



ADVANCING TOWARDS LITTER - FREE
ATLANTIC COASTAL COMMUNITIES
BY PREVENTING AND REDUCING
MACRO AND MICRO LITTER

D2.2.3. HARMONISED PROTOCOLS FOR SAMPLE PREPARATION: MICROPLASTICS EXTRACTION AND ISOLATION

WORK PACKAGE 2: ASSESSING AND MONITORING MICRO AND MACRO
LITTER CONTAMINATION. ACTIVITY 2: IMPROVED MICROPLASTICS
MONITORING, TASK 3: OPTIMISING SAMPLE PREPARATION PROTOCOLS
TO EXTRACT AND ISOLATE MICROPLASTICS FOR THEIR
CHARACTERISATION



Work package	WP2: Assessing and Monitoring micro and macro litter contamination
Activity and task	A2: Improved microplastics monitoring Task 3: Optimising sample preparation protocols to extract and isolate microplastics for their characterisation
Date	15 / 09 / 2026
Version	2
Authors	CIIMAR: SARA COUTO LOURENÇO, VÂNIA FREITAS, SABRINA RODRIGUES, RÚBEN PEREIRA, FRANCISCA ESPINCHO, DIOGO SILVA, CLÁUDIA MOURA, SANDRA RAMOS, C. MARISA R. ALMEIDA
Participants	ARDITI, CEDRE, IEO, IFREMER

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1. BACKGROUND

Primary and secondary microplastics are increasingly becoming the focus of concern due to their pervasive nature in the environment (Kamel et al., 2024). Despite the numerous studies already published on microplastic monitoring, there is still an urgent need to establish harmonised guidelines for the collection, quantification and reporting of plastic particles, so that comparisons among different locations can be made and effective actions can take place (Bessa et al., 2019; Bhardwaj et al., 2024; Borja & Elliott, 2021).

Microplastic monitoring involves several steps that must be fully optimised and validated to obtain reliable reports on the presence and characterisation of microplastics, thereby contributing to baseline assessments, for instance, within the framework of the Marine Strategy Framework Directive (MSFD) (MSFD Technical Group on Marine Litter, 2023). However, monitoring micro-litter remains a major challenge, as its small size makes it harder to detect, being more pervasive and associated with numerous potential impacts that are still poorly understood (Ramos et al., 2024). Over recent years, growing efforts have focused on improving the detection and monitoring of micro-litter pollution, with attempts to standardise sampling, sample processing and analytical methods (Bessa et al., 2019; OSPAR, 2024). However, further work is still required to strengthen monitoring capabilities through harmonised protocols.

In this context, Activity 2 of Work Package 2 (WP2) of the Free LitterAT project aims to propose harmonised guidelines for microplastics monitoring in the aquatic environment across multiple matrices, including water, sediment and biota. Specifically, it focuses on optimising protocols for sampling, sample processing and preparation for microplastic analysis and to test, enhance, and refine both established and innovative methods for microplastic quantification and characterisation. These developments will contribute to obtaining validated, comparable and reproducible data on microplastic contamination in the aquatic environment, thereby supporting the establishment of robust baselines in line with the requirements of the MSFD.

This Deliverable addresses the complexities and methodological challenges of sample preparation for micro-litter extraction and isolation. It follows WP2 Deliverable 2.2.1. *Harmonised Sampling Guidelines to Monitor Microplastics in Different Matrices* which addresses the complexities and methodological challenges of micro-litter sampling and precedes Deliverable 2.2.4. *Guidelines for Microplastics Quantification and Analysis*, which presents guidelines for microplastic quantification and characterisation.



All three deliverables are available online on the Free LitterAT project website (<https://freelitterat.eu/assessing-and-monitoring-marine-litter/>) and in Zenodo:

- D2.2.1. - <https://doi.org/10.5281/zenodo.21035158>;
- D2.2.3 - <https://doi.org/10.5281/zenodo.22771751>;
- D2.2.4. - <https://doi.org/10.5281/zenodo.22771805>.

The document provides harmonised guidelines for sample preparation procedures across different environmental matrices, outlining appropriate methods (including material, equipment and reagents) tailored to different sample types (water, sediment and biota), while ensuring the integrity of the micro-litter. It also describes appropriate procedures depending on sample characteristics and suitable filter selection based on the intended characterisation technique. Additionally, it includes a dedicated section focused on quality assurance and control to minimise contamination and ensure the reliability and comparability of results across studies.

A matrix-based approach is followed throughout the document in which similar sections are repeated across matrices but adapted to the specific characteristics of each sample type. This approach allows users to easily identify and apply the procedures relevant to the matrix of interest without the need to consult the entire document.

The content of this Deliverable is built upon the European Union's Guidelines on Marine Litter Monitoring in European Seas [with inputs by the MSFD Technical Group on Marine Litter (MSFD Technical Group on Marine Litter, 2023)] which address marine waters, seafloor and coastal sediments, and biota. These are complemented by other official guidelines, including GESAMP (GESAMP, 2019), focusing on marine waters, seabed sediments and biota, NOAA (Masura et al., 2015) focusing primarily on marine and freshwater waters and sediments, and the OSPAR Microplastic Expert Group (OSPAR, 2024) with emphasis on seafloor sediments, marine surface waters and biota. In addition, it is supported by a comprehensive literature review (Bessa et al., 2019; Dris et al., 2024; Frias et al., 2018; Mahapatra et al., 2024; Withana et al., 2024; Zhao et al., 2024) and input from the practical expertise gained by the project team (Espincho et al., 2024; Moura et al., 2025; Rivoira et al., 2020; Rodrigues et al., 2023; Rodrigues et al., 2019; Silva et al., 2022) and its partners (Cadiou et al., 2020; Filgueiras et al., 2019; Michela et al., 2021; Olga et al., 2021) to include other matrices not mentioned in the referred guidelines.

By encompassing diverse environmental matrices, recognising current methodological limitations and indicating harmonised and cost-effective solutions, this



Deliverable introduces a novel and comprehensive framework to enhance microplastic monitoring capacity and comparability.



WATER

2. WATER SAMPLE PREPARATION

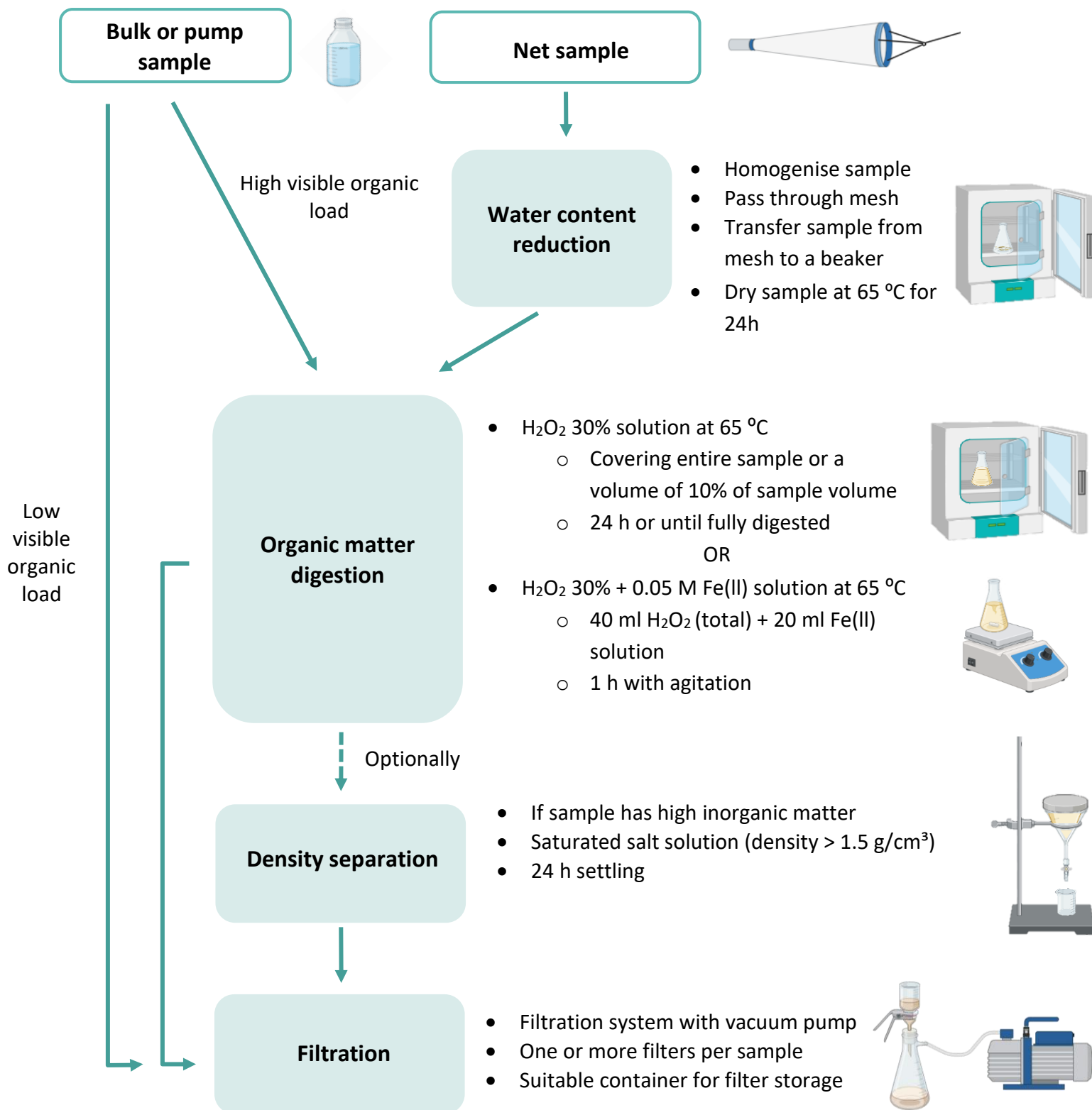


Figure 1. Suggested workflow of the procedural steps for water sample preparation.



WATER

2.1. Material, equipment and reagents

The following lists include materials, equipment and reagents that may be required depending on the selected protocol. The appropriate items should be selected according to the sample type, procedure and subsequent particle characterisation technique.

