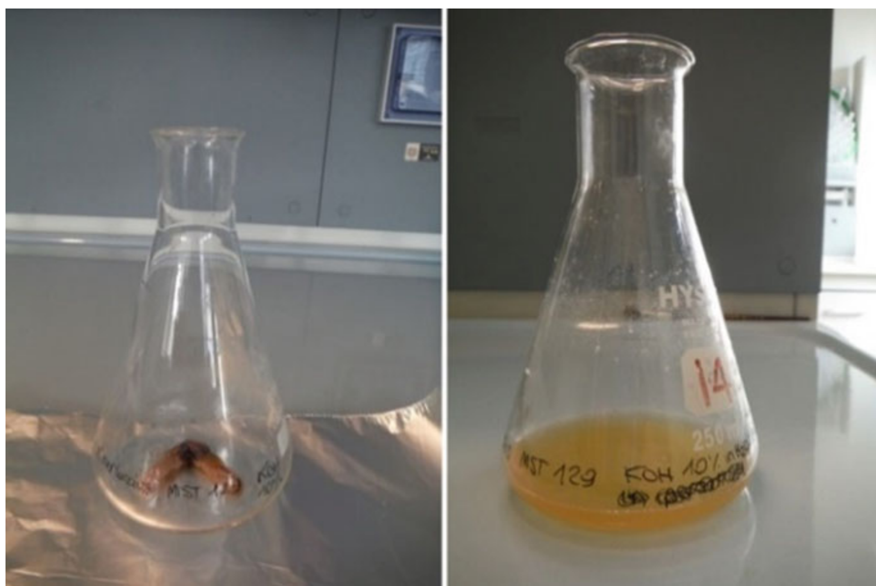


Fig. 8.13 Digestion of the gastrointestinal tract of the red mullet using 10 % KOH solution

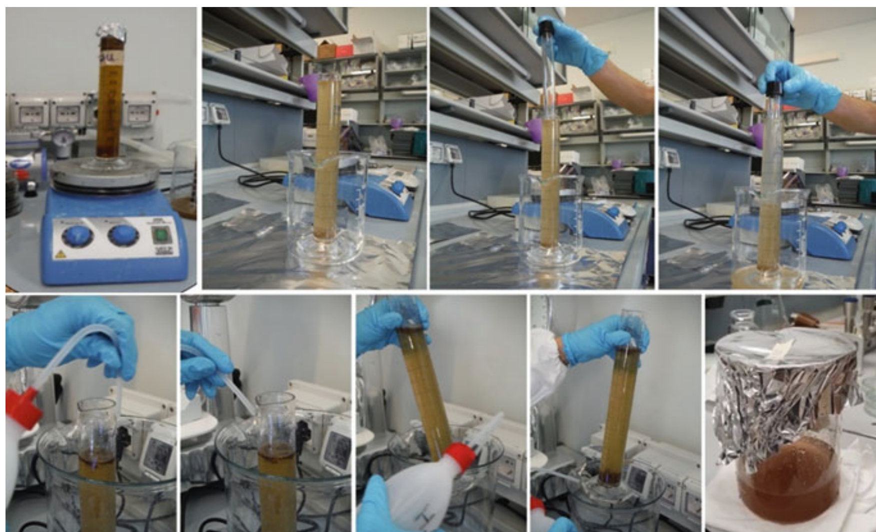


Fig. 8.14 Density separation procedure and recovery of supernatant after the digestion of the gastrointestinal tract of red mullet

Processing of Fatty Tissues

When the tissues to be analyzed are particularly fatty, the digestion with KOH is not suitable, because formation of oil droplets in the solution hampers the density separation and the filtration steps. In these conditions, it is suggested to dry the tissues, instead of digesting, before the density separation and subsequent filtration. The procedure consists of placing the sample in a petri dish and leaving it to dry in oven at a maximum temperature of 50 °C. Once it is dried, sample is gently triturated using a mortar to obtain a powder (Fig. 8.15). The powder is then put in a 100-ml glass cylinder to carry out the density separation according to procedure described above (Figs. 8.14 and 8.15).

