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Technical Guide

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Do's and don'ts of microplastic research: a comprehensive guide

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Abstract

Microplastics are environmental contaminants consisting of small plastics ≤ 5 mm. Concerns over the adverse effects of microplastics have led to a rapid growth in the available literature despite the lack of harmonized methods and materials. Therefore, the field is becoming increasingly daunting to new researchers. A state-of-the-art guide was assembled following a comprehensive literature review of microplastics research with the intent of addressing contemporary challenges, prioritized based on a survey, and introducing best practices. The lack of standardized methods and reference materials, the lack of access to analytical equipment, and the difficulty in working with lower environmental concentrations in laboratory tests (e.g., toxicity assays) remain a great challenge. The present work addresses these issues across three main sections: definitions, sampling, and evaluation of adverse effects. Harmonized methods and greater collaboration were identified as opportunities in this rapidly evolving field. A review of available interlaboratory comparison tests was also conducted to support additional recommendations.

Keywords: Plastic pollution, environmental monitoring, environmental impact, toxicity assays

INTRODUCTION

Microplastics are small plastics ≤ 5 mm that are either fragmented in the environment or resulting from human products and activities[1]. Until recently, most research has been focused on the marine environment, where they were first identified[2]. Concerns have been raised regarding the persistence, ubiquity, and irreversibility of microplastics in the environment[3]. However, the adverse effects of microplastics remain a controversial issue[4]. The lack of standardized methods and reference particles further complicates result interpretation. Still, research continues to proliferate with the term "microplastics" retrieving 16,083 publications in the Web of Science as of January 2024, of which 4,056 were published in 2023 (25,2%). Researchers now entering the field are bewildered and daunted by the large literature base.

A small survey (n = 37) has been conducted during a webinar to summarize difficulties felt by researchers, translating common challenges faced by researchers, worthy of addressing [Supplementary Materials]. The greatest challenges in microplastics sampling and analysis were the lack of standardized methods (78.4%), followed by contamination control (51.4%), lack of specialized equipment in the laboratory (51.4%), and difficulty in identifying microplastics and fibers (35.1%). The greatest challenges in (eco)toxicity studies of microplastics were access to adequate microplastics to be tested (37.8%), quantification of microplastics to prepare solutions or confirm nominal concentrations (37.8%), and use of environmentally relevant concentrations (37.8%). As a result, the present article was idealized with the objective of addressing contemporary challenges and best practices in working with microplastics through a comprehensive literature review.

CHALLENGES IN MICROPLASTICS DEFINITION

What are microplastics?

Plastics are synthetic materials made of long chains of organic polymers, extensively used in a plethora of familiar applications. While thermoset plastics cannot be melted or reshaped by heat once solidified, thermoplastics can be repeatedly melted and molded into different forms. Of the 390.7 Mt of plastics produced globally in 2021, 44% were used in packaging[5]. It has been estimated that 79% of plastics produced as of 2015 persist in landfills or in the natural environment[6]. These plastics can break down into small particles known as microplastics (≤ 5 mm). The term "microplastics" was first used by Dr. Richard Thompson to describe microscopic plastic debris found in seawater samples near Plymouth, UK[7]. Although previous reports of similar particles have been published as early as the 1970s[8], the use of the term microplastics only became popular after Thompson's 2004 publication. Currently, microplastics comprise a class of contaminants characterized by being particles of organic synthetic polymers[9]. Indeed, each microplastic is a unique combination of polymer type, additives, adsorbed contaminants, degradation state, color, size, shape, and biofouling (i.e., accumulation of biogenic matter and organisms on its surface).

How are microplastics defined?

The definition of microplastics is based on size, with the most common upper limit being 5 mm. This limit has been supported by organizations such as the National Oceanic and Atmospheric Administration (NOAA)[10] based on the particle's behavior (e.g., the possibility of being ingested by organisms by accident) and their visibility to the public. The lower limits have been defined as 1 μm based on detection limits of common equipment (e.g., micro-spectroscopy) used in detection and characterization[1]. Therefore, microplastics can be defined as plastic particles of 1 μm to 5 mm in at least one dimension. However, definitions of 1 μm - 1 mm in size have been proposed based on the International System of Units[11]. In these cases, nanoplastics would be defined as plastic particles < 1 μm, although this definition is still debated

considering nanoparticles are defined as particles with their size ranging from 10 to 100 nm in at least one of its dimensions[11,12].

Microplastics are also classified based on their origins [Figure 1]. Primary microplastics are those intentionally produced by the industry with ≤ 5 mm, while secondary microplastics originate from the fragmentation of larger plastics[11,13,14]. This classification was challenged when a report classified primary microplastics as those particles reaching the environment with ≤ 5 mm, despite degradative origins (e.g., microplastics from tire wear)[15]. This classification likely originates from particulate matter, which is classified as primary when directly released as particles and secondary when produced in the environment from chemical precursors (e.g., nitrogen oxides)[16]. Since it is not possible to determine if the fragmentation occurred prior to or after arriving at the environment, this classification is not suitable for microplastics. Instead, identifying if microplastics originate from intentional production or fragmentation enables intervention either by banning industrial uses (e.g., microbead ban in cosmetics) or preventing the fragmentation of larger plastics (e.g., avoiding littering)[17].

Another aspect to consider is what physicochemical properties define microplastics. Questions arise about the classification of modified natural polymers (e.g., cellophane), surface coatings (e.g., acrylic paints), and composites as microplastics. As a rule, microplastics should be solid particles, insoluble in water, and made of a synthetic polymeric matrix[1]. Therefore, the following materials should also be considered as microplastics since they contain modified or synthetic polymers in their composition: modified natural polymers (rubber, rayon, cellophane), inorganic or hybrid polymers (e.g., silicone), copolymers [e.g., acrylonitrile-butadiene-styrene (ABS)], composites (e.g., graphite-reinforced epoxy), surface coatings containing synthetic polymers (e.g., acrylic resins), and tire wear particles[11].

How are microplastics formed?

As discussed in the previous point, microplastics may be classified as primary when industrially produced as plastics ≤ 5 mm to be used in products (e.g., exfoliants in cosmetics) or industries (e.g., abrasives to clean ship's hulls). Moreover, microplastics may be produced through the fragmentation of larger plastic objects. Solar ultraviolet radiation initiates an oxidation reaction mainly responsible for plastic degradation[18]. As a result of photooxidation, carbonyl and hydroxyl functional groups are produced[19]. Photooxidation can be delayed by the presence of antioxidant additives in plastics. Plastics may become yellow through the accumulation of degradation products and fragment as a result of mechanical forces (e.g., abrasion with sand) or surface cracking following photooxidation[18]. Other reactions may involve thermal degradation, hydrolysis, and biodegradation[13]. Degradation is an ongoing process, meaning that microplastics are continuously fragmenting into smaller and smaller particles. Nonetheless, complete degradation into simple molecules (e.g., CO_2, H_2O) is expected to take hundreds of years (e.g., 450 years for polyethylene)[20].

Are microfibers also microplastics?

Demand for synthetic fibers has grown, reaching 55.2 million tons in 2014, of which 46.1 million tons is for polyester (a.k.a., polyethylene terephthalate)[21]. Demand for clothing is expected to reach 160 million tons in 2050, of which 63% will be for synthetic fibers (estimated 100 million tons)[22]. The fragmentation of these synthetic fibers (i.e., made of synthetic organic polymers) produces microfibers, which are considered microplastics and are characterized by a minimum 1:3 aspect ratio (i.e., "rod" shape)[23]. Sources of microfibers include textiles, but also fishing equipment and packaging[23]. An estimate of 20%-35% of microplastics are microfibers, although local concentrations vary greatly depending on sources[24]. Due to their shape, fibers may be able to penetrate deeply into the tissue, accumulate, and cause adverse effects to organisms.