MATERIAL AND EQUIPMENT

1. Glass beakers
2. Glass funnels
3. Funnel support
4. Metal mesh
5. Metal tools
6. Aluminium foil
7. Stirring magnet
8. Filters (choose according to the intended particle characterisation technique)
 - a. nitrate cellulose
 - b. glass fibre
 - c. quartz fibre
 - d. PTFE membranes
 - e. polycarbonate filters
 - f. aluminium oxide (Anodisc) membranes
 - g. zinc selenide windows
 - h. silicon filters
9. Glass Petri dishes
10. Vacuum filtration system
11. Oven
12. Heating plate

REAGENTS

1. Deionised water
2. Hydrogen peroxide (H_2O_2 , 15% or 30%)
3. Potassium hydroxide (KOH , $\leq 10\%$)
4. Ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)
5. Sulfuric acid (H_2SO_4 , concentrated)
6. Enzymes
7. Saturated salt solution (choose one suitable for your lab work)
 - a. sodium chloride (NaCl)
 - b. calcium chloride (CaCl_2)
 - c. zinc chloride (ZnCl_2)
 - d. sodium iodide (NaI)
 - e. sodium Tungstate (Na_2WO_4)
 - f. potassium carbonate (K_2CO_3)
 - g. sodium bromide (NaBr)
 - h. potassium formate (HCOOK)
8. Ethanol or isopropanol

2.2. Suggested procedure

See Figure 1 for a suggestion of the water sample preparation workflow based on the works of Cadiou et al. (2020), Dris et al. (2024), Galgani et al. (2013), Mahapatra et al. (2024), Masura et al. (2015), Nayebi et al. (2023) and Rodrigues et al. (2019). Bulk or pump samples with low turbidity and no visible biological material may be filtered directly, proceeding to Section 2.2.4 *Filtration*. Samples with a high organic load should be subject to an organic matter digestion step prior to filtration, following the procedure



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described in Section 2.2.2 *Organic matter digestion*. For samples collected using a net-based method, preparation should begin with Section 2.2.1 *Water content reduction*, followed by organic matter digestion, as net samples generally concentrate planktonic and detrital organic material.

2.2.1. *Water content reduction*

This step is required when processing net samples, as these typically contain higher water amounts. Reducing the water content facilitates the subsequent digestion of the organic matter and improves processing efficiency.

Suggested protocol

1. Homogenise the water samples by stirring
2. Pass the sample through a mesh size appropriate to the study objective (the mesh size should be defined according to the target particle size)
3. Using a metal spatula, transfer the material retained on the mesh to a beaker and cover it with aluminium foil
4. Place the beaker in an oven at 65 °C for 24h
 - a. For faster drying, samples may be dried at 90 °C overnight for a maximum of 12 h (Rodrigues et al., 2019)
5. Proceed to Section 2.2.2. *Organic matter digestion*

2.2.2. *Organic matter digestion*

Select one of the following treatment types based on sample characteristics (Table 1):

- **Oxidative:** H₂O₂ 30% solution;
- **Fenton oxidation:** H₂O₂ 30% + 0.05 M Fe(II) solution.

NOTE:

- a. Acid digestion is not recommended, as strong acids may affect synthetic polymers and compromise microplastic identification.
- b. Use a validated protocol that preserves microplastics integrity.



WATER

TABLE 1: DIGESTION OF ORGANIC MATTER - TREATMENT SELECTION BASED ON SAMPLE CHARACTERISTICS

Sample characteristics	Recommended treatment	Observations
High organic content	Oxidative (H_2O_2) or Fenton oxidation [$H_2O_2 + Fe(II)$]	Preserves most polymers Fenton oxidation is less time-consuming
Mineral-rich	Oxidative (H_2O_2)	May require additional density separation

Suggested protocols

- **Oxidative digestion - recommended for bulk and pump samples**
 1. Add directly to the water sample a volume of H_2O_2 30% solution equivalent to 10% of the sample volume
 2. Incubate the samples in an oven at 65 °C for 24 h (do not exceed the referred time)
 - a. The temperature and time of exposure were tested to ensure microplastics integrity (Pereira et al., 2026; Rodrigues et al., 2019; Silva et al., 2022)
 3. Remove the sample from the oven immediately after digestion is complete
 4. Proceed to filtration (Section 2.2.4. *Filtration*) or perform a density separation (Section 2.2.3. *Density separation*) if needed
- **Fenton oxidation - recommended for net samples (Rodrigues et al., 2019)**
 1. Add 20 mL of aqueous 0.05 M Fe(II) solution (7.5 g of $FeSO_4 \cdot 7H_2O$ in 500 mL of water and 3 mL of concentrated sulfuric acid) directly to the sample
 2. Add 20 mL of 30% H_2O_2 solution to the sample
 3. Heat the mixture in a hotplate at 65 °C with agitation for 30 min (until gas bubbles appear on the surface)
 - a. If no reaction occurs, the temperature can be increased up to 75 °C

- b.** If the reaction is excessive (intense bubbling rising rapidly), remove the sample from the heating plate until it stabilises to prevent overflow and particle loss
- 4.** When the reaction stops, add another 20 ml of H₂O₂ 30% solution to the sample
- 5.** Heat at 65 °C with agitation for 30 min
 - a.** If the reaction is excessive (intense bubbling rising rapidly), remove the sample from the heating plate until it stabilises to prevent overflow and particle loss
- 6.** Proceed to filtration (Section 2.2.4. *Filtration*) or perform a density separation (Section 2.2.3. *Density separation*) if needed

If the digested sample contains a significant amount of inorganic matter, apply a density separation to isolate the microplastics. When not necessary, skip to Section 2.2.4 *Filtration*.

- sodium chloride (NaCl);
- calcium chloride (CaCl_2);
- zinc chloride (ZnCl_2);
- sodium iodide (NaI);
- sodium polytungstate (Na_2WO_4);
- potassium carbonate (K_2CO_3);
- sodium bromide (NaBr);
- potassium formate (HCOOK).

- a. NaCl: As the lowest-density salt option, ensure that no particles are retained within the settled inorganic material. May corrode metal equipment.
- b. CaCl_2 : Effective for density separation but may corrode metal equipment.
- c. ZnCl_2 : Highly effective due to its high density, but incompatible with KOH due to possible flocculation. Handle with extreme care due to its corrosive and toxic properties. Solutions containing ZnCl_2 must be treated as hazardous waste and disposed of accordingly.
- d. NaI: Effective and suitable for use with KOH. May corrode metal equipment when in solution.



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Suggested protocol

1. Add sufficient salt to the digested sample to saturate the solution - amount depends on salt used (see notes below)
2. Stir gently with a metal spoon or non-metallic utensil
3. Transfer the saturated solution to the density separator (e.g., funnels)
4. Let the inorganic matter settle for 24 h (microplastics will float)
5. Recover the separated fractions
 - a. Collect the settled bottom fraction and visually inspect it for potentially trapped microplastics, using a stereomicroscope when needed. If microplastics are observed, recover them where possible or repeat the density separation. If no microplastics are detected, discard this fraction.
 - b. Collect the suspended fraction separately and proceed to Section 2.2.4. *Filtration*.

Suggestion

- When using NaCl, add 3 g of salt per 10 ml sample solution
- If microplastics remain in the residue, repeat the density separation using a denser salt, with a density $> 1.5 \text{ g/cm}^3$

NOTE:

- a. Use a pro analysis grade salt to minimise potential microplastic contamination from the salt itself. The presence of microplastics in the salt can be assessed by dissolving a known mass of salt in a selected volume of deionised water, filtering the solution and check of microplastic presence in the filter.
- b. It is suggested to measure and record the density of the saturated solution after fraction recovery, using a hydrometer or a density meter to confirm that the density was within the expected range for the salt used.

2.2.4. Filtration

The samples should be filtered immediately after organic matter digestion or density separation. Select the adequate filter type according to the intended analytical technique for subsequent visual characterisation of particles and polymer identification (Table 2):

- **For visual and fluorescence characterisation:** cellulose nitrate membrane filters (0.45 μm), glass fibre filters (0.3 μm , 0.7 μm or 1.2 μm) or quartz fibre filters are



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recommended for visual characterisation; aluminium oxide filters or black polycarbonate filters are more recommended for fluorescence characterisation;

- **For polymer identification:** select a filter compatible with the chosen analytical technique, considering whether the particles will be manually transferred to the equipment or analysed directly on the filter surface.

TABLE 2: RECOMMENDED FILTER TYPES BASED ON PARTICLE VISUALISATION AND POLYMER IDENTIFICATION TECHNIQUES

Characterisation technique	Recommended filter types		
Visual analysis with particles manually transferred to the equipment	Nitrate cellulose	Glass fibre	Quartz fibre
Fluorescence	Aluminium oxide membrane (Al ₂ O ₃)	Black polycarbonate membrane	
Raman spectroscopy	Silicon membranes	Metal-coated polycarbonate (Au/Al, dark-field)	Polytetrafluoroethylene (bright-field)
IR spectroscopy	Gold-coated polycarbonate (reflection)	Aluminium oxide membrane (Al ₂ O ₃)	Zinc selenide, silicon (transmission)
GC/MS, Pyrolysis-GC/MS	Glass fibre	Quartz fibre	Metallic filter (particles can be recovered)

Suggested protocol

1. Filter the entire digested sample (or the suspended fraction, if density separation was performed) using a glass filtration system, a vacuum pump and the selected filter
2. Rinse the sample container thoroughly with deionised water to ensure that no particles remain attached
3. Use one filter per sample (more if necessary)



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4. Carefully remove the filter from the filtration system using metal tweezers
5. Place the filter in a pre-cleaned glass Petri dish or other suitable container, depending on the analytical technique selected for particle characterisation
6. Allow the filters to dry at room temperature in a glass container with a lid (not sealed) to avoid airborne contamination
7. Store in a dry and dark environment until particle characterisation



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3. SEDIMENT SAMPLE PREPARATION