Filtration of Supernatant

Filter under vacuum the supernatant through a nitrate cellulose. The pore size of filter should be chosen according to the detection limit of the analytical method used for the subsequent chemical identification of microplastics: 8-μm pore size is suitable for the μ-FTIR spectroscopy. Filtration can be speeded up mixing the supernatant with a metal spatula during procedure.

At the end of filtration, recover the filter and put it in a petri dish adding 15 % H_2O_2 solution, useful to digest the organic material eventually remained as residue, irrespective of digestion and density separation steps. Petri dish is left in oven at a maximum temperature of 50 °C until the end of digestion and the drying of filter. To avoid the formation of salt crystals on dried filter, ultrapure/distilled water can be used to rinse the filter before its recovery at the end of filtration (Fig. 8.16).

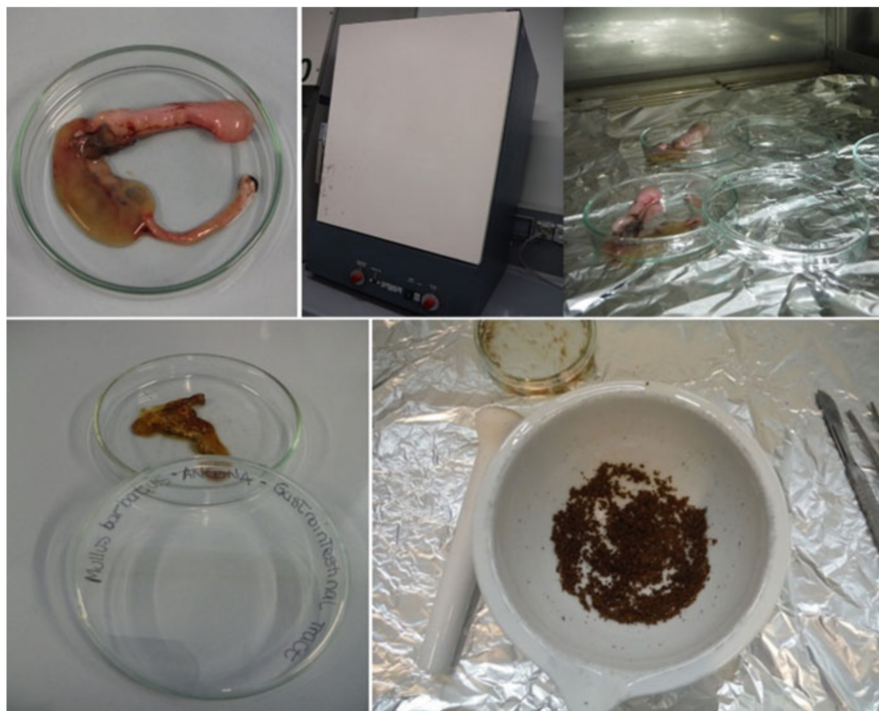


Fig. 8.15 Drying of gastrointestinal tract and trituration before density separation and filtration as an alternative procedure to KOH digestion method for processing fatty tissues

Dried filters are observed under a stereomicroscope for the manual isolation of all particles resembling microplastics and fibers (Fig. 8.17a–e), which are classified based on shape and color, and then measured using an image analysis software for categorization in size classes (Fig. 8.17 and Table 8.3). The chemical identification will be performed later on single particles and fibers, for example, using μ-FTIR or μ-Raman spectrometry (Fig. 8.20a).

QA/QC Procedures

To reduce and monitor the potential contamination of sample by external microplastics and microfibers during all phases' protocol, be sure to follow recommendations as follows:

- Perform dissection and various processing steps in a dedicated room with closed windows and restricted access.
- Clean all laboratory surfaces and materials used for processing samples with ultrapure water and ethanol.
- Dress only cotton wear. It is also recommended to register colors of clothes worn underneath the lab coats.