Figure 1. Primary microplastic (transparent bead), secondary microplastic (blue fragment), and mesoplastic (white fragment) found on Matosinhos beach, Portugal, in September 2023.

CHALLENGES IN MICROPLASTICS ANALYSIS

Organizations have previously attempted to provide guidance on methodologies (e.g., NOAA, Marine Strategy Framework Directive technical subgroup[10,25]), but mostly focused on larger microplastics in the marine environment. A new attempt of standardization (or harmonization) is under development by the ISO (e.g., ISO/FDIS 24,187 Principles for the analysis of microplastics present in the environment).

Objective, experimental design and statistical analysis

A research question precedes any scientific study, as all planning depends on it. It must be clearly stated as an objective or hypothesis, which is essential to define the scope, provide a foundation for communication, and avoid dispersion to other related topics. Research questions are generally motivated by knowledge gaps arising from previous works, either original articles or literature reviews. It will determine what strategy and methods are necessary to answer this question, also considering the feasibility of its implementation. Defining variables and categories from the start will help in the production of a harmonized database. Ideally, categories should be based on literature to improve comparability between results. For instance, the shape of microplastics could be classified as sphere, spheroid, cylindrical pellet, fragment, film, fiber, and other[11].

Sampling strategies have included different sections, transects, or the use of quadrants[26]. Spatial sampling can include: (i) random sampling, randomly selecting points in an area if a homogeneous distribution is expected (e.g., sampling four random 1 m^2 quadrants in a beach); (ii) systematic sampling, using grids, gradients or transects limited to an area allowing to detect gradients (e.g., sampling a 100 m transect perpendicular to the shoreline); or (iii) stratified random, which subdivides different zones where concentrations are expected to be different and random sampling is conducted inside each zone or stratum (e.g., sampling four random 1 m^2 quadrant in three transects in the beach, each 10 m wide, starting from the shoreline). The objective is to include representative sampling areas. The United States Environmental Protection Agency (EPA) suggests a beach sampling protocol consisting of 12 samples inside 1 m^2 quadrant

collected over a 100 m sampling area, collecting samples from four random transects over three lines parallel to the water (high tide line, middle beach, and back beach) [Figure 2][27].

Statistical analysis may be conducted to address the need for a different sampling strategy. For instance, the coefficient of variation, calculated as the standard deviation divided by the geometric mean of replicates from a sampling area, can express data dispersion coming from different samples. A coefficient of variation of ≤ 20% is commonly accepted[28]. If methodological aspects are excluded from causing variation (i.e., variation is not coming from errors in sample collection, processing, and analysis), which can be excluded by running spiked samples, a high coefficient of variation (i.e., high data dispersion) may indicate the need to apply a different sampling strategy. A lower coefficient of variation may be needed for the detection of smaller effect sizes. It is worth considering that microplastic may suffer spatiotemporal variations, for instance, depending on meteorological or seasonal events[29] or on sampling depth[30]. To understand the dynamics of microplastics in the environment, the following aspects should be considered: (i) tridimensional distribution; (ii) measures at regular intervals to account for temporal variation; (iii) source appointment; (iv) particle characteristics related to distribution; and (v) mechanisms influencing distribution[31].

Sampling design, including the number of samples and replicates, depends on the objective of the work. Ideally, statistical approaches should be defined a priori to ensure the data collected serves its function. Data should be recorded in long format, with each row pertaining to a particle (for characterization) or to a replicate (for quantification) and each column to a variable to be assessed (e.g., identification code, color, size, shape, polymer type). Comparisons and conclusions should always be based on a statistical analysis of the data. The chi-square test is commonly used for two categorical variables (e.g., does color vary significatively between areas?). Analysis of quantitative data generally involves t tests or analysis of variance (ANOVA) for parametric datasets, or the corresponding Mann-Whitney U and Kruskal-Wallis tests for non-parametric datasets (e.g., does concentration vary between different areas?). Probability results (i.e., *P*-values) should be reported and compared to the tested significance level (e.g., $\alpha = 0.05$). Categorical variables (e.g., color) should be reported as absolute and/or relative frequencies (i.e., number and/or percentage), and quantitative data should be reported as a mean for parametric datasets, or median for non-parametric datasets, accompanied by a measure of dispersion (e.g., variance, standard deviation, interquartile range).

How to collect samples of microplastics?

The first step is to collect samples containing microplastics, which may include matrices as diverse as water, sediment or soil, air, and biological matter [Figure 3]. The choice of sample collection method depends on the work's objectives. For instance, surface water collection may not be a good estimate of benthic organisms' exposure, especially considering that surface and deep waters may contain different microplastics depending on each particle's sinking behavior (e.g., dependent on density and biofouling)[32]. Moreover, recent publications are mainly focused on collecting the whole size range of microplastics (i.e., 1-5,000 μm). Since smaller microplastics are more abundant (e.g., 92% are < 300 μm in surface water in the South China Sea[33]), smaller sample volumes or weights may be collected. Reporting these smaller microplastics is needed for risk assessment since particles < 150 μm have a higher probability of being internalized by organisms[34]. The choice of sampling methods should also take into consideration principles of green analytical chemistry, which include reducing sample treatment, saving energy and reagents, automation, reducing analytical waste, and avoiding the use of hazardous reagents[35].

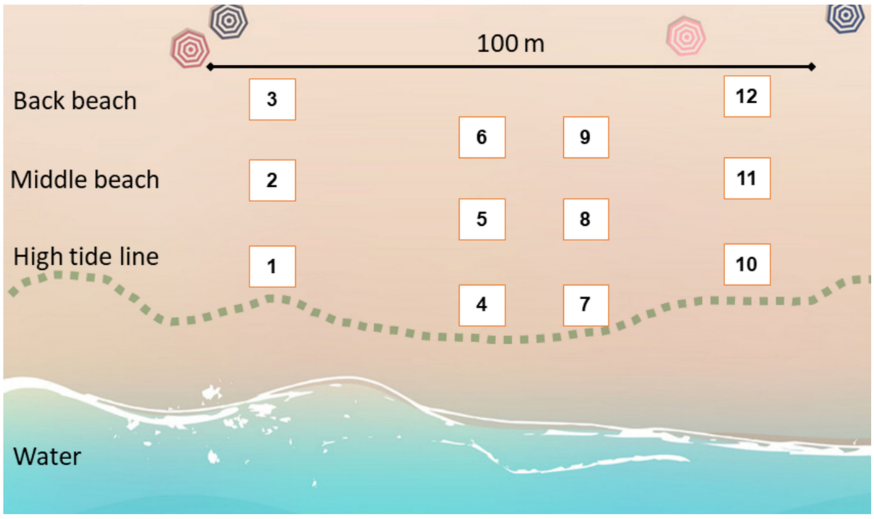

Figure 2. Beach sampling protocol according to the United States Environmental Protection Agency (original artwork).