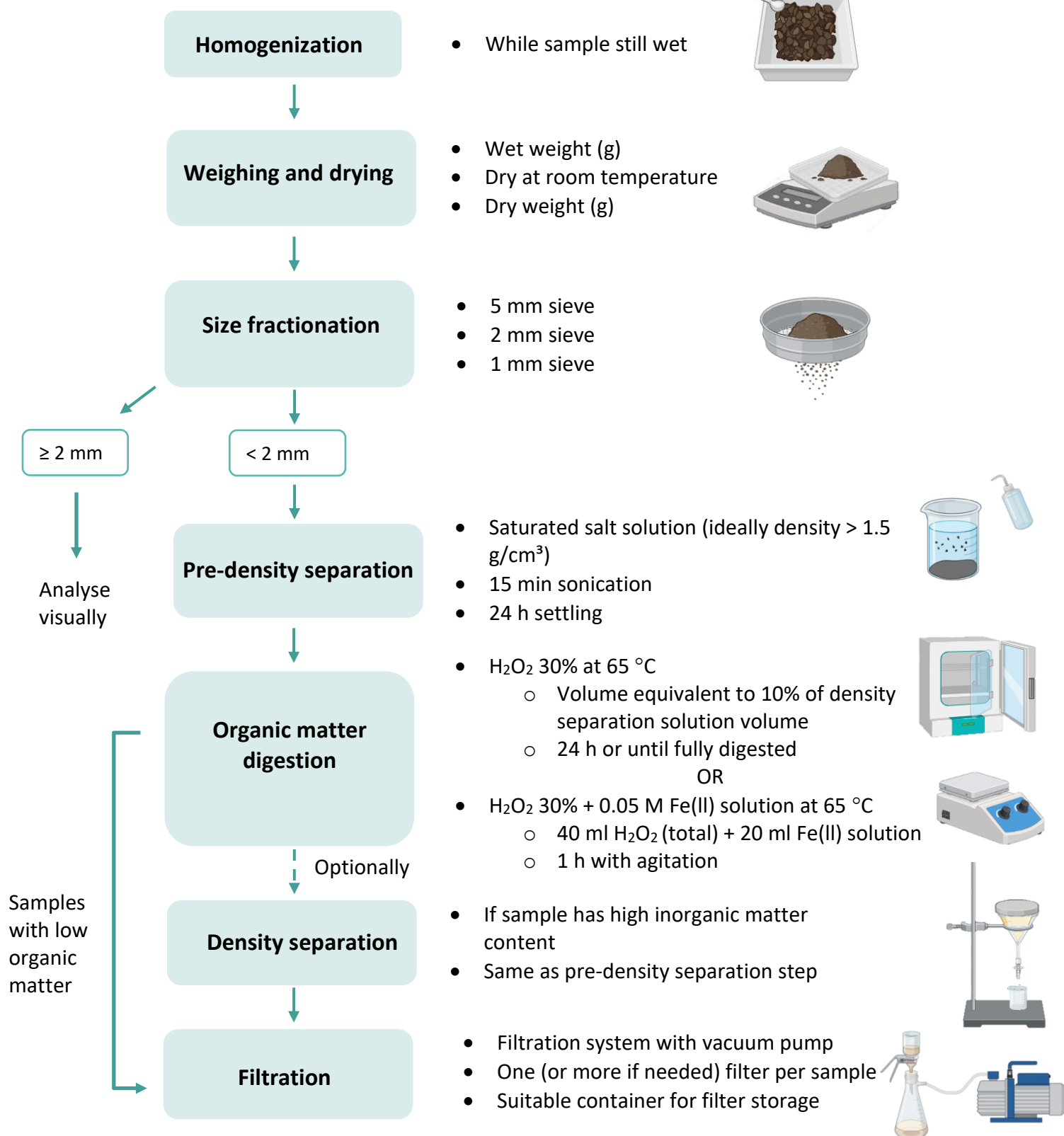


Figure 2. Suggested workflow of the procedural steps for sediment sample preparation.



SEDIMENT

3.1. Material, equipment and reagents

The following lists include materials, equipment and reagents that may be required depending on the selected protocol. The appropriate items should be selected according to the sample type, procedure and subsequent particle characterisation technique.

MATERIAL AND EQUIPMENT

1. Glass beakers
2. Glass funnels
3. Funnel support
4. Metal sieves
5. Metal tools
6. Trays/containers
7. Aluminium foil
8. Analytical balance
9. Oven
10. Heating plate
11. Stirring magnet
12. Filters (choose according to the intended particle characterisation technique)
 - a. nitrate cellulose
 - b. glass fibre
 - c. quartz fibre
 - d. PTFE membranes
 - e. polycarbonate filters
 - f. aluminum oxide (Anodisc) membranes
 - g. zinc selenide windows
 - h. silicon filters
13. Glass Petri dishes
14. Vacuum filtration system

REAGENTS

1. Ethanol or isopropanol
2. Deionised water
3. Triton X-100
4. Saturated salt solution (choose one suitable for your lab work)
 - a. sodium chloride (NaCl)
 - b. calcium chloride (CaCl₂)
 - c. zinc chloride (ZnCl₂)
 - d. sodium iodide (NaI)
 - e. sodium Tungstate (Na₂WO₄)
 - f. potassium carbonate (K₂CO₃)
 - g. sodium bromide (NaBr)
 - h. potassium formate (HCOOK)
5. Hydrogen peroxide (H₂O₂, 15% or 30%)
6. Ferrous sulfate heptahydrate (FeSO₄·7H₂O)
7. Sulfuric acid (H₂SO₄, concentrated)
8. Potassium hydroxide (KOH, ≤10%)
9. Enzymes

3.2. Suggested procedure

See Figure 2 for an overview of the suggested sediment sample preparation workflow based on the works of Almeida et al. (2023), Cadiou et al. (2020), Filgueiras et al. (2019),



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Frias et al. (2018), G rigny et al. (2024), Mahapatra et al. (2024), Masura et al. (2015), Michela et al. (2021), Olga et al. (2021), OSPAR (2024) and Rivoira et al. (2020). The method was optimised as part of this work and the details of the optimisation process are provided in the “Sediment Protocol Optimization Report” in the Annex.

NOTE: Within the framework of Free LitterAT, a complementary protocol was proposed for the specific preparation of sediment samples to be analysed by pyrolysis-GC/MS. The protocol involves digestion with H₂O₂, followed by density separation using CaCl₂ and filtration of the extract onto a quartz filter. The filters are subsequently dried and grinded before a sub-sample is analysed by pyrolysis-GC/MS. Further details are provided in Deliverable 2.5.5.

3.2.1. Homogenization

1. Transfer each sample to a separate metal container
2. Homogenise each sample by mixing thoroughly the sediment while still wet, using a metal spatula or spoon (carefully to avoid breaking microplastics)
3. Proceed to Section 3.2.2. *Weighing and drying*

3.2.2. Weighing and drying

1. Weigh the entire sample, ensuring that there is sufficient sediment mass to obtain the required number of replicates
2. Record the wet weight (g)
3. Dry the sample at room temperature (~20 °C), protected from external contamination

NOTE:

- a. Drying may take several days - if necessary, dry the sediment in an oven at 40 °C to accelerate the process.
 - b. For sediments with low organic matter content, the drying step may be omitted to avoid increasing the adhesion of microplastics to sediment particles. In this case, skip to step 5.
4. Once fully dried, weigh the dry sample and record the dry weight (g) - to calculate the water percentage of the sample
 5. Optionally, perform sediment characterisation on a separate replicate, including:
 - a. Carbonate content (%) and organic carbon (%);

b. Organic content (%; to be determined by loss on ignition at 550 °C);

c. Grain-size distribution expressed as: silt and clay (<63 µm, %); fine sand (≥63–250 µm, %); medium sand (>250–1000 µm, %); coarse sand (>1000–2000 µm, %); gravel (>2000 µm, %).



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- b. CaCl_2 : Effective for density separation but may corrode metal equipment.
 - c. ZnCl_2 : Highly effective due to its high density, but incompatible with KOH due to possible flocculation. Handle with extreme care due to its corrosive and toxic properties. Solutions containing ZnCl_2 must be treated as hazardous waste and disposed of accordingly.
 - d. NaI: Effective and suitable for use with KOH. May corrode metal equipment when in solution.
- Filter the saturated salt solution with the same type of filter used for the samples, to remove any impurities and have a clear solution (recommended)

2. Density separation

- For each sample, weigh 100 g of sieved sediment into a small glass beaker - the same beaker will be used for the subsequent density separation step

NOTE: If the sediment is highly muddy, with low grain size, it is highly recommended to reduce the mass to 20 g and prepare multiple analytical replicates to reach the 100 g.

- Add the saturated salt solution to each sediment replicate until $\frac{3}{4}$ of the beaker is full
 - Optionally, add one drop of Triton X-100 to each beaker to reduce surface tension and reduce sediment aggregation
- Stir gently using a metal spoon and sonicate for 15 min at 35 kHz (avoid overheating)

NOTE:

- a. This step, as well as the adding of the surfactant, is suggested to ensure that particles are completely dissociated from organic and inorganic matter, increasing the recovery of microplastics.
 - b. The sonication step was tested for microplastics integrity and efficiency in microplastics recovery (see “Sediment Protocol Optimization Report” in annex).
- Clean the exterior of the beaker containing the sediment sample with deionised water and place it inside a clean, larger beaker
 - Carefully, add additional saturated salt solution until the inner beaker is nearly full
 - Allow to settle for 24 h or until the supernatant becomes visibly clear



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- If necessary, gently stir the floating layer during this period to facilitate particle release

3. Overflow

- After 24h, carefully add more saturated salt solution to the inner beaker to overflow the floating fraction into the outer beaker
 - If necessary, slightly tilt the inner beaker to facilitate overflow
- Collect the overflow for further processing

NOTE: If the overflow sample contains substantial visible debris:

- a. Filter the solution through a fine filter cloth with a mesh size $<30\ \mu\text{m}$, made of a suitable material for particle recovery. Be aware that this step will result in the loss of the small particles, depending on the mesh used.
 - b. Rinse the material retained on the cloth with deionised water into a clean beaker and store for further organic matter digestion, which should be done following the Fenton oxidation procedure (see Section 3.2.5. *Organic matter digestion*).
- Proceed to Section 3.2.5. *Organic matter digestion*

3.2.5. *Organic matter digestion*

Select one of the following treatments based on sample characteristics and study objectives (Table 3):

- **Oxidative:** H_2O_2 30% solution;
- **Fenton oxidation:** H_2O_2 30% + 0.05 M Fe(II) solution.