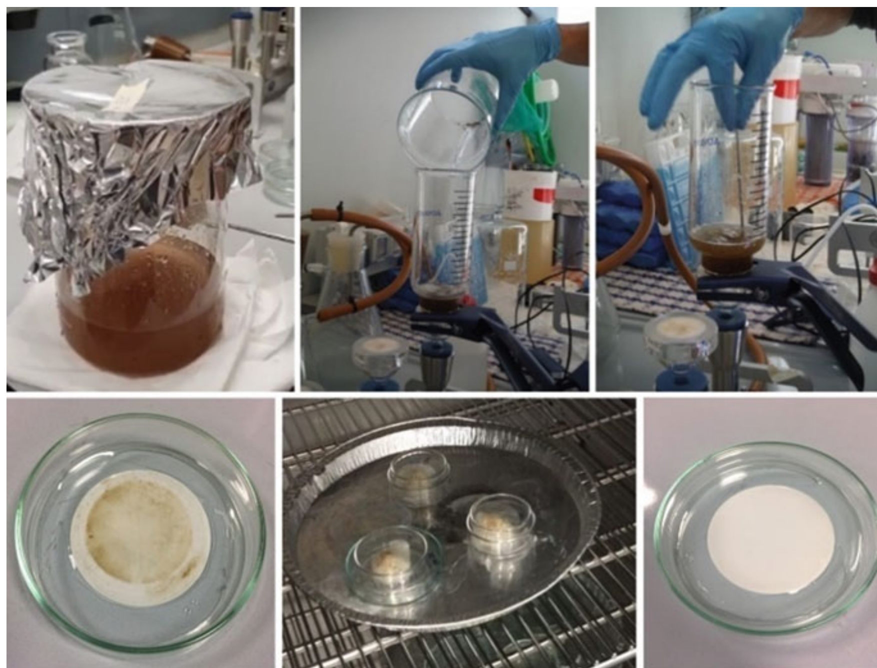


Fig. 8.16 Filtration of supernatant after the density separation step and digestion of recovered filters with 15 % H_2O_2 in oven to eliminate residual organic material

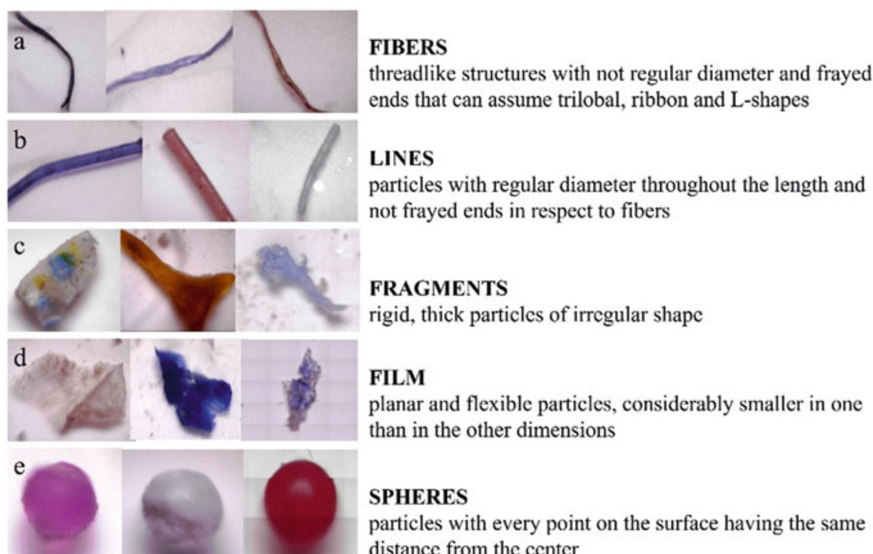


Fig. 8.17 Example of visual guidelines for the identification of particles based on shape

Table 8.3 Example of datasheet showing classification of each particle extracted per sample into the main categories of physical characteristics

Sample code	Particle ID	*Shape*					*Color*					*Size classes* (mm)			
		Fiber	Line	Fragment	Film	Sphere	Black	Blue	Red	Transparent	Others	5–1	1–0.5	0.5–0.1	0.1–0.01
S1	S1.1	x					x					x			
	S1.2			x			x					x			
	S1.3				x		x					x			

- Cover the top of glass containers with aluminum foil, especially during the filtration step.
- Prefilter working solution before their use with 0.45-μm pore size filter.
- Include a blank sample starting from dissection: hold an open beaker with distilled water on the workbench while dissecting and process it according to the same procedures applied to samples.