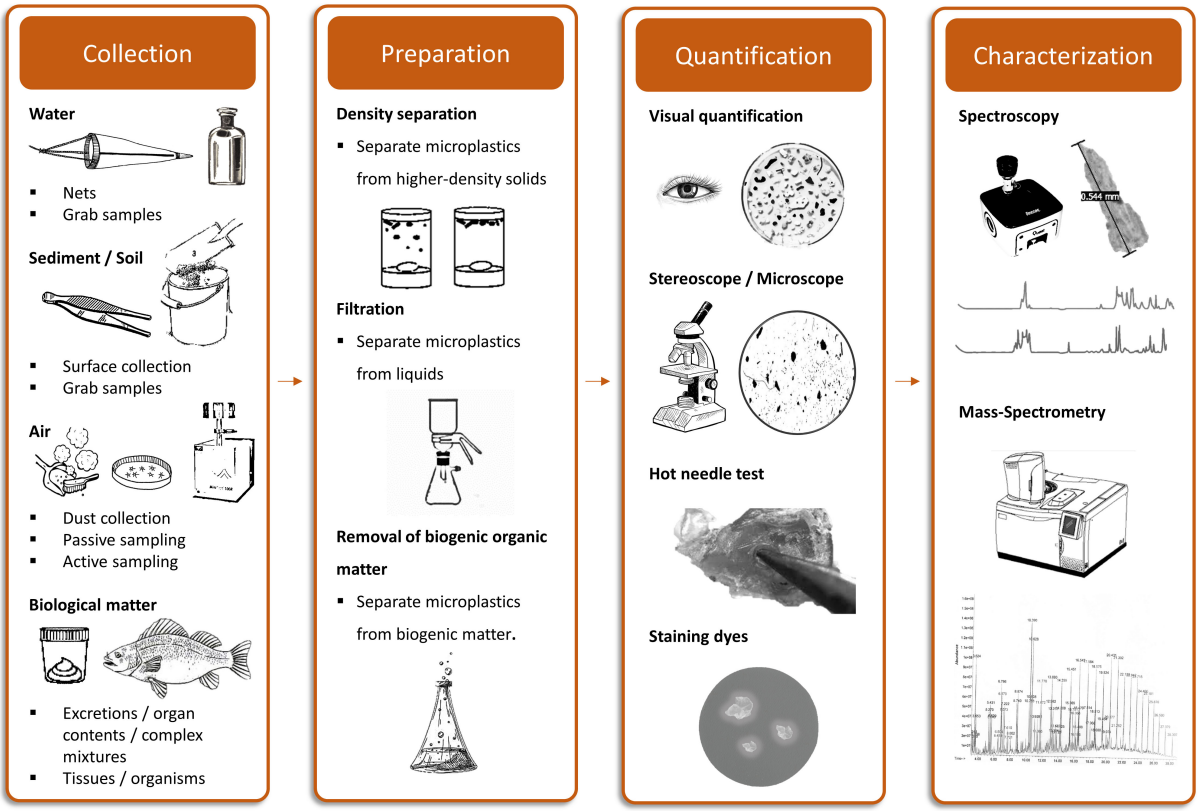

Figure 3. Summary of methods for sampling and analysis of microplastics.

Sampling water

For water, the choice is between using nets (e.g., 330 μm manta trawls) or collecting samples using bottles (i.e., grab samples) to be filtered in the laboratory. Nets may vary in configuration, for instance, sampling either surface water in the case of manta trawls or at a depth of 40-50 cm in the case of neuston nets[26]. Since

microplastics may be differentially distributed on the water column (e.g., based on density), the choice of sampling methodology may create a bias on the types of particles found. While nets sample high volumes of water (m^3) with relative ease, they require water flow (e.g., towed by boat), contamination control is difficult, and the precise volume of water filtered is prone to considerable error. Collecting grab samples limits volume (L), either due to transportation or clogging, but allows sampling of smaller particles that are more abundant and more likely to induce adverse effects on organisms. This may include manually using buckets and bottles on surface water or using specialized equipment to collect underwater samples (e.g., Niskin sampler)[36]. Other works have used sieving and/or pumps to collect samples[37]. Grab samples are generally filtered in the laboratory, which may use filter membranes of different compositions and pore sizes. Filtering water through a 1 μm pore filter is expected to retain 40 times more microplastics than using a manta trawl[38]. Moreover, reliable results were produced by collecting 1 L grab samples in four replicates and assessing microplastics > 50 μm[28].

Sampling sediment or soil

For sediments or soil, collection generally requires defining a surface area (e.g., 1 m^2) where the top layer is collected (e.g., top 5 cm) using metal shovels and non-plastic containers (e.g., aluminum, glass). Samples will be taken to the laboratory and separated by density by filtering the supernatant after mixing with saturated solutions. Plastics' densities range between 0.90 and 1.45 $g \cdot cm^{-3}$, whereas sediments possess higher densities, approximately 2.65 $g \cdot cm^{-3}$. Saturated solutions of NaCl, while inexpensive and non-hazardous, produce the lowest recovery rates (75.71%) compared to higher recovery rates (> 85%) of NaBr and $CaCl_2$ [39]. This occurs due to the saturated NaCl solution's lower density (1.2 $g \cdot cm^{-3}$), which is insufficient to separate higher-density plastics (e.g., polyvinyl chloride)[39]. Extraction with high-density solutions might need to be repeated to separate all particles from the sediment matrix. Conversely, higher-density solutions may present toxicity and higher costs, which can be alleviated by reusing the solution after filtration, thereby reducing costs and waste production[35]. Other techniques involve the use of complex apparatus for density extraction[40], elutriation[41], or oil-based extraction[42]. Alternatively, microplastics may be collected with tweezers directly from the surface, underwater samples collected by submergible claws (e.g., Van Veen grab), or with sediment core samplers. Results should be reported by the number and weight of microplastics per area and the weight of samples. Moreover, results should be reported in dry weight by drying the sample until no variation in weight occurs (or calculating dry weight based on the weight loss in a subsample). Studies should include details on sediment or soil characterization (e.g., sediment's grain size), since this may help highlight deposition patterns.

Sampling air

For air, samples are collected actively on a filter using an air sampling pump (e.g., flow rate of 5 $L \cdot min^{-1}$ for 24 h[43]), passively by deposition on the surface of a known area (e.g., funnel, Petri dish), or by collecting dust from surfaces using brushes. Samples are generally collected at a human breathing height (1.5 m). Passive sampling has the advantage of not needing specialized equipment but requires longer sampling periods and may not directly translate airborne concentrations (i.e., larger particles are more likely to sediment). Results from passive sampling (e.g., particles$\cdot m^{-2} \cdot h^{-1}$) allows to estimate airborne contamination of other matrices (e.g., water, soil), whereas active sampling (particles$\cdot m^{-3}$) is useful to estimate respiratory exposure, and dust collection allows to estimate the amount of microplastics relative to the total amount of dust collected (e.g., particles$\cdot g^{-1}$). Only particles with aerodynamic diameters < 10 μm (capable of reaching the deep lung) should be considered in the estimation of airborne exposure based on human tidal volume (6 $L \cdot min^{-1}$)[44]. Separation of < 10 μm can be achieved in active sampling by using a selective inlet.

Sampling biological matter

For biological matter, samples may be constituted by excretion products and organ contents (e.g., feces, digestive contents), tissues (e.g., digestive system, food), organisms (e.g., invertebrates), or complex mixtures (e.g., wastewater). Collection will vary according to each case and separation mainly relies on digestion and filtration. It is recommended to limit the amount of sample to reduce filter clogging and physically remove hard tissues (e.g., bones, chitin, or carbonaceous shells). Biological matter is often rich in fat, which must be removed manually, using detergents or ethanol[44]. Moreover, lipids will be subjected to saponification in the presence of alkali solutions (e.g., KOH), which may be removed using hot water. Wastewater samples may be collected as grab samples or through high-volume filtration using sieves[46]. Overall, biological matter is the most challenging matrix to work with. Ideally, new methods will be developed to identify the precise location of microplastics in tissues using histological techniques. Several features of the biological samples should be taken into account: (i) trophic transfer; (ii) correlation with concentrations found in environmental matrices; (iii) microplastics in internal organs (besides the digestive system) as evidence of translocation; (iv) possible accumulation of microplastics in organisms; and (v) presence of related contaminants (e.g., plastic additives). More research on microplastics is needed, especially addressing terrestrial ecosystems.