NOTE:

- a. Acid digestion is not recommended, as strong acids may affect synthetic polymers and compromise microplastic identification.
- b. Use validated protocols that preserve microplastics integrity.



SEDIMENT

TABLE 3: DIGESTION OF ORGANIC MATTER - TREATMENT SELECTION BASED ON SAMPLE CHARACTERISTICS

Sample characteristics	Recommended treatment	Observations
High organic content	Oxidative (H_2O_2) or Fenton oxidation [$H_2O_2 + Fe(II)$]	Preserves most polymers Fenton oxidation is less time consuming
Mineral-rich	Oxidative (H_2O_2)	May require additional density separation

Suggested protocols

- Oxidative digestion**

1. Add 30% H_2O_2 solution directly to the sample at a volume corresponding to 10% of the sample volume (e.g., 10 ml of H_2O_2 for 100 ml of sample) - record the volume added
2. Incubate the samples in an oven at 65 °C for up to 48 h- do not exceed the referred time
 - a. The temperature and time of exposure were tested to ensure microplastics integrity (Pereira et al., 2026; Rodrigues et al., 2023; Silva et al., 2022)

NOTE: Samples with a high organic matter content may generate excessive foaming during the oxidative reaction. It is advisable to allow the samples to react at room temperature for a few hours before placing them in the oven. If foam forms, carefully rinse it down with deionised water (a minimal volume should be added to prevent diluting H_2O_2 solution) to prevent overflow from the beaker.

3. Remove the sample from the oven immediately after digestion is complete
4. Proceed to filtration (Section 3.2.7. *Filtration*) or perform a density separation (Section 3.2.6. *Density separation*) if needed

- Fenton oxidation (adapted from Rivoira et al., 2020)**

1. Add 20 mL of aqueous 0.05 M Fe(II) solution (7.5 g of $FeSO_4 \cdot 7H_2O$ in 500 mL of water and 3 mL of concentrated sulfuric acid) directly to the sample



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2. Add 20 mL of 30% H₂O₂ solution to the sample
3. Heat the mixture in a hotplate at 65 °C with agitation for 30 min (until gas bubbles appear in the surface)
 - a. If no reaction occurs temperature can be increased up to 75 °C
 - b. If the reaction is excessive (intense bubbling rising rapidly), remove the sample from the heating plate until it stabilises to prevent overflow and particle loss
4. When the reaction stops, add another 20 ml of H₂O₂ 30% solution to the sample
5. Heat at 65 °C with agitation for 30 min
 - a. If the reaction is excessive (intense bubbling rising rapidly), remove the sample from the heating plate until it stabilises to prevent overflow and particles loss
6. Proceed to filtration (Section 3.2.7. *Filtration*) or perform a density separation (Section 3.2.6. *Density separation*) if needed

- **Oxidative digestion (Gerigny et al., 2024)**

1. Add H₂O₂ 30% with a volume of 10% of the sample volume (e.g., 10 ml H₂O₂ for 100 ml of sample) - record the volume
2. Heat to 40°C, stirring, for 8 to 12 h
3. Proceed to filtration (Section 3.2.7. *Filtration*) or perform a density separation (Section 3.2.6. *Density separation*) if needed

NOTE: Samples with a high organic matter content may generate excessive foaming during the oxidative reaction. It is advisable to allow the samples to react at room temperature for a few minutes (10-20 min) before heating. Prepare a cold-water bath and a drip tray if necessary to monitor the reaction. If foam forms, it may be carefully rinsed with deionised water to prevent overflow from the beaker.

3.2.6. *Density separation (if needed)*

Apply a density separation if the digested sample has a high inorganic matter content.

Suggested protocol

1. Add sufficient salt to the sample to saturate the solution - amount depends on salt used



SEDIMENT

2. Stir gently with a metal spoon
3. Sonicate samples for 15 min at 35 kHz (avoid overheating)
4. Transfer the saturated solution to the density separator (e.g., funnels or beakers)
5. Let the inorganic matter settle for 24 h (microplastics will float)
6. Recover the separated fractions
 - a. Collect the settled bottom fraction and visually inspect it for potentially trapped microplastics, using a stereomicroscope when needed. If microplastics are observed, recover them where possible or repeat the density separation. If no microplastics are detected, discard this fraction
 - b. Collect the suspended fraction separately and proceed to Section 3.2.7. *Filtration*

NOTE:

- a. Use a pro analysis grade salt to minimise potential microplastic contamination from the salt itself. The presence of microplastics in the salt can be assessed by dissolving a known mass of salt in a selected volume of deionised water, filtering the solution and check of microplastic presence in the filter.
- b. It is suggested to measure and record the density of the saturated solution after fraction recovery, using a hydrometer or a density meter to confirm that the density was within the expected range for the salt used.

3.2.7. Filtration

The samples are to be filtered after organic matter digestion or density separation. Select the adequate filter type according to the intended analytical technique for visual characterisation of particles and polymer identification (Table 4):

- **For visual and fluorescence characterisation:** cellulose nitrate membrane filters (0.45 µm), glass fibre filters (0.3 µm, 0.7 µm or 1.2 µm) or quartz fibre filters are recommended for visual characterisation; aluminium oxide filters or black polycarbonate filters are more recommended for fluorescence characterisation;
- **For polymer identification:** select a filter compatible with the chosen analytical technique, considering whether the particles will be manually transferred to the equipment or analysed directly on the filter surface.



SEDIMENT

TABLE 4: RECOMMENDED FILTER TYPES BASED ON PARTICLE VISUALISATION AND POLYMER IDENTIFICATION TECHNIQUES

Characterisation technique	Recommended filter types		
Visual analysis with particles manually transferred to the equipment	Nitrate cellulose	Glass fibre	Quartz fibre
Fluorescence	Aluminium oxide membrane (Al ₂ O ₃)	Black polycarbonate membrane	
Raman spectroscopy	Silicon membranes	Metal-coated polycarbonate (Au/Al, dark-field)	Polytetrafluoroethylene (bright-field)
IR spectroscopy	Gold-coated polycarbonate (reflection)	Aluminium oxide membrane (Al ₂ O ₃)	Zinc selenide, silicon (transmission)
GC/MS, Pyrolysis-GC/MS	Glass fibre	Quartz fibre	Metallic filter (particles can be recovered)

Suggested protocol

1. Filter the sample using a glass filtration system, a vacuum pump and the selected filter
2. Rinse the sample container thoroughly with deionised water to ensure that no particles remain attached
3. Use one filter per sample (more if necessary)
4. Carefully remove the filter from the filtration system using a metal tweezer
5. Place the filter in a pre-cleaned glass Petri dish or other suitable container, depending on the analytical technique selected for particle characterisation
6. Allow the filters to dry at room temperature in a container with a lid (not sealed) to avoid airborne contamination
7. Store in a dry and dark environment until particle characterisation



4. BIOTA SAMPLE PREPARATION

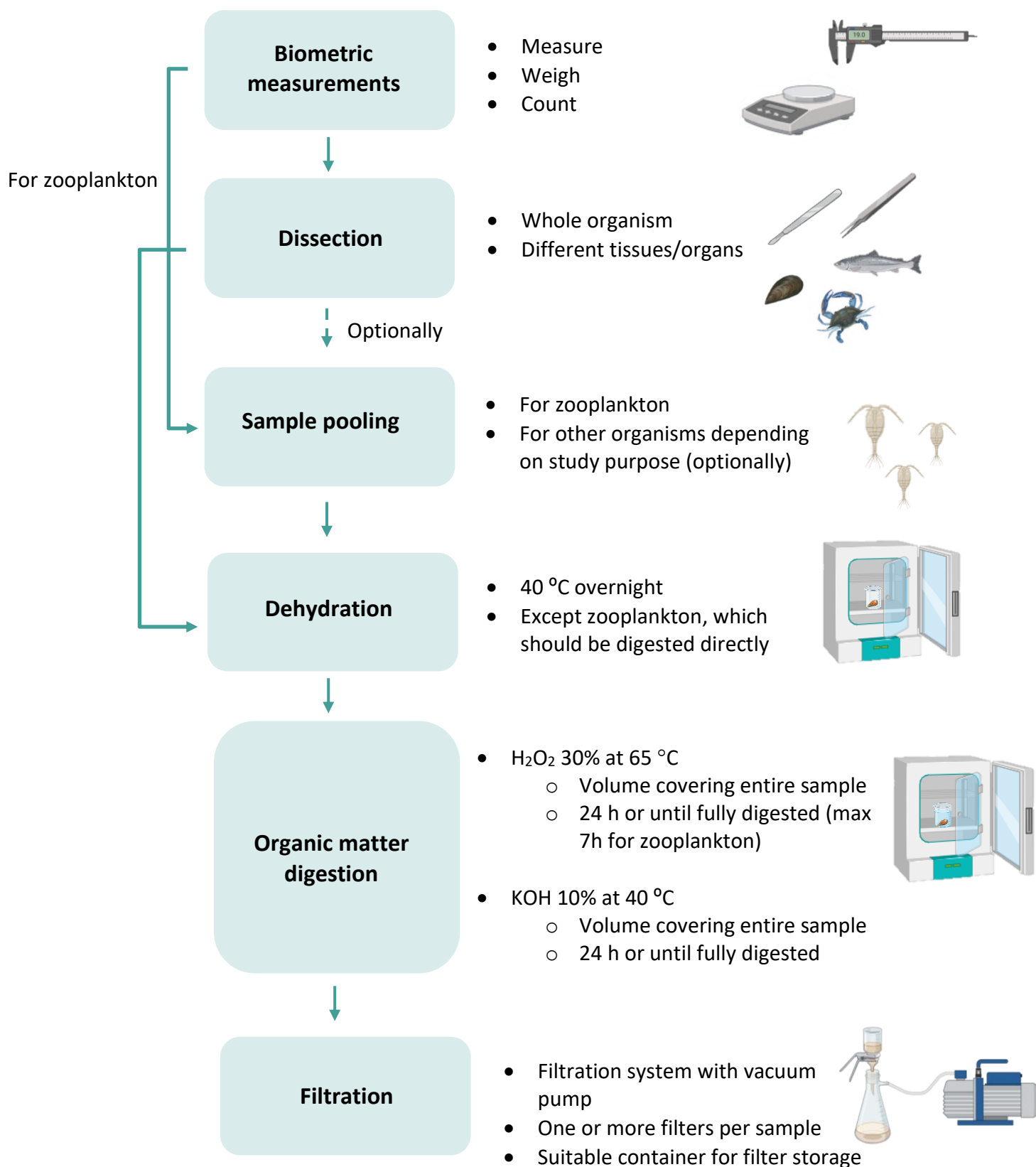


Figure 3. Suggested workflow of the procedural steps for biota sample preparation.