8.4 Analytical Techniques for Microplastic Characterization

After extraction and isolation, particles need to be accurately characterized in terms of number, size, shape, surface texture, color, and chemical typology of polymers. This information is relevant to trace microplastic sources, origins, weathering, and residence time in the field, as well as to highlight those typologies more available for biota. Chemical analysis can also allow to identify additives and associated contaminants or impurities on microplastic surface, like organic and inorganic material. Various techniques can be used in sequence or in association with their own advantages and limitations, often dependent on dimensions of microplastics to be detected: the actual challenge is to implement existing tools or develop new approaches to overcome the characterization of smallest microplastics and nanoplastics.

8.4.1 Physical Characterization

8.4.1.1 Microscopy Techniques

Physical characterization through microscopy techniques is primarily used to identify and classify microplastics preserved on a filter or in petri dishes or jars (Cutroneo et al. 2020).

Optical microscopy (OM) is suitable to visually examine particles of submillimeter size retaining the 3D shape and color of suspected microplastics. OM allows to distinguish between plastics and other organic/inorganic compounds by analyzing detailed surface textures and structural information (Jung et al. 2021). Visual guidelines can help the operator in the identification of suspected microplastics, including bright and unnaturally colored particles, fragments with sharp geometrical shapes, shiny surfaces, and featureless fibers with a consistent width (Fig. 8.17). Physical and tactile guidelines include the particle holding its shape or stretched when poked and resistance to easy breakage (Primpke et al. 2020). Once particles were identified, they are measured using an image analysis software and categorized by shape, color, and size classes (Table 8.3). The main advantage of light microscopy in microplastic characterization is that it is a relatively cheap and easy approach. However, visual sorting under a stereomicroscope can be difficult for microplastics with no specific color, and it requires considerable time and resources in terms of researchers involved in counting hundreds of particles (Campanale et al. 2020). Since this technique does not provide information on the chemical

composition of objects, further characterization is necessary to confirm the plastic nature of particles.

Scanning electron microscopy (SEM) can also be used, allowing to visualize nanometer-sized particles. Discrimination of surface structures of plastics and other materials can be integrated with an energy-dispersive X-ray probe (SEM-EDX) to provide further information on the elemental composition of organic and inorganic species, particularly useful for environmental samples. However, SEM-EDX is expensive and requires substantial time and effort for sample preparation and examination, which limits the number of samples that can be handled in routinary analyses (Jung et al. 2021).