Sample preparation: removal of biogenic organic matter

Almost all samples require some sort of sample preparation to reduce the number of particles and clean the surface of microplastics [Figure 4]. This is mostly accomplished through digestion (i.e., natural organic matter degradation). The most common solutions used are H_2O_2 (alone or as Fenton's reagent when combined with Fe as a catalyzer), HNO_3, and KOH. H_2O_2 [e.g., 30% (v/v)] is mostly used on environmental samples (e.g., water, sediment) and KOH [e.g., 10% (w/v)] on biological matter (e.g., tissues). Acids are generally not recommended due to the risk of damaging microplastics in the sample[47]. Although enzymes degrade with a high efficiency without damaging microplastics, they are also often prohibitively expensive. Reactions may be accelerated by heating, although temperatures > 60 °C are not recommended as they may destroy microplastics[48]. The use of organic solvents to further clean the surface of microplastics may also cause damage. For instance, acetone may melt polystyrene into a paste[49]. Therefore, it is recommended that sample preparation protocols are tested using positive controls with various representative polymer types (i.e., spiked samples).

Sample preparation: separation of microplastics

Filter membranes are often used in microplastics analysis to separate particles from liquids. The type and pore size of these filter membranes can influence the retained fraction of microplastics, the amount of residual matrix, and the ease of filtration (depending on vacuum pressure). The choice will also depend on treatment and analysis, as it may benefit from materials that are inert (e.g., glass fiber filter), degradable (e.g., cellulose), or present low signal interference in spectroscopic analysis (e.g., silver). Filter structure can also influence the size, shape, and number of particles recovered (e.g., multilayered filters are better at retaining fibers)[50]. While the choice of filter membrane may lead to underestimation, it can also introduce contamination if no decontamination procedure is conducted before use. Similarly, sieve use will depend on pore size and decontamination measures.

Emerging methods for microplastics separation involve chromatographic techniques, such as field flow fraction, which consists of applying a field (e.g., electrical, hydraulic) perpendicular to the flow of particles in a narrow channel leading to the accumulation of particles in one of the walls, and hydrodynamic chromatography, which separates particles by sizes by passing the sample through a column with solid beads, carried by a mobile phase[51]. Dielectrophoretic separation is based on an electric field that separates

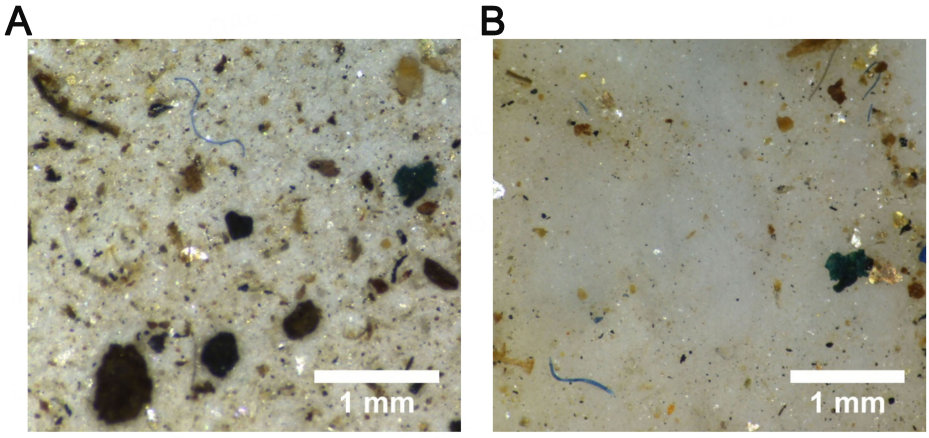

Figure 4. Sediment sample (A) before and (B) after biogenic organic matter removal using 10 mL of 30% hydrogen peroxide and 10 mL of 0.05 M of iron catalyst for 15 min.

particles of different dielectric signatures into microchannels[52]. Alternatively, flow cytometry can be used for sorting and counting and characterizing microplastics based on optical properties, with the advantage of having a high particle throughput[53]. It can be combined with fluorescent dyes and preconcentration steps to improve accuracy. Fractioning could also be used to separate microplastics from liquids, but smaller and less dense particles will require centrifuging at higher speeds for longer periods (e.g., > 3,500 rpm, > 5 min[54]), ideally inside glass tubes to avoid plastic contamination.

How to identify microplastics under the microscope?

Successful microplastics identification depends on proper sample preparation, which is easier in less complex matrices (e.g., in water than in food). Screening microplastics under a stereoscope or microscope is one of the simplest and cheapest methods available. However, it is also subjective and can lead to misclassification of other materials such as microplastics (up to 70%)[54]. Exposing particles to heat (130 °C, 3-5 s)[56] or touching them with a hot needle[57] helps to further separate large melting particles as thermoplastics. The use of a soldering iron instead of a hot needle and a classification criterion (positive if particle melts, bends, or curls) results in ≥ 82% correct classification[58]. While the hot needle test may also be performed, synthetic fibers are visually classified based on their regular and smooth surface, following a classification diagram[59]. Size, shape, and color must be recorded for each particle. Analysis may be aided by image processing software (e.g., ImageJ).

The use of staining dyes provides a more objective criterion if they are selective for plastics or if other stainable particles have been removed (during digestion). Nile Red is the staining dye most frequently used in microplastics screening [Figure 5]. Plastics stained with Nile Red produce a red-to-yellow fluorescence under the blue light (470 nm) when seen through an orange filter or under the UV light (250 nm)[60]. This method seems to reduce the number of false positives, as 90% of fluorescent particles in sediment samples (> 630 µm)[61] and 100% in bottled water samples (> 100 µm)[62] were confirmed as plastics by chemical characterization. Nile Red allows quantification of microplastics > 1 µm when coupled with fluorescence microscopy and can be used as a selection criterion before chemical identification, improving sample throughput[63]. Moreover, staining dyes are inexpensive. Grids or guiding markers are recommended when working with small particles under the microscope to avoid counting them twice.

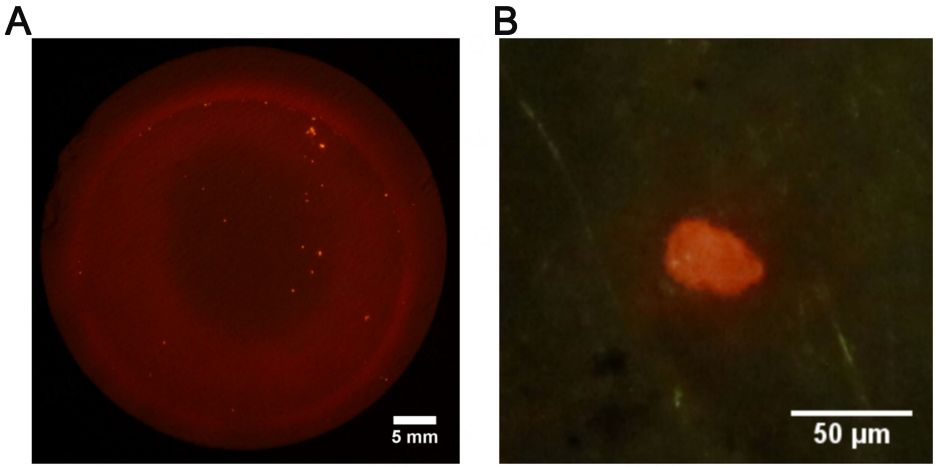

Figure 5. Fluorescent microplastics after Nile Red staining and under 470 nm with an orange lens filter: full glass fiber filter (47 mm) spiked with (A) microplastics and (B) suspected microplastics in river water under the microscope.