BIOTA

4.1. Material, equipment and reagents

The following lists include materials, equipment and reagents that may be required depending on the selected protocol. The appropriate items should be selected according to the sample type, procedure and subsequent particle characterisation technique.

MATERIAL AND EQUIPMENT

1. Dissecting tools
2. Analytical balance
3. Stereomicroscope
4. Glass beakers/vials
5. Aluminium foil
6. Oven
7. Heating plate
8. Stirring magnets
9. Filters (choose according to the intended microplastics characterisation technique)
 - a. nitrate cellulose
 - b. glass fibre
 - c. quartz fibre
 - d. PTFE membranes
 - e. polycarbonate filters
 - f. aluminium oxide (Anodisc) membranes
 - g. zinc selenide windows
 - h. silicon filters
10. Glass Petri dishes
11. Vacuum filtration system

REAGENTS

1. Deionised water or filtered seawater
2. Hydrogen peroxide (H_2O_2 , 15% or 30%)
3. Potassium hydroxide (KOH, $\leq 10\%$)
4. Enzymes
5. Ethanol or isopropanol
6. Liquid silicone (antifoam)

4.2. Suggested procedure

See Figure 3 for an overview of the workflow for biota sample preparation based on the works of Bessa et al. (2019), Espincho et al. (2024), MSFD Technical Group on Marine Litter (2023), Hermesen et al. (2018), Lusher et al. (2017), Moura et al. (2025), Rivoira et al. (2020), Rodrigues et al. (2023) and Silva et al. (2022). The procedures are adapted for different taxonomic groups, covering tissue collection, recording of biometric data and sample processing.



BIOTA

4.2.1. Crustaceans, echinoderms and bivalves

- **Tissue - depending on the study aim**
 - Whole organism
 - Isolated tissues or organs
- **Biometric data**
 - Total weight (wet weight)
 - Dry weight, to be determined using additional replicate samples
 - Tissue or organ weight (wet weight)
 - Length, width and other relevant measurement
 - Sex (optional)
 - Optional tissue composition, to be determined using additional replicate samples: lipid content (wet weight) and protein content (wet weight)

Suggested protocol

1. Weigh the whole organism (wet weight)
2. Measure length, height and width
3. Rinse the exterior of the organism with deionised water to ensure that no particle is attached
4. Remove the shell or exoskeleton, as appropriate
5. Collect the tissues of interest
6. Weigh the eviscerated tissues
7. Place the collected tissues in a clean glass container
 - a. Or wrap each sample in aluminium foil and store at -20 °C until further analysis
8. Proceed to Section 4.2.6. *Dehydration* or Section 4.2.7. *Organic matter digestion*

NOTE:

- a. Pools of organisms may be prepared when individual specimens are too small for analysis or when pooling is justified by the study objective.
- b. Preliminary tests should be performed to establish the maximum feasible pooled mass that can be processed reliably.



BIOTA

4.2.2. Cephalopods

- **Tissue - depending on the study aim**
 - Whole organism
 - Head/mantle
 - Tentacles/legs
- **Biometric data**
 - Total weight (wet weight)
 - Dry weight, to be determined using additional replicate samples
 - Tissue or organ weight (wet weight)
 - Length, width and other relevant measurements
 - Sex (optional)

Suggested protocol

1. Weigh the whole organism (wet weight)
2. Separate the head/mantle from the tentacles/legs
3. Remove all internal content from the head/mantle and discard it
4. Rinse the head/mantle tissue and tentacles/legs thoroughly with deionised water to remove any attached particles
5. Weigh the tissues collected (wet weight)
6. Place the collected tissues in a clean glass container
 - a. Or wrap each sample in aluminium foil and keep at -20 °C until further analysis
7. Proceed to Section 4.2.6. *Dehydration* or Section 4.2.7. *Organic matter digestion*

NOTE:

- a. Pools of organisms may be prepared when individual specimens are too small for analysis or when pooling is justified by the study objective.
- b. Preliminary tests should be performed to establish the maximum feasible pooled mass that can be processed reliably.

4.2.3. Teleosts

- **Tissue - depending on the study aim**
 - Muscle
 - Gastrointestinal tract (GT)



BIOTA

- Other organs
- **Biometric data**
 - Total weight (wet weight)
 - Dry weight, to be determined using additional replicate samples
 - Eviscerated weight (wet weight)
 - Tissue or organ weight (wet weight)
 - Total length - tip of snout to end of caudal fin
 - Fork length - tip of snout to fork of caudal fin
 - Standard length - tip of snout to base of caudal fin, excluding the caudal fin
 - Sex (optional)

Suggested protocol

1. Weigh the entire fish (wet weight)
2. Rinse the exterior of the fish thoroughly with deionised water to remove any attached particles
3. Dissect the fish and collect the GT
4. Weigh the eviscerated organ
5. Remove surrounding organs
 - a. Collect and weigh them if necessary for study purposes
6. Weigh the eviscerated fish
7. Collect fish muscle
8. Weigh the tissue
9. Rinse each sample with deionised water
10. Place the collected tissues in a clean glass container
 - a. Or wrap each sample in aluminium foil and keep at -20 °C until further analysis

NOTE:

- a. Pools of organisms may be prepared when individual specimens are too small for analysis or when pooling is justified by the study objective.
- b. Preliminary tests should be performed to establish the maximum feasible pooled mass that can be processed reliably.



4.2.4. Zooplankton

- Tissue
 - Whole organism

Suggested protocol (Rodrigues et al., 2019)

1. Place the organisms in a clean glass petri dish
2. Isolate each organism and examine individually under a stereomicroscope
3. Rinse each organism with deionised water to remove any externally attached particles
4. Sort organisms by species or taxonomic group, according to study objectives
5. Combine pools of organisms of the same species/group to obtain sufficient biomass for analysis
6. Transfer each pool into clean glass scintillation vials
7. Proceed directly to Section 4.2.7. *Organic Matter Digestion*
 - a. If necessary, store at -20°C until further processing

4.2.5. Macroalgae

- Tissue
 - Whole organism

Suggested protocol (Pereira et al., 2026)

1. Place each seaweed in a clean glass beaker
2. Wash seaweed with Triton X-100 solution, 1 % v/v, for 30 min to remove adsorbed microplastics
 - a. Use a wash bottle filled with the same Triton X-100 solution to assist washing
3. Filter the resulting washing solution to assess seaweed contamination through adsorption (proceed to Section 4.2.7. *Filtration*)
4. Dry the washed seaweed in an oven at 60°C until completely dried
5. Manually grind the dried seaweed as finely as possible and homogenise
6. Transfer 0.5 g of homogenised seaweed into a clean glass bottle
7. Proceed directly to Section 4.2.7. *Organic Matter Digestion*



4.2.6. Dehydration

This step is applied to reduce moisture content to improve organic matter digestion efficiency and reduce excessive foaming during oxidative treatments. It can be applied to all organisms except for zooplankton and macroalgae (see details in Sections 4.2.4 and 4.2.5, respectively).

Suggested protocol

1. Transfer the samples to glass containers and place them in an oven
 - a. The containers should be covered loosely with aluminium foil
2. Dry the samples at 40 °C overnight (maximum 24 h)
3. Proceed to Section 4.2.7. *Organic matter digestion*

4.2.7. Organic matter digestion

Select one of the following treatment types based on sample characteristics and research objectives (see Table 5):

- **Oxidative:** H₂O₂ 30% solution;
- **Fenton oxidation:** H₂O₂ 30% + 0.05 M Fe(II) solution;
- **Alkaline:** KOH at maximum 10%;
- **Enzymatic:** e.g., cellulase;
- **Combination** of the treatments above.

NOTE:

- a. Acid digestion is not recommended, as strong acids may affect synthetic polymers and compromise microplastic identification.
- b. Use validated protocols that preserve microplastics integrity.
- c. Optionally, add one drop of liquid silicone (prevents foam formation).