8.4.1.2 Light-Scattering Technique

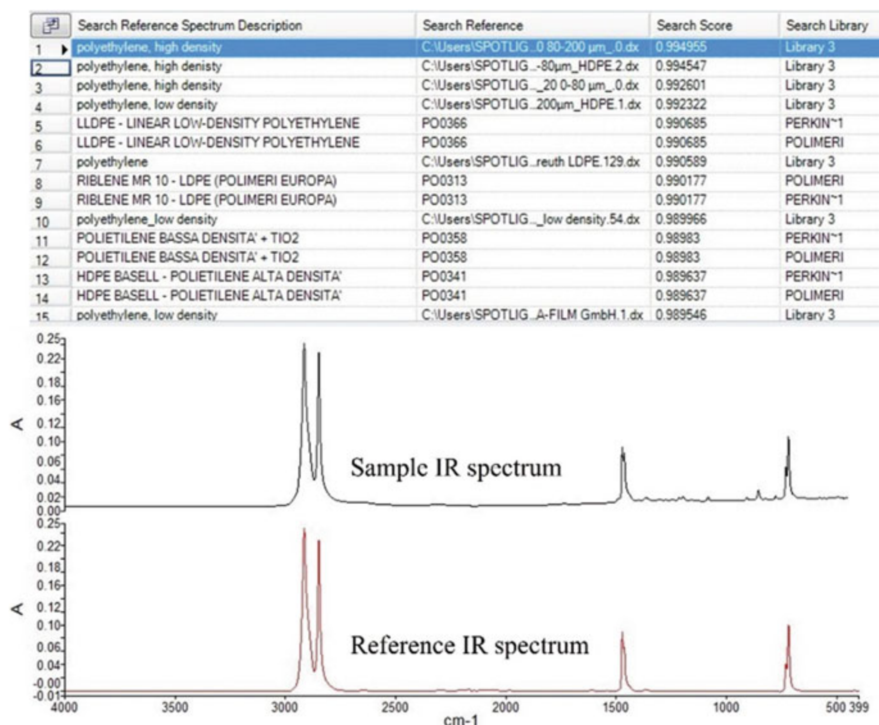
Multiple methods apply the scattering of laser light on particles to obtain information on physical properties like particle size and particle-size distribution. Dynamic light scattering (DLS), the most widely used, measures particle sizes in the range from 1 nm to 3 mm based on the fluctuation of intensity of a laser beam that passes the suspension. These particle size analyzers calculate total particle-size distribution without distinguishing microplastics and other particles. Therefore, microplastic particle size and distribution can be measured using light diffraction and/or dynamic light scattering only when these particles have been previously isolated and represent the only present in the solution matrix: in this respect, a rigid sample pre-treatment is fundamental to completely remove all other organic/inorganic particles. DLS may provide different results from those obtained by visual inspection and might not detect small-size particles that are masked by the effect of larger particles on strongly scattered light (Lee and Chae 2021).

Nanoparticle tracking analysis (NTA) can represent another approach on characterization of environmental matrices. NTA gives information on size profile, recording scattered laser light with a microscope and a digital camera. NTA visualizes nanoparticles and particle concentration in the solution and derives size of particles correlating their hydrodynamic diameter due to its Brownian motion (Schwaferts et al. 2019). The size distribution obtained with NTA may be less sensitive to the presence of large particles and aggregates than DLS. NTA can detect particles up to 30 nm but not particles larger than 2 μm: analysis using NTA is more time-consuming (up to 1 h) than DLS (several minutes; Lee and Chae 2021).

8.4.2 Chemical Characterization

8.4.2.1 Spectroscopy Methods: FTIR and Raman

The spectroscopic methods, including Fourier-transform infrared (FTIR) spectroscopy and Raman spectroscopy, are the most common approaches in the chemical identification of microplastics, being also recommended by the MSFD-TSGML. These methods are based on the energy absorption by characteristic functional groups of polymer particles, resulting in a vibrational spectrum which is unique for every polymer type. The chemical identification of particles is obtained by



	Search Reference Spectrum Description	Search Reference	Search Score	Search Library
1	polyethylene, high density	C:\Users\SPOTLIG...0 80-200 µm_0.dx	0.994955	Library 3
2	polyethylene, high denisty	C:\Users\SPOTLIG...-80µm_HDPE.2.dx	0.994547	Library 3
3	polyethylene, high density	C:\Users\SPOTLIG..._20 0-80 µm_0.dx	0.992601	Library 3
4	polyethylene, low density	C:\Users\SPOTLIG...200µm_HDPE.1.dx	0.992322	Library 3
5	LLDPE - LINEAR LOW-DENSITY POLYETHYLENE	PO0366	0.990685	PERKIN~1
6	LLDPE - LINEAR LOW-DENSITY POLYETHYLENE	PO0366	0.990685	POLIMERI
7	polyethylene	C:\Users\SPOTLIG...reuth LDPE.129.dx	0.990589	Library 3
8	RIBLENE MR 10 - LDPE (POLIMERI EUROPA)	PO0313	0.990177	POLIMERI
9	RIBLENE MR 10 - LDPE (POLIMERI EUROPA)	PO0313	0.990177	PERKIN~1
10	polyethylene_low density	C:\Users\SPOTLIG..._low density.54.dx	0.989966	Library 3
11	POLIETILENE BASSA DENSITA' + TIO2	PO0358	0.98983	PERKIN~1
12	POLIETILENE BASSA DENSITA' + TIO2	PO0358	0.98983	POLIMERI
13	HDPE BASELL - POLIETILENE ALTA DENSITA'	PO0341	0.989637	PERKIN~1
14	HDPE BASELL - POLIETILENE ALTA DENSITA'	PO0341	0.989637	POLIMERI
15	polyethylene, low density	C:\Users\SPOTLIG...A-FILM GmbH.1.dx	0.989546	Library 3