How to analyze microplastics by chemical characterization?

Vibrational spectroscopy techniques in microplastic characterization

Visual identification and staining dyes only provide information on suspected microplastics. Chemical characterization is needed for confirmation and polymer identification. The most common techniques are based on vibrational spectroscopy. Fourier transform infrared spectroscopy (FTIR) relies on the detection of the absorption, transmittance, or reflectance of infrared light based on the change in the dipole moment, whereas Raman spectroscopy detects the inelastic scattering of light caused by changes in polarizability within the molecule. It is recommended to characterize at least 10% of suspected microplastics > 100 µm[25]. Micro-spectroscopy techniques (i.e., coupled with microscopy) allow the characterization of particles down to 1-20 µm[64], ideally of a specific area of the filter when scan mode (or mapping) is available or of a group of pre-selected suspected particles (which should be representative of the sample). However, micro-FTIR and micro-Raman spectroscopy are time-consuming (e.g., 6-8 h per sample[65]) and require expensive equipment, which researchers may only have access to through collaborations. Therefore, these techniques are generally not suited for quantification (which still relies on microscopy or staining dyes). Moreover, the presence of contaminants or pigments on microplastics may cause interference, such as titanium dioxide (TiO_2)[66] or black pigments (e.g., carbon black)[67]. Generally, FTIR is more lenient when interference is present, while photo-bleaching (i.e., fluorophore undergoes photo-destruction when the absorption/emission limits are surpassed) may be attempted in micro-Raman spectroscopy[64].

Another challenge in vibrational spectroscopy is the analysis of the fingerprint spectrum. Each peak or band corresponds to vibrations of specific chemical bonds [Figure 6]. Spectra may be subjected to baseline correction, which enables the removal of peaks not in the sample (e.g., CO_2 infrared peak at ~2,360 cm^{-1}). Polymer identification may rely on a personal database of known plastics, on the equipment's database, or on open online databases (e.g., OpenSpecy[68], SiMPle[69]). Besides reporting the relative frequency of different polymer types, the carbonyl and methyl index can be calculated from infrared spectra, which are used as indicators for the weathering state. Carbonyl groups (–C=O) are produced during plastic's degradation through photooxidation, translated as an infrared band between 1,850-1,550 cm^{-1}, centered in 1,715 cm^{-1} (ketone)[70]. Thus, the Carbonyl Index (CI) can be calculated as the ratio between the carbonyl peak (1,715 cm^{-1}) and the reference peak for the polymer (e.g., 1,471 cm^{-1} for polyethylene, 1,458 cm^{-1} for polypropylene, 1,452 cm^{-1} for polystyrene)[19]. In polypropylene, the first stages of degradation lead to the production of volatile oxidation products (e.g., acetone), which are lost by evaporation, resulting in a

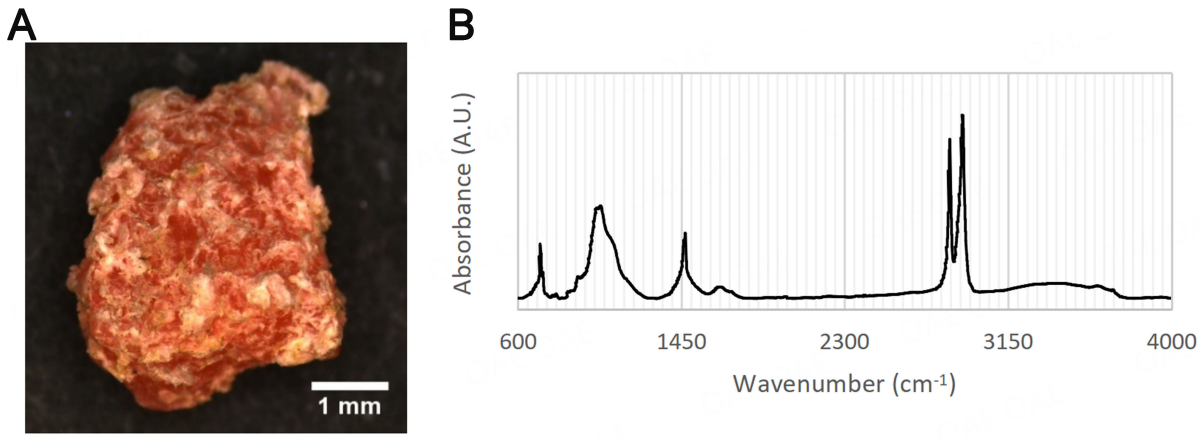

Figure 6. (A) Microplastics collected on a beach in Portugal, and identified as polyethylene based on (B) its infrared spectra.

reduction in methyl groups (-CH_3). Thus, the Methyl Index (MI) may be calculated for polypropylene as the ratio between the methyl group (1,377 cm^{-1}) and the reference peak (1,458 cm^{-1})[71]. Comparison between CI should only be conducted for the same polymer type. Particles should be thoroughly cleaned to remove biological debris that may translate into an increased carbonyl band (e.g., using cyclohexane at 80 °C for 48-96 h[72]).

Mass spectrometry techniques in microplastic characterization

Vibrational spectroscopy techniques are non-destructive and allow chemical and physical characterization of particles, which is highly relevant for risk assessment. However, they are time-consuming. Alternatives based on mass spectrometry (MS) [e.g., pyrolysis-gas chromatography-MS (Py-GC-MS), thermoextraction and desorption coupled with gas chromatography-MS, liquid chromatography-MS] allow to gather information from complex matrices without pre-selection, quickly, and even for smaller microplastics sizes[73]. The cons of these methods involve the need for calibration curves for each compound (including considerations on degraded states), sample destruction, and loss of information regarding physical parameters (i.e., only reported as mass, e.g., $\mu g \cdot mg^{-1}$).

Py-GC-MS has increased in popularity due to its ability to detect smaller particle sizes (even nanoplastics), with higher sensitivity and lower vulnerability to interference, and being compatible with direct analysis and/or with most sample preparation methods[74]. This technique is based on the thermal decomposition of the sample's molecules into simpler fragments which are separated by a capillary column and detected by mass spectrometry. A specific indicator (fragment) is required for each polymer type to properly detect and quantify them based on calibration curves. An optimized Py-GC-MS method for microplastics has been proposed using a pyrolysis temperature of 700 °C, split ratio of 5, and 300 °C as injector temperature, allowing limits of detection of 1 μg for microplastics[75]. This method performed well when tested on particles previously identified by micro-Raman spectroscopy and correctly identified > 70% of environmental microplastics. Worst results were found for fibers due to their low weight, which could be improved by decreasing the limit of detection using single ion monitoring or pre-concentrating the sample.

Scanning electron microscopy in microplastic characterization

The determination of total organic carbon analysis can be used in the quantification of the total mass of plastics in complex samples after biogenic organic matter removal[76]. Equations are available to make estimate conversions between different types of reporting units[77]. Scanning electron microscopy may be

used to characterize the surface of microplastic, while coupling with energy-dispersive X-ray spectroscopy allows for chemical characterization. However, most of the elemental composition of plastics is similar to biological matter (e.g., C, O, H), which may hinder identification.