TABLE 5: DIGESTION OF ORGANIC MATTER - TREATMENT SELECTION BASED ON SAMPLE CHARACTERISTICS

Sample characteristics	Recommended treatment	Observations
High organic content	Oxidative (H_2O_2), Fenton oxidation [$H_2O_2 + Fe(II)$] or Alkaline (KOH)	Preserves most polymers Fenton oxidation is less time consuming
High organic content (cellulose-, protein- or lipid- rich)	Enzymatic (e.g., cellulase, protease, lipase) or combined (e.g., cellulase and H_2O_2)	High preservation of microplastics More expensive and time- consuming
Mineral-rich samples	Oxidative (H_2O_2)	May require additional density separation

Suggested protocols - crustaceans, echinoderms, bivalves, cephalopods and teleost

- **Oxidative digestion (Silva et al., 2022)**
 1. Add H_2O_2 30% solution until the sample is fully covered (record the volume)
 2. Incubate the samples in an oven at 65 °C for up to 48 h until complete digestion of the organic matter (do not exceed the referred time)
 - a. Samples with a high organic matter content may produce excessive foaming during the oxidative reaction. If needed, leave the samples at room temperature for a few hours before putting them in the oven. Once in the oven, monitor the reaction regularly.
 - b. A drop of liquid silicone can be added to prevent excessive foam formation (from team's own experience)
 - c. The temperature and time of exposure were tested to ensure microplastics integrity (Pereira et al., 2026; Rodrigues et al., 2019; Silva et al., 2022)
 3. Proceed to Section 4.2.8. *Filtration*



BIOTA

- **Alkaline digestion (Phuong et al., 2017; Lusher et al., 2017)**
 1. Add KOH 10% ensuring the sample is fully covered (record the volume)
 2. Heat at 60 °C for 24 h while stirring at 145 rpm
 3. Proceed to Section 4.2.8. *Filtration*

Suggested protocol - zooplankton

- **Oxidative digestion (Rodrigues et al., 2023)**
 1. Add 2 ml of H₂O₂ 30% to each scintillation vial ensuring the organisms are fully submerged and close the vial
 - a. Add a higher volume if necessary
 2. Incubate the samples in an oven at 65 °C for a maximum of 7 h
 - a. The temperature and time of exposure were tested to ensure microplastics integrity (Rodrigues et al., 2023)
 3. Remove the sample immediately after digestion is complete
 4. Proceed to Section 4.2.8. *Filtration*

Suggested protocol – macroalgae

- **Mixed digestion (Pereira et al., 2026)**
 1. Prepare a cellulase solution by diluting 0.5 g cellulase from *Aspergillus niger* in 50 mL acetate buffer (0.1 M, pH 5.0)
 2. Add 50 mL of 1% w/v cellulase solution to the glass bottle
 3. Place samples in the oven at 50 °C for 24 h
 - a. Manually agitate samples periodically during incubation
 4. After 24 h, add 100 mL of 30% v/v H₂O₂ solution
 5. Place samples in the oven at 65 °C for up to 72 h
 - a. Manually agitate samples periodically during digestion
 - b. Remove samples earlier if digestion is complete

NOTE: The protocol was tested to ensure microplastics integrity being suitable for most common microplastics. Rayon and acrylic show however some degradation (Pereira et al., 2026).

 6. Proceed to Section 4.2.8. *Filtration*



4.2.8. Filtration

The samples should be filtered after organic matter digestion. Select the adequate filter type according to the intended analytical technique for visual characterisation of particles and polymer identification (Table 6):

- **For visual and fluorescence characterisation:** cellulose nitrate membrane filters (0.45 µm), glass fibre filters (0.3 µm, 0.7 µm or 1.2 µm) or quartz fibre filters are recommended for visual characterisation; aluminium oxide filters or black polycarbonate filters are more recommended for fluorescence characterisation;
- **For polymer identification:** select a filter compatible with the chosen analytical technique, considering whether the particles will be manually transferred to the equipment or analysed directly on the filter surface.

TABLE 6: RECOMMENDED FILTER TYPES BASED ON PARTICLE VISUALISATION AND POLYMER IDENTIFICATION TECHNIQUES

Characterisation technique	Recommended filter types		
Visual analysis with particles manually transferred to the equipment	Nitrate cellulose	Glass fibre	Quartz fibre
Fluorescence	Aluminium oxide membrane (Al ₂ O ₃)	Black polycarbonate membrane	
Raman spectroscopy	Silicon membranes	Metal-coated polycarbonate (Au/Al, dark-field)	Polytetrafluoroethylene (bright field)
IR spectroscopy	Gold-coated polycarbonate (reflection)	Aluminium oxide membrane (Al ₂ O ₃)	Zinc selenide, silicon (transmission)
GC/MS, Pyrolysis-GC/MS	Glass fibre	Quartz fibre	Metallic filter (particles can be recovered)



BIOTA

Suggested protocol

1. Filter the sample using a glass filtration system, a vacuum pump and the selected filter
NOTE: For samples with high fat content:
 - a. Keep samples at a warmer temperature (~40 °C) to facilitate the filtration (filtration immediately after removing each flask from the oven);
 - b. If a layer of fat is formed at the surface, remove it for observation under a stereomicroscope while still warm and filter the remaining sample (Silva et al., 2022).
2. Rinse the sample container thoroughly with the minimum quantity of deionised water to ensure that no particles remain attached
3. Use one filter per sample (more if necessary)
4. Carefully remove the filter from the filtration system using a metal tweezer
5. Place the filter in a pre-cleaned glass Petri dish or other suitable container, depending on the analytical technique selected for particle characterisation
6. Allow the filters to dry at room temperature in a container with a lid (not sealed) to avoid airborne contamination
7. Store in a dry and dark environment until particle characterisation



5. LABORATORY QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance and control (QA/QC) measures are essential to ensure the accuracy, reliability and comparability of results obtained during microplastics analysis. The procedures described in this section follow the recommendations of the MSFD Technical Group on Marine Litter (MSFD Technical Group on Marine Litter, 2023), complemented by the extensive laboratory experience of our team.

5.1. Quality Assurance

Table 7 summarises the recommended laboratory quality assurance practices, including workspace management, operator precautions, material and equipment handling, and sample and solution control procedures. These measures should be consistently applied during sample preparation.

TABLE 7: LABORATORY QUALITY ASSURANCE	
Category	Best Practices
Workspace	Clean the laboratory regularly
	Minimise air circulation
	Use laminar flow chamber or fume hood if possible
	Clean workspace with filtered 70% ethanol solution
Operators	Wear cotton lab coats (preferably bright or uncommon colours)
	If using gloves previously wash them with deionised water and/or ethanol
	Avoid plastic fibre face masks
Material & Equipment	Use glass and metal tools
	Avoid plastics/synthetics
	Clean all equipment/tools with filtered 70% ethanol solution and deionised water before use and in-between samples
	Cover instruments with aluminium foil when not in use
Samples & Solutions	When possible, minimise sample handling steps
	Pre-filter all solutions with a pore size much smaller than the smallest target particle
	Keep samples and solutions always covered



QUALITY ASSURANCE AND QUALITY CONTROL

5.2. Quality Control

Table 8 summarises the recommended laboratory quality control procedures, including the use of blanks and controls, definition of limits of detection, assessment of protocol efficiency and recovery tests using reference samples.

TABLE 8: LABORATORY QUALITY CONTROL

Category	Recommendation
Blanks	Analyse blank samples alongside real samples (it is recommended that the number of blanks should be at least 10% of total sample number)
	Define the limit of detection (LOD) (mean + 3 × standard deviation of particle concentration) based on the number of microplastic particles found in field and laboratory blank samples
	Report both blank results and uncorrected sample results (generally it is not recommended to subtract these values but to report them)
Controls	Place a Petri dish with deionised water near your workspace during sample processing steps to monitor airborne contamination
	Use one Petri dish for each sample (or pool of samples) in each step of the protocol
	Petri dishes should only be open while the corresponding samples are exposed Report any airborne contamination
Protocol Efficiency	Always use a protocol that has been tested for efficiency and microplastics integrity
	If available, analyse reference samples to test protocol efficiency
Reference Samples (if available)	Spike samples with a known number of synthetic polymer particles (different sizes, shapes and polymers): - at least 50 reference particles for both fragments and fibres 3 types of polymers (different densities) 3 morphologies - sizes like the lower cut-off size (e.g., 100 µm)
	Process the reference samples in the same way as the other samples
	Calculate the recovery ratio (%) as the mean value of re-detected reference particles, accounting for size, morphology and polymer composition
	Minimum recovery ratio should be 80% for particles >100 µm



REFERENCES

6. REFERENCES

Bessa, F., Frias, J. P. G. L., Kögel, T., Lusher, A., Andrade, J. M., Antunes, J., Sobral, P., Pagter, E., Nash, R., O'Connor, I., Pedrotti, M. L., Keros, E., León, V., Tirelli, V., Suaria, G., Lopes, C., Raimundo, J., Caetano, M., Gago, J., & Gerdt, G. (2019). Harmonized protocol for monitoring microplastics in biota (D4.3 BASEMAN report). JPI-Oceans BASEMAN Project.

Bhardwaj, L. K., Rath, P., Yadav, P., & Gupta, U. (2024). Microplastic contamination, an emerging threat to the freshwater environment: a systematic review. *Environmental Systems Research*, 13(1), 8. <https://doi.org/10.1186/s40068-024-00338-7>

Borja, A., & Elliott, M. (2021). From an economic crisis to a pandemic crisis: The need for accurate marine monitoring data to take informed management decisions. In C. Sheppard (Ed.), *Advances in Marine Biology* (Vol. 89, pp. 79-114). Academic Press. <https://doi.org/10.1016/bs.amb.2021.08.002>

Cadiou, J. F., Gerigny, O., Š, K., Zeri, C., Kaberi, H., Alomar, C., Panti, C., Fossi, M. C., Adamopoulou, A., Digka, N., Deudero, S., Concato, M., Carbonell, A., Bains, M., Galli, M., & Galgani, F. (2020). Lessons learned from an intercalibration exercise on the quantification and characterisation of microplastic particles in sediment and water samples. *Marine Pollution Bulletin*, 154, 111097. <https://doi.org/10.1016/j.marpolbul.2020.111097>

Dris, R., Beaupaire, M., Bouzid, N., Stratmann, C., Nguyen, M. T., Bordignon, F., Gasperi, J., & Tassin, B. (2024). Chapter 3 - Sampling and analyzing microplastics in rivers: What methods are being used after a decade of research? In E. Y. Zeng (Ed.), *Microplastic Contamination in Aquatic Environments (Second Edition)* (pp. 65-91). Elsevier. <https://doi.org/10.1016/B978-0-443-15332-7.00013-2>

Espincho, F., Pereira, R., Rodrigues, S. M., Silva, D. M., Almeida, C. M. R., & Ramos, S. (2024). Assessing Microplastic Contamination in Zooplanktonic Organisms from Two River Estuaries. *Water*, 16(7), 992. <https://doi.org/10.3390/w16070992>

Filgueiras, A. V., Gago, J., Campillo, J. A., & León, V. M. (2019). Microplastic distribution in surface sediments along the Spanish Mediterranean continental shelf. *Environmental Science and Pollution Research*, 26(21), 21264-21273. <https://doi.org/10.1007/s11356-019-05341-5>