Fig. 8.18 Result of acquisition of an infrared spectrum of a microparticle extracted from sample (sample IR spectrum) and of matching with a reference polymer in a database (reference IR spectrum): the match factor threshold of 0.99 (search score) validates the polymer identification as polyethylene (photo credit, L. Pittura)

comparing the spectrum of the investigated sample with spectra of known polymers by matching them to spectral libraries through database comparison algorithms (Fig. 8.18). In coupling the spectrometer (FTIR or Raman) to a microscope, small microplastics are measurable through the "micro"-spectroscopy (μ-FTIR and μ-Raman): μ-Raman spectroscopy can characterize microplastic samples higher than 1 μm, while μ-FTIR spectroscopy could identify microparticles higher than 10–20 μm (Silva et al. 2018). These techniques are nondestructive and can be coupled with other methodologies to obtain additional and complementary information on the composition of plastic polymers (Campanale et al. 2020). Atomic force microscopy (AFM) combined with either FTIR or Raman spectroscopy is a potential candidate for nanoplastic analysis: AFM probes can be operated in both contact and noncontact modes with objects providing images at nanometer resolutions, while FTIR or Raman spectroscopy determines the chemical composition of the object (Shim et al. 2017).

Transmission, reflectance, and attenuated total reflectance (ATR) are acquisition modes available in FTIR analysis (Fig. 8.19). In transmission mode, the FTIR

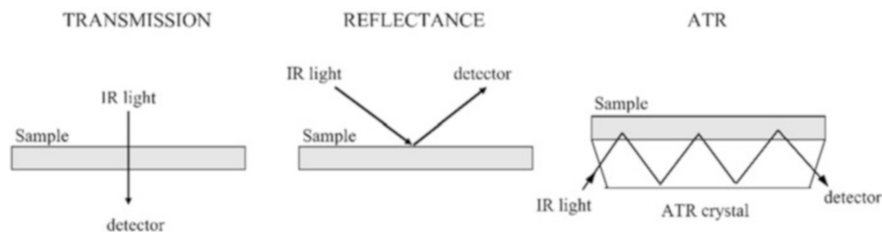


Fig. 8.19 Acquisition modes of FTIR analysis

spectrometer records the IR light that passes through the sample. Working in the transmission mode makes the characterization of microplastics difficult for two main reasons: (i) if the particle is too thick, the IR beam does not pass making the characterization impossible; (ii) if the particle is not clean, there is disturbance, and the IR spectrum is difficult to interpret. In addition, the transmittance mode needs IR transparent filters (e.g., aluminum oxide), and it is limited, owing to total absorption patterns, by a certain thickness of microplastic samples. The reflectance mode records the IR signal that is reflected from the sample: the disadvantage is that measurements of irregularly shaped microplastics may result in non-interpretable spectra due to refractive error. The use of micro-ATR accessory in combination with microscopy can prevent these problems since IR spectra are collected at the surface of a particle (Löder and Gerdts 2015): the sample is in contact with a crystal of high refractive index, and the IR light passes throughout the crystal hitting the sample several times and finally reaches the detector obtaining the IR spectrum. The pressure produced by the ATR probe may, however, damage highly weathered or fragile microplastics, and tiny plastic particles can be pulled from the filter paper by adhesion to or electrostatic interaction with the probe tip. On the other side, an ATR probe made of germanium can be easily damaged by contact analysis with hard and sharp inorganic particles like those possibly remained on a filter paper from a sandy sample (Shim et al. 2017).