Other techniques in microplastic characterization

Other techniques of microplastics analysis have been proposed to address challenges in traditional approaches. Hyperspectral imaging is based on spectral fingerprints (e.g., on near-infrared spectrum), which may allow differentiation between polymer types[78]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, which involves ionization and mass-to-charge ratio (m/z) measurement, may be useful in the detection of microplastics, including weathered particles[79]. Dynamic light scattering and nanoparticle tracking analysis are based on light scattering and Brownian motion and can be complementary in the characterization and quantification of particles[80]. Atomic force microscopy scans the sample surface by registering attractive and repulsive forces between the surface and the cantilever[81]. The techniques described in the last two sentences may also be suitable for nanoplastics analysis.

Characterization of nanoplastics

Nanoplastics are more challenging due to the smaller particle sizes (< 1 μm) below most limits of detection and are prone to contamination. Sample preparation also involves the removal of natural organic matter, separation and preconcentration (e.g., filtration, centrifugation), and identification using spectroscopy or mass spectrometry[82]. However, so far, only a few studies have been able to detect nanoplastics in real environmental samples. While scanning electron microscopy has been used to characterize very small plastics (< 10 μm)[83], quantification based on the detection of particles containing carbon has been questioned due to the potential contamination with other similar organic and inorganic materials[84].

How to avoid contamination of microplastics samples?

Microplastics are so ubiquitous that they can easily contaminate samples. Researchers must be aware of the two main sources of contamination, namely, the deposition of airborne microplastics in samples and cross-contamination from solutions and materials used. Reducing airborne contamination requires: (i) regular laboratory cleaning schedules; (ii) proper ventilation and reduced movement; (iii) capping all samples and solutions (e.g., with glass lids or aluminum foil); (iv) conducting work under the fume hood, or ideally, the laminar flow hood; (v) wearing cotton lab coats. Reducing cross contamination requires: (i) filtering all solutions used in cleaning or to be added to the solution; (ii) washing materials between samples, ideally in acid followed by running filtered distilled water; (iii) using only glass and metal materials (i.e., no plastics); (iv) decontaminating inorganic filters (e.g., 3 h at 450 °C). Finally, a relevant number of blanks must be conducted during sample collection (i.e., field blanks) and processing (i.e., procedural blanks and open filters to evaluate airborne deposition). Microplastics found in blanks can be subtracted from results or samples only considered when statistically different from concentrations in blanks.

What can we learn from existing interlaboratory comparison tests?

Interlaboratory tests have been conducted despite not reaching a definitive conclusion regarding recommended methodologies [Table 1]. Since most tests were conducted with pristine microplastics, results may vary when working with environmental samples. For instance, micro-Raman spectroscopy works better with pristine samples since it is susceptible to fluorescence interference and may cause polymer degradation as a result of heating[64]. Studies seem to agree that errors vary greatly between research groups[85,86], which supports conclusions that most are a result of human error[85,87]. Nonetheless, variation is also attributed to microplastics' characteristics, such as particle size (i.e., higher for sizes < 20 μm), shape, and color or matrix properties[85]. While attempts have also been made to harmonize the production of reference materials[88], these are not yet widely available or frequently used. Sample preparation seems to

Table 1. Main conclusions arising from interlaboratory tests conducted on the methods for quantification and characterization of microplastics

Test name	Published date	*n*	Samples	Main conclusions	Ref.
Isobe *et al*., 2019	2019	12	Two bottles with different concentrations of PP, LDPE, HDPE, and NOM in seawater (400-5,700 μm)	High uncertainty (> 50%) likely results from human error (i.e., misidentification or measuring errors) Sample preparation (i.e., chemical treatment, density separation) reduced uncertainty from 40% to 20% Underestimation of 20% for microplastics < 1 mm (spectroscopy recommended)	[87]
Müller *et al*., 2020	2020	17	Sugar water spiked with PE, PVC, PET, PMMA, and PS (8-140 μm)	Better identification of polymer type by micro-Raman spectroscopy and Σ-GC/MS than by micro-FTIR Quantification using microscopy and micro-FTIR worked best	[90]
Becker *et al*., 2020	2020	9	Sediment sample spiked with PP, PS, PE, PET, and suspended particulate matter (2,000-3,000 μm)	Py-GC-MS, TED-GC-MS, TGA-FTIR correctly identified all polymers and produced reasonable results The mean absolute deviation from the median was 14% for PE and PS and > 40% for PP and PET High variability in the analysis of PET likely results in the use of adsorber materials and stationary phase adapted for non-polar compounds Additives in PE could influence the formation of decomposition products and interfere with readings TGA-MS not recommended since it did not confirm the presence of the four polymer types	[93]
Cadiou *et al*., 2020	2020	5	Clean water or sediment spiked with PET, PVC, PC, HDPE, and PP (300-5,000 μm)	Error was < 25% of the absolute value for microplastic counts (60% underestimation and 40% overestimation) Errors in quantification were higher in sediment compared to water samples (18% *vs.* 14% root mean square errors) Laboratories varied greatly in the error of detection of microplastics (2%-3% *vs.* 23%-30%) Regarding shape, films are generally overestimated while others are correctly counted or underestimated Color also influences the correct estimation of microplastics Underestimation might be explained by lack of detection, loss of laboratory material, or loss by being blown out during handling.	[85]
JRC's interlaboratory test	2021	98	One sample of PET in salt water (30-200 μm)	Methods had a high variability depending on research groups Many groups efficiently detected the correct number of plastics in the samples using multiple techniques, including microscopy, micro-FTIR, and micro-Raman spectroscopy Some contamination control measures (i.e., the use of cleaning paper and gloves) seem to be related to the underestimation of microplastics	[86]
WEPAL-QUASIMEME/NORMANs	2021	30	11 samples comprised each of the most common polymer types (PC, PS, PP, PET, LDPE, EPS, PVC) and one blank (2,270-4,310 μm)	Correct chemical identification > 80% (except for low-density polyethylene) Consistent performance of micro-Raman spectroscopy Quantification was often flawed in terms of absolute number and the high dispersion of data (i.e., relative standard deviation of 57%-91%)	[91]
The Plastic Busters MPAs	2021	4	Fish gastrointestinal tract and mussel tissues spiked with PE, PP, and PET (> 200 μm)	The coefficient of variation after digestion and counting under the microscope was < 11% for most samples Recovery rates were 96.7% for 5 mL/g of 10% KOH and 88.8% for 20 mL/g of 15% H_2O_2	[89]

				Changes due to sample preparation were observed as fragmentation (7.1%), discoloration (2.1%), deformation (9.2%), and degradation (17.1%) Microplastics correctly identified by FTIR varied between 47.8% and 69.5%	
De Frond *et al*., 2022	2022	22	Three samples of PE, PS, PVC, PET, and NOM in drinking water and one blank (1-5,000 μm)	Microscopy allowed for a mean recovery rate of 92% for microplastics > 20 μm and 32% for < 20 μm The mean blank sample count was 91 particles Micro-FTIR accurately identified 95% of microplastics and misidentified 8% of NOM as microplastics Micro-FTIR accuracy was 33% for microplastics < 20 μm Micro-Raman spectroscopy accurately identified 91% of microplastics and misidentified 68% of NOM as microplastics Micro-Raman spectroscopy performed poorly on dyed cellulose fibers due to the interfering fluorescence.	[92]

PP: Polypropylene; LDPE: low-density polyethylene; LDPE: low-density polyethylene; NOM: natural organic matter; PE: polyethylene; PVC: polyvinyl chloride; PET: polyethylene terephthalate; PMMA: polymethyl methacrylate; Σ-GC/MS: thermo-extraction-and-desorption- or pyrolysis- combined with gas chromatography coupled to mass spectrometry; micro-FTIR: micro-Fourier transform infrared spectroscopy; Py-GC-MS: pyrolysis gas chromatography-mass spectrometry; TED-GC-MS: thermal extraction desorption followed by gas chromatography coupled to mass spectrometry; TGA-FTIR: thermogravimetry-infrared spectroscopy; TGA-MS: thermogravimetry coupled to mass spectrometry; PS: polystyrene; EPS: expanded polystyrene.