Frias, J., Pagter, E., Nash, R., O'Connor, I., Carretero, O., Filgueiras, A., Viñas, L., Gago, J., Antunes, J., Bessa, F., Sobral, P., Goruppi, A., Tirelli, V., Pedrotti, M. L., Suaria, G., Aliani, S., Lopes, C., Raimundo, J., Caetano, M., & Gerdt, G. (2018). *Standardised protocol for monitoring microplastics in sediments* May 2018. <https://doi.org/10.13140/RG.2.2.36256.89601/1>



REFERENCES

MSFD Technical Group on Marine Litter, Galgani, F., Ruiz-Orejón, L. F., Ronchi, F., Tallec, K., Fischer, E. K., Matiddi, M., Anastasopoulou, A., Andresmaa, E., Angiolillo, M., Bakker Paiva, M., Booth, A. M., Buhhalko, N., Cadiou, B., Clarò, F., Consoli, P., Darmon, G., Deudero, S., Fleet, D., Fortibuoni, T., Fossi, M.C., Gago, J., Gérigny, O., Giorgetti, A., González-Fernández, D., Guse, N., Haseler, M., Ioakeimidis, C., Kammann, U., Kühn, S., Lacroix, C., Lips, I., Loza, A. L., Molina Jack, M. E., Norén, K., Papadoyannakis, M., Pragnel-Raasch, H., Rindorf, A., Ruiz, M., Setälä, O., Schulz, M., Schultze, M., Silvestri, C., Soederberg, L., Stoica, E., Storr-Paulsen, M., Strand, J., Valente, T., van Franeker, J., van Loon, W. M. G. M., Vighi, M., Vinci, M., Vlachogianni, T., Volckaert, A., Weiel, S., Wenneker, B., Werner, S., Zeri, C., Zorzo, P., and Hanke, G., Guidance on the Monitoring of Marine Litter in European Seas An update to improve the harmonised monitoring of marine litter under the Marine Strategy Framework Directive, EUR 31539 EN, Publications Office of the European Union, Luxembourg, 2023, ISBN 978-92- 68-04093-5, doi:10.2760/59137, JRC133594 <https://doi.org/10.2760/59137>

Gerigny, O., Blanco, G., Lips, U., Buhhalko, N., Chouteau, L., Georges, E., Meyers, N., Vanavermaete, D., Galgani, F., Ourgaud, M., Papillon, L., Sempéré, R., & De Witte, B. (2024). Comparative analysis of microplastics detection methods applied to marine sediments: A case study in the Bay of Marseille. *Marine Pollution Bulletin*, 207, 116787. <https://doi.org/10.1016/j.marpolbul.2024.116787>

Kamel, A. H., Hefnawy, A., Hazeem, L. J., Rashdan, S. A., & Abd-Rabboh, H. S. M. (2024). Current perspectives, challenges, and future directions in the electrochemical detection of microplastics. *RSC Adv*, 14(3), 2134-2158. <https://doi.org/10.1039/d3ra06755f>

Mahapatra, S., Maity, J. P., Singha, S., Mishra, T., Dey, G., Chandra Samal, A., Banerjee, P., Biswas, C., Chattopadhyay, S., Patra, R. R., Patnaik, S., & Bhattacharya, P. (2024). Microplastics and nanoplastics in environment: Sampling, characterization and analytical methods. *Groundwater for Sustainable Development*, 26, 101267. <https://doi.org/10.1016/j.gsd.2024.101267>

Masura, J., Baker, J., Foster, G., Arthur, C., & Herring, C. (2015). Laboratory Methods for the Analysis of Microplastics in the Marine Environment: Recommendations for quantifying synthetic particles in waters and sediments. *Silver Spring, MD, NOAA Marine Debris Division*(NOAA Technical Memorandum; NOS-OR&R-48), 31. <https://doi.org/10.25607/OBP-604>

Michela, A., Olivia, G., Tommaso, V., Marie-Claire, F., Eric, T., Elodie, R., Francoise, C., Leonardo, T., Anne, V., Boris, D., & François, G. (2021). Distribution of seafloor litter and its interaction with benthic organisms in deep waters of the Ligurian Sea (Northwestern Mediterranean). *Science of The Total Environment*, 788, 147745. <https://doi.org/10.1016/j.scitotenv.2021.147745>



REFERENCES

- Moura, C., Silva, D. M., Espincho, F., Rodrigues, S. M., Pereira, R., Almeida, C. M. R., Ramos, S., & Freitas, V. (2025). Assessing Microplastic Contamination and Depuration Effectiveness in Farmed Pacific Oysters (*Crassostrea gigas*). *Environments*, 12(8), 254. <https://doi.org/10.3390/environments12080254>
- Olga, C., Jesús, G., & Lucía, V. (2021). From the coast to the shelf: Microplastics in Rías Baixas and Miño River shelf sediments (NW Spain). *Marine Pollution Bulletin*, 162, 111814. <https://doi.org/10.1016/j.marpolbul.2020.111814>
- OSPAR. (2024). CEMP Guidelines for the monitoring of microlitter (including microplastics) in seafloor sediments for the OSPAR maritime area. OSPAR Agreement 2024-06. <https://www.ospar.org/documents?v=57834>
- Ramos, S., Rodrigues, S. M., Pereira, R., Silva, D., & Almeida, C. M. R. (2024). 6.14 - Floatables and Plastic Debris in Estuarine and Coastal Marine Environments. In D. Baird & M. Elliott (Eds.), *Treatise on Estuarine and Coastal Science (Second Edition)* (pp. 467-511). Academic Press. <https://doi.org/10.1016/B978-0-323-90798-9.00111-6>
- Rivoira, L., Castiglioni, M., Rodrigues, S. M., Freitas, V., Bruzzoniti, M. C., Ramos, S., & Almeida, C. M. R. (2020). Microplastic in marine environment: reworking and optimisation of two analytical protocols for the extraction of microplastics from sediments and oysters. *MethodsX*, 7, 101116. <https://doi.org/10.1016/j.mex.2020.101116>
- Rodrigues, S. M., Espincho, F., Elliott, M., Almeida, C. M. R., & Ramos, S. (2023). Methodology optimization to quantify microplastic presence in planktonic copepods, chaetognaths and fish larvae. *MethodsX*, 11, 102466. <https://doi.org/10.1016/j.mex.2023.102466>
- Rodrigues, S. M., R. Almeida, C. M., & Ramos, S. (2019). Adaptation of a laboratory protocol to quantify microplastics contamination in estuarine waters. *MethodsX*, 6, 740-749. <https://doi.org/10.1016/j.mex.2019.03.027>
- Silva, D. M., Almeida, C. M. R., Guardiola, F., Rodrigues, S. M., & Ramos, S. (2022). Optimization of an Analytical Protocol for the Extraction of Microplastics from Seafood Samples with Different Levels of Fat. *Molecules*, 27(16), 5172. <https://doi.org/10.3390/molecules27165172>
- Withana, P. A., Senadheera, S. S., He, D., Bank, M. S., Gu, C., Hwang, S. Y., & Ok, Y. S. (2024). Soil microplastic analysis: a harmonized methodology. *Critical Reviews in Environmental Science and Technology*, 54(15), 1138-1163. <https://doi.org/10.1080/10643389.2023.2301052>
- Zhao, X., Liu, Z., Zuo, J., Cai, L., Liu, Y., Han, J., & Zhang, M. (2024). Comparison of Oil Extraction and Density Extraction Method to Extract Microplastics for Typical Agricultural Soils in China. *Agronomy*, 14(6), 1193. <https://doi.org/10.3390/agronomy14061193>



ADVANCING TOWARDS LITTER - FREE
ATLANTIC COASTAL COMMUNITIES
BY PREVENTING AND REDUCING
MACRO AND MICRO LITTER

SEDIMENT PROTOCOL OPTIMIZATION REPORT

Annex to D2.2.3: Harmonised Protocols for Sample Preparation: Microplastic Extraction and Isolation

WORK PACKAGE 2: ASSESSING AND MONITORING MICRO AND MACRO
LITTER CONTAMINATION. ACTIVITY 2: IMPROVED MICROPLASTICS
MONITORING, TASK 3: OPTIMISING SAMPLE PREPARATION PROTOCOLS
TO EXTRACT AND ISOLATE MICROPLASTICS FOR THEIR
CHARACTERISATION



Work package	WP2: Assessing and Monitoring micro and macro litter contamination
Activity and task	A2: Improved microplastics monitoring Task 3: Optimising sample preparation protocols to extract and isolate microplastics for their characterisation
Date	15 / 09 / 2026
Version	1
Author/s	CIIMAR: SARA COUTO LOURENÇO, RÚBEN PEREIRA, C. MARISA R. ALMEIDA
Participants	

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1. INTRODUCTION

This report is intended as an annex to Deliverable 2.2.3 *Harmonised Protocols for Sample Preparation: Microplastics Extraction and Isolation*, in which the protocol for microplastic extraction in sediment is described.

The reliable extraction of microplastics (MPs) from sediment matrices requires an optimised procedure that maximises particle recovery while minimising sample losses. Given the complexity and heterogeneity of sediment samples, optimisation tests were essential to ensure that the selected protocol is efficient and reproducible for harmonised application.

The referred protocol comprises four main steps: pre-density separation, organic matter digestion, density separation and filtration. The present report describes the optimisation tests performed in environmental sediment samples to evaluate the efficiency of the procedure for extracting MPs and to identify potential improvements, particularly for density separation and digestion methods, as well as the optimal number of replicates required.

MP recovery efficiency was compared across different experimental conditions to assess the performance, robustness, and reproducibility of the tested procedures. These tests provide the experimental basis for the selection and refinement of the harmonised protocol presented in Deliverable 2.2.3.

2. SAMPLE COLLECTION AND PREPARATION

Sediment samples were collected from the Lima Estuary in November 2024. Following collection, the samples were placed in an aluminium tray, homogenised with a stainless-steel spatula and air-dried at ambient temperature in the tray covered with aluminium foil. Once dried, sediments were sieved through a 2 mm stainless-steel mesh, and the fraction < 2 mm was used for further processing. For each protocol tested, 20 g of sediment were weighted per replicate.