Instruments available on the market differ mainly by the type of microscope coupled to the spectrometer and the mode of particle acquisition, being manual or automated. A manual sample placement means that there is a single-point acquisition and particles must be positioned singularly. More expensive instruments have the possibility of fully automated measurements of multiple particles in a sample and to map or generate spatial chemical images of whole-membrane filters through the motorized movement of the sample table of the microscope (Fig. 8.20a–b). Micro-Raman imaging theoretically allows for the spectral analysis of whole-membrane filters at a spatial resolution below 1 μm. Focal plane array (FPA)-based FTIR imaging allows for detailed and unbiased high-throughput analysis of total microplastics on a sample filter. This technique enables the simultaneous recording of several thousand spectra within an area with a single measurement and thus the generation of chemical images (Löder and Gerdts 2015). The main disadvantages of these automated methods are extended processing time to map an entire filter (9 h to

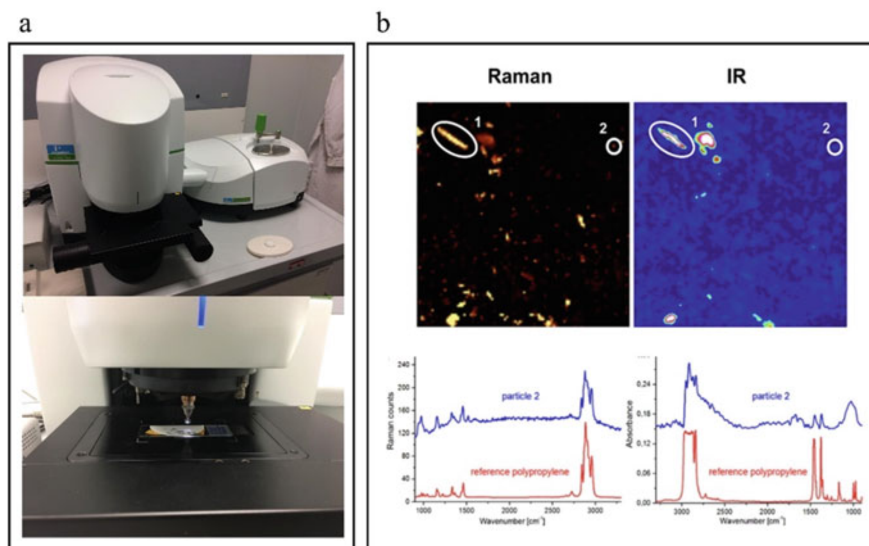


Fig. 8.20 (**a**) Single-point analysis of particles through μATR-FTIR spectroscopy (photo credit, L. Pittura) and (**b**) Raman and IR chemical images with false coloring denoting the spectral intensity of particles and spectra identification in comparison with a reference (modified from Araujo et al. 2018)

scan one filter paper; Shim et al. 2017), refractive errors during measurement of irregularly shaped microplastic particles, lack of information on associated organic additives to MPPs (microplastics), and overlap of polymer bands given by organic and inorganic contaminations that can disturb identification of particles (Campanale et al. 2020).

8.4.2.2 Thermoanalytical Methods: Py-GC-MS

Pyrolytic gas chromatography in combination with mass spectrometry (Py-GC-MS) can be used to assess the chemical composition of potential microplastic particles by analyzing their thermal degradation products. In following a pyrolytic process, decomposition products characteristic of each polymer are trapped on a solid-phase adsorbent and thermally desorbed. Volatile compounds are then separated by gas chromatography and identified by mass spectrometry (Campanale et al. 2020).