reduce the errors in the identification of microplastics by removing confounding particles[87,89]. Microscopy, micro-FTIR, and micro-Raman spectroscopy are associated with high recovery rates (> 90%) and spectroscopy methods may also provide information on polymer type[90-92]. Nonetheless, higher errors are expected for smaller microplastic sizes[87]. Alternatively, pyrolysis gas chromatography-mass spectrometry, thermogravimetry-infrared spectroscopy, and thermal extraction desorption followed by gas chromatography coupled to mass spectrometry are also efficient in the quantification and characterization of some polymer types[93]. These techniques also present limitations such as the specificity of adsorber materials and stationary phase compounds for some polymer types or being influenced by additives in plastics[93]. Surprisingly, the most frequently found variation in results was underestimation and not overestimation (expected due to sample contamination)[85-87]. This underestimation likely results from errors in detection or losses to laboratory material or to the air when handling these samples[85]. Therefore, frequent tests are recommended to evaluate the recovery rates of the group's methodologies using a diverse array of microplastics and matrices and improve techniques to reach an acceptable variation.

CHALLENGES IN EVALUATING THE ADVERSE EFFECTS OF MICROPLASTICS

How do microplastics enter the environment?

Microplastics originate from virtually any object made of plastic [Figure 7]. Some relevant sources of microplastics include pellets, microbeads in personal care products, paint, textiles (including garments, household textiles, personal protective equipment, geotextiles used in agriculture), wastewater effluents, synthetic pavement and artificial turf, vehicle tire wear, fishing gear, and littered or mismanaged plastic waste[94-96]. Lack of proper waste management and wastewater treatment greatly contributes to the amount of (micro)plastics entering the environment. Most microplastics are thought to originate from terrestrial areas, especially related to high anthropogenic pressures (e.g., high population density, high industrialization), and then be transported by wind, runoff, or rivers.

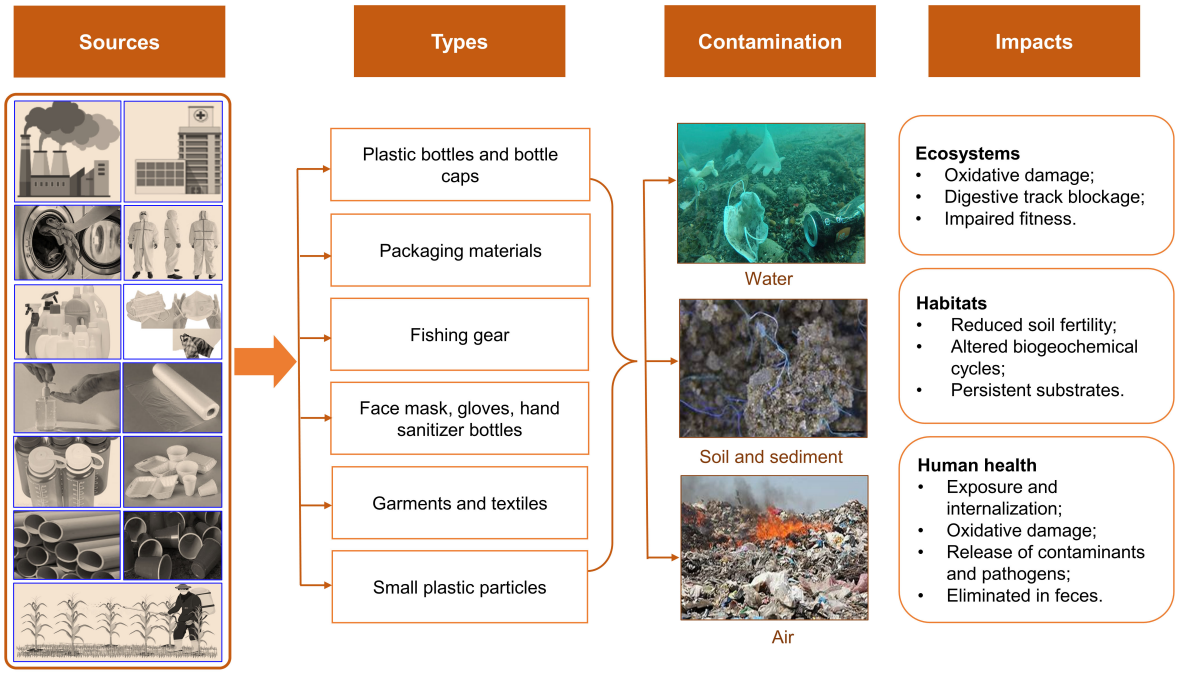

Figure 7. Examples of sources, types, contamination, and impacts of microplastics in the environment.

Estimations indicate that most microplastics reaching the sea originate from river transport[97] whereas atmospheric transport may contaminate remote regions, such as the Tibetan Plateau[98]. The distribution of microplastics may also depend on their surface coverage with organic matter and organisms (i.e., biofouling), for instance, modulating their distribution in the water column[99]. Microplastics may also accumulate in environmental matrices, such as sediments or soils, and can be incorporated into geological formations, being considered markers for the Anthropocene[100]. However, few works have collected data to support the identification of sources and pathways of microplastics in the environment due to the previously addressed methodological challenges.

What are microplastics' effects on the environment?

Biotic effects of microplastics

The presence of microplastics in the environment leads to the exposure of organisms, with these particles being found in the gastrointestinal system of invertebrates (e.g., *Chironomus riparius* larvae)[101] and vertebrates (e.g., *Seriolella violacea* fish)[102]. These studies are often limited to the detection of larger microplastics (e.g., > 500 μm) in the gastrointestinal lumen or tissue. Nonetheless, microplastics in the gut may translocate to internal tissues (e.g., hemolymph of *Mytilus edulis*[103]) and be transferred through the food chain[104]. However, evidence for bioaccumulation and biomagnification in the wild is insufficient[105,106]. Bioindicators are species that can be used to monitor environmental contamination. Bivalves, as filter feeders, have already been suggested as bioindicators for microplastics[107], supported by a significant correlation between concentrations in water and in tissues of *Mytilus edulis* and *Perna viridis*[108]. The use of bioindicator species can provide insights into the spatial distribution of microplastics and their interaction with organisms within the ecosystem.

Laboratory tests on aquatic organisms reveal effects on feeding (e.g., digestive tract blockage), endocrine disruption, metabolism, and gene expression, leading to reduced growth and survival[109]. These likely stem

from reactive oxygen species, seemingly the molecular initiating event across different animal species[110]. A species sensitivity distribution determined a threshold effect concentration (HC_5) of 11-521 microplastics·L^{-1}, representing 28% of locations included in the study[38]. Despite the large amount of available literature, most assays have used concentrations 2 to 7 orders of magnitude higher than environmental levels, instead of environmentally relevant concentrations (e.g., ≤ 1 μg·L^{-1} for water)[111]. However, preparing lower concentrations may be challenging due to the lack of methods to quantify microplastics in small volumes, which often involve spectrofluorometry (i.e., when using fluorescent particles) or the use of Neubauer counting chambers[112].