3. MICROPLASTIC EXTRACTION PROTOCOLS

3.1. Reference method adaptation

Based on the method of Rivoira et al. (2020), 10 sediment samples replicates (20 g each) were subjected to a pre-density separation step using a saturated sodium chloride (NaCl) solution (8 g of NaCl per 100 ml deionised water). Each replicate was placed in a 100 ml glass beaker and filled to approximately $\frac{3}{4}$ of the volume with the NaCl saturated solution. Although it is not used in method of Rivoira et al. (2020), one drop of a surfactant (e.g Triton X-100) was added to reduce sediment aggregation. The mixture was stirred gently and left to settle for 24 hours in the beakers covered with aluminium foil.

After this period, each beaker was filled with additional NaCl saturated solution to create an overflow, which was carefully transferred to a larger beaker and stored. The sediment samples were then left to settle for another 24 hours, after which the overflow process was repeated. Unlike Rivoira et al. (2020), the overflow solution was not passed through a filter cloth (mesh <0.03 mm) to avoid the loss of smaller particles in the process. The overflow solutions obtained from both pre-density separation steps were combined and subjected to oxidative digestion of organic matter on a hot plate at 75°C by adding 30% (v/v) hydrogen peroxide (H_2O_2) and 0.05 M iron(II) [Fe(II)] solution, the Fenton's reagent (20 ml solution per 100 ml NaCl overflow solution).

Following organic matter digestion, an additional NaCl density separation step was carried out (adding 6 g per 20 ml of digested solution), followed by settling for 24 hours. The supernatant solution was then filtered under vacuum through cellulose nitrate filters ($0.45\ \mu\text{m}$). The filters were stored in pre-cleaned glass Petri dishes for subsequent visual analysis under a stereomicroscope (Leica EZ04).

Results: A total of 10 replicates were analysed, with an average of 21 ± 8 MPs (mean $\pm$ SD) per sample (Section 6, Table 1). Results showed a high variability in the total number of MPs recovered among replicates. The variability was even higher for the type of MPs found in lower amounts, such as spheres, films or even fragments. This indicates the need to carry out a high number of replicates to guarantee a correct MPs characterisation in sediments. In all replicates, fibres were the predominant morphology. The colour composition varied across replicates, though colourless and blue were the most frequent ones. Most particles size was in the range 0.5-2 mm, followed by those <0.5 mm.



3.2. Pre-density separation optimization

To further optimise MPs separation from sediment, an additional pre-density separation step was performed using calcium chloride (CaCl_2) saturated solution in the same 10 sediment samples replicates previously tested (in section 3.1. Reference method adaptation). This step was done after the pre-density separations with NaCl saturated solution, to verify whether MPs remained in the residual sediment. CaCl_2 solution has a higher density than NaCl solution, potentially promoting a higher MPs suspension. Therefore, a CaCl_2 saturated solution (50 g of CaCl_2 per 100 ml deionised water) was added to each beaker, that contained only the sediment, filling approximately three-quarters of the total volume. The mixtures were stirred gently and left to settle for 24 hours.

After this period, each beaker was refilled with the same CaCl_2 saturated solution and the overflow was transferred to a larger beaker for organic matter digestion on a hot plate (75 °C) by adding 30 % (v/v) H_2O_2 and 0.05 M Fe(II) solution (20 ml solution per 100 ml CaCl_2 overflow solution). Density separation was not performed on these digested samples, the samples being immediately filtered under vacuum through cellulose nitrate filters (0.45 μm). The filters were stored in pre-cleaned glass Petri dishes for subsequent visual analysis under a stereomicroscope (Leica EZ04).

NOTE: An additional test was conducted to evaluate the possibility of recovering and reusing the CaCl_2 saturated solution by skipping the digestion step and filtering the sample immediately after density separation. However, the filters became severely clogged due to the high organic content, demonstrating that this approach is not feasible and that organic matter digestion is essential prior to filtration to ensure MPs recovery.

Results: The samples had an average of 33 ± 13 (mean $\pm$ SD) MPs (Section 6, Table 2). Results showed that there was a higher MP recovery in this test which indicates that pre-density separation with NaCl saturated solution alone may not be sufficient for complete MP extraction from sediment and that a pre-density separation with CaCl_2 saturated solution improves the MPs recovery efficiency. As in the previous test, there was a high variability in the total number of MPs recovered among replicates. Fibres remained the predominant morphology and the colour distribution was similar, with colourless and blue fibres being the most common one, although one more colour, pink, was detected. Particles size distribution was identical among tests.



3.3. Organic matter digestion protocols comparison

An alternative digestion procedure was performed on 5 additional sediment samples replicates to optimise this step. After the two consecutive pre-density separations with NaCl (as described in Section 3.1 *Reference method adaptation*), 30 % (v/v) H₂O₂ solution (at a volume of 10 % of overflow solution volume) was added and incubated in an oven at 65 °C for 24 hours. Post-digestion density separation and filtration were identical to the reference procedure (see Section 3.1. *Reference method adaptation*).

Results: In the 5 replicates analysed there was an average of 6 ± 4 (mean $\pm$ SD) MPs per sample (Section 6, Table 3). Again, a high variability in the total number of MPs recovered among replicates was observed. As in the previous tests, fibres were the predominant morphology. The colour composition was consistent with earlier results, with colourless and blue fibres being the most frequent and particles size range was identical to that previously observed.

Given the different number of replicates, it is not possible to directly compare the digestion methods in terms of MPs recovery efficiency. Although an identical number of fragments and spheres were observed, the number of fibres was lower in the current test. Previous tests carried out by the team (Rodrigues et al., 2019; Silva et al., 2022) have shown that MPs are not significantly affected by either organic matter digestion protocol, so the low number of fibres is probably related with the heterogeneity of the sediment sample rather than degradation of these MPs. In practical terms, the Fenton digestion is faster, while the oven incubation method requires less reagents. The choice between methods ultimately depends on the availability of equipment and materials in each laboratory, but both should be suitable, although further tests were made to make a significant comparison (see Section 3.5. *Improved protocol*)

3.4. Ultrasonication step

An ultrasonication step, based on the approach described by Angiolillo et al. (2021), was tested prior to pre-density separation to evaluate whether it improved the release of MPs from sediment aggregates. To reduce the number of processing steps and further assess the separation efficiency of the CaCl₂ saturated solution, only this salt was used for the pre-density separation. The sediment replicates were divided into sonicated and non-sonicated treatments for comparison. For that purpose, 10 replicates were placed in glass beakers, filled to approximately $\frac{3}{4}$ of the total volume with CaCl₂ saturated



solution and one drop of Triton X-100. The mixture was stirred gently and 5 of the 10 replicates were sonicated for 15 min at 35 kHz (Elma Transsonic T460). The sediment was then left to settle for 24 hours alongside the other 5 replicates that were not sonicated.

After this period, each beaker was refilled with the same saturated solution, and the overflow was transferred to a larger beaker for organic matter digestion in a hot plate with the Fenton reagent and filtration under the same conditions as described in Section 3.1. *Reference method adaptation.*

NOTE: To ensure that sonication did not affect the integrity of the particles, a preliminary test was carried out using acrylic fabric, lycra fabric, rayon fabric, cellulose acetate fragments and polyamide fragments. The particles were placed in solution and sonicated for 15 min at 35 kHz, being afterwards observed under the stereomicroscope. Although sonication did not visibly affect most tested materials, rayon fibre bundles loosened after the treatment, suggesting that sonication may cause fibre bundle disaggregation or fragmentation. This should be considered when interpreting fibre abundance.

Results: The non-sonicated samples had an average of 5 ± 2 (mean $\pm$ SD) MPs per sample (Section 6, Table 4), while the sonicated samples presented a higher average of 9 ± 6 (mean $\pm$ SD) MPs per sample (Section 6, Table 5). Again, a high variability among replicates was observed indicating that a higher number of replicates may be more suitable to ensure representative and reliable results. The analysed replicates showed similar results to the previous treatments regarding microplastic morphology, with fibres being predominant, and particles size range. Regarding colour, a higher diversity of colours was observed in sonicated replicates. These results suggest that ultrasonication may enhance MPs recovery efficiency, likely by promoting the disaggregation of sediment particles and the release of MPs trapped within them.

3.5. Improved protocol

The optimised steps mentioned above were combined to establish an improved protocol, testing again the two organic matter digestion procedures. At this time only three replicates were prepared for each procedure. Initially, all six sediment samples replicates were subjected to a pre-density separation using CaCl_2 saturated solution. For that, each sediment replicate was placed in a 100 ml glass beaker, which was then filled



to approximately $\frac{3}{4}$ of its total volume with the CaCl_2 saturated solution and a drop of Triton X-100. The mixture was gently stirred, sonicated for 15 min at 35 kHz (Elma Transsonic T460) and left to settle for 24 hours.

After this period, each beaker was refilled with the same saturated solution, and the overflow was collected in larger 250 ml beakers. Two different organic matter digestion procedures were then applied:

- a. Hot plate digestion – adding a 30 % (v/v) H_2O_2 with 0.05 M Fe(II) solution (20 ml solution per 100 ml of overflow solution volume), placed in a hot plate at 75 °C, with agitation for 1 hour
- b. Oven digestion – using a 30 % (v/v) H_2O_2 solution (10% of overflow solution volume), with incubation at 65 °C for 24 hours

The samples were then directly filtered using cellulose nitrate filters (0.45 μm) with the aid of a vacuum pump. All filters were stored in pre-cleaned glass Petri dishes for subsequent MPs characterisation.

Results: The results showed a higher MP recovery efficiency for the hot plate digestion, with an average of 42 ± 10 (mean $\pm$ SD) MPs (Section 6, Table 6), compared to 16 ± 4 (mean $\pm$ SD) MPs obtained with the oven digestion (Section 6, Table 7). Fibres remained the predominant morphology in both treatments, with colourless and blue colours being the most common. Filters from oven digestion had higher amounts of residual material, potentially masking MPs and compromising their visual detection under the stereomicroscope. This limitation could partly explain the lower number of MPs observed in the oven-treated samples. To overcome this limitation, filtration through more than one filter could be used.

Overall, these findings suggest that