The pyrolysis of plastic polymers results in characteristic pyrograms, which facilitate the polymer identification by comparing combustion products with reference pyrograms of known virgin-polymer samples (Löder and Gerdts 2015). Contrarily to Raman or FTIR technique, which only investigates the surface of a particle, Py-GC-MS allows the analysis of the whole particle, enabling to simultaneously identify polymer types and associated organic plastic additives. Although Py-GC-MS has the advantage that individual sorting of particles is not needed, the limit is the amount of sample (e.g., 0.35–7 mg) that can be analyzed (Shim et al. 2017). This

quantity may compromise the representativeness of the sample composition when complex environmental samples are analyzed, as it may not be homogenous on a small scale. Variants of this technique have been used to develop new methods, such as thermo-extraction and desorption coupled with GC-MS which combines thermogravimetric analysis (TGA) and thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS; Silva et al. 2018). Thermal analysis provides an alternative method to spectroscopy for chemical identification of polymer types but, as a destructive method, prevents the possibility of additional characterization of particles.

8.5 Data Expression

The expression and normalization of obtained results should be harmonized for comparison among studies.

For water samples, data are usually provided as the number of microplastics (MPPs) per unit of volume (MPPs L^{-1} or MPPs m^{-3}) or per unit area (MPPs m^{-2}; Gago et al. 2018).

For both intertidal and subtidal sediments, it is recommended reporting the number of microplastics (MPPs) per unit area (MPPs m^{-2}) and per unit dry mass (MPPs kg^{-1} dry). In addition, since samples collected are a function of the length, breadth, and thickness of the collected area, also the number of particles per cubic cm or m should be provided (MPPs cm^{-3}/m^{-3}; Uddin et al. 2020; Frias et al. 2018).

Data on ingestion of microplastics by biota should be presented containing, at least, the following information for each investigated species:

(a) The frequency of ingestion given as the percentage of specimens containing one or more particles on the total of analyzed specimens.
(b) The average number of particles calculated on organisms positive to ingestion (reporting only the average number of particles per individual might be misleading if it is not specified whether organisms without particles [i.e., 0 values] were included in the average).
(c) The number of analyzed specimens.

Frequency of ingestion reflects the probability for organisms to interact with microplastics in their own habitat, appearing to be a more appropriate index than the number of ingested items to monitor microplastics in natural population and to better highlight differences among sampling areas and species (Avio et al. 2020). Expression of data as number of particles per weight of tissues is not recommended since microplastics are not homogeneously distributed among and within tissues and because tissues are often subjected to marked weight variations (Bessa et al. 2019).

In most cases, microplastic numbers are not sufficient to make a mass determination. However, if a mass determination is possible, mass of MPPs is also provided: for water and sediment, it is normalized on abovementioned units; for biota, the mass of MPPs is usually given per unit mass of tissue (MP g^{-1} tissue; Uddin et al. 2020).

As previously mentioned, it would be appropriate to represent separately quantification data on microplastic particles (MPPs) and those of textile microfibers (MFs): for the latter, data on natural microfibers should also be provided using the same reporting units for MPPs.

In addition to data on total quantification, it is important to provide a detailed representation of all physical and chemical characteristics of particles extracted from environmental samples. Each typology of size classes, shape, color, and polymer can be easily provided as relative contribution to the total number of microplastics extracted from sample. Presentation of data on extracted microplastics as percentage distribution into size classes is particularly important, as this improves the understanding of the size distribution of microplastics in the marine environment and highlights dimensions more available for biota. Regarding the shape, inter-study comparison is often hampered by the absence of standardized definitions and categories for microplastic characteristics; in this respect, it is suggested to always provide the applied definition of shapes and to provide a corresponding photo of extracted microplastics. Results of studies reporting microplastics without a chemical characterization should not be considered reliable.

It is important to stress that the adequate presentation of obtained results is of key importance to trace origin, distribution and fate of microplastics, as well as their biological impact and risk for the marine environment.

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