Organisms and microplastics should be selected simultaneously, based on the likelihood of environmental exposure and the availability of international guidelines (e.g., OECD Test Guidelines). Tested microplastics may be acquired (e.g., Sigma-Aldrich; Cospheric; plastic industry) or produced in the laboratory (e.g., using cryomilling or a simple coffee grinder), followed by adequate particle characterization [Figure 8]. Microplastics may also transport adsorbed contaminants or pathogens[113]. Therefore, more complex tests involve aging microplastics under ultraviolet light, producing leachates by incubating them in a pristine solution, or contaminating microplastics with other substances or microorganisms. The contribution of each characteristic to toxicity should be identified (e.g., chemical composition, size, shape).

Abiotic effects of microplastics

Little information is available on those other than marine organisms (e.g., plants) and abiotic effects. Indeed, microplastics may cause abiotic effects on ecosystems, such as favoring species using them as substrates[114], altering the distribution of substances and/or biogeochemical cycles[115], and changing thermal diffusivity and permeability of sediments or soils[116,117]. Thus, marine plastic pollution is already considered a planetary boundary threat due to its accumulation, long-lasting nature, irreversibility, and capacity to disrupt natural processes[3]. Future study designs should encompass the information needed to conduct risk assessment, enabling informed decisions about control and mitigation strategies (i.e., risk management). Work objectives should attempt to fill knowledge gaps following a DPSIR framework (i.e., drivers, pressures, state, impact, and responses)[118].

Plastisphere: life on microplastics

The plastisphere is a unique ecosystem of microorganisms living on the surface of microplastics. It is formed when the surface of particles is covered with biofilms, facilitating further colonization by microorganisms and forming communities with metabolic interactions that might include species with diverse functions (e.g., plastic degradation), which also depends on microplastics' characteristics (e.g., polymer type)[119]. Therefore, microplastics can be considered a novel niche habitat, with longer environmental persistence than natural organic substrates and with hydrophobic surfaces prone to colonization and biofilm formation[120]. A concerning fact is that the microbial communities associated with microplastics differ from those in the surrounding ecosystem, potentially giving rise to microbial invasion, and some contain opportunistic pathogens (e.g., *Vibrio*)[121,122]. Therefore, there is a growing need for research on the plastisphere, not only due to its potential adverse impacts on ecosystems and health but also for the prospects of identifying naturally occurring plastic-degrading species (or communities) that could contribute to mitigating plastic pollution.

Effects of microplastics on human health

Microplastics enter the food chain through water, feed, food, and atmospheric contamination. Moreover, smaller microplastics in suspension in the atmosphere can be inhaled. Human exposure to microplastics through ingestion and inhalation has been estimated as 2.93×10^{10} microplastics·$year^{-1}$[123]. However,

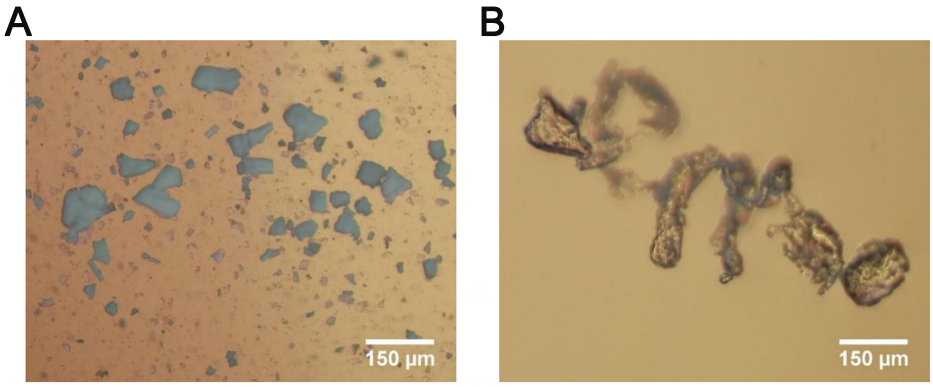

Figure 8. (A) Commercially available polystyrene microplastics; and (B) polypropylene microplastics produced in a coffee grinder in the laboratory.

estimations vary depending on the food types included in the model and the methods used in the original articles (e.g., size limit of detection). An accurate estimation based on uniform methods and estimated daily exposure models is needed. Inhalation is often overestimated due to the use of results from total suspended particulates instead of inhalable or respirable particles (< 100 µm or < 10 µm, respectively). Dermal exposure is not relevant due to the thickness of the stratum corneum. A study of 102 participants found significant correlations between the concentrations of microplastics in feces with exposure to dust and the consumption of bottled water and take-away food[121], supporting inhalation and diet as the main exposure routes.

After exposure of the digestive or respiratory system, only a small amount of smaller microplastics is translocated to internal tissues (< 1%)[122,124]. Some microplastics will find their way into the blood and become systemically distributed until they get caught into narrow capillary beds (e.g., < 5 µm in human lungs)[125] or are removed by the macrophages of the reticuloendothelial system[126]. Microplastics are then eliminated into the bile or the gastrointestinal lumen, where they will be excreted in the feces[127]. Concentrations as high as 139 microplastics·g^{-1} have been found in human feces[128] Additionally, microplastics have been found in human samples including saliva, sputum, bronchoalveolar lavage fluid, pulmonary tissue, blood, liver, kidney, placenta, and breast milk[129].

Despite knowledge gaps, there is no evidence to support the impacts of microplastics on human health[130]. For instance, a single intravenous injection of millions of 3-12 µm microplastics in dogs did not induce significant health effects for up to 4 weeks[131]. However, studies have generally been conducted using pristine microplastics and under acute exposure conditions. The release of monomers, additives, degradation products, adsorbed contaminants, and transport of pathogens may result in different effects of environmental exposure to microplastics, requiring further research. Moreover, microplastics can have adverse effects even when not internalized. Indeed, microplastics may change the human gut microbiome, which is recognized for its importance to human health, leading to dysbiosis[132]. The distribution and toxicity of microplastics may be further clarified by testing radiolabeled particles. Potential pathways of toxicity for microplastics could involve oxidative stress, cytotoxicity, and inflammation.

CONCLUSION

The findings gathered in the "Do's and Don'ts of Microplastic Research" webinar envisaged the generation of a beginner's guide for researchers working with microplastics. Another objective was to identify current challenges related to microplastics contaminants in this rapidly emerging field. Overall, the major identified

challenges include: (i) the absence of standardized methods; (ii) lack of reference materials; (iii) hindered access to highly expensive equipment; and (iv) resolution problems when working with smaller environmental concentrations (e.g., toxicity assays). To overcome these critical issues, the implementation of a harmonized guideline or standard is warranted and may be achieved through more active and widespread cooperation between researchers of different fields and institutions with paired expertise. During the guideline or standard development, the reference materials should be clearly defined, and thus a comprehensive list of their properties will ensure manufacturing consistency. The highly expensive and specific equipment required for the advanced analysis of microplastics may be circumvented in the short term through a broader collaboration between institutions. Nevertheless, higher funding should be oriented to this pivotal area by governments and funding agencies, for a sustained generation of ubiquitous high-quality microplastic research. With up-to-date equipment, the resolution limitations (i.e., smaller sizes) may be mitigated. Therefore, higher interconnectivity between researchers and institutions is extremely important and should be actively fomented. Moreover, researchers should be involved in dissemination campaigns to increase the public and government's awareness of the importance of microplastic research.

DECLARATIONS

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Authors' contributions

Substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data for the work: Prata JC

Drafting the work or revising it critically for important intellectual content: Prata JC, Padrão J, Khan MT, Walker TR

Final approval of the version to be published: Prata JC, Padrão J, Khan MT, Walker TR

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: Prata JC, Padrão J, Khan MT, Walker TR

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Ethical approval and consent to participate

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Consent for publication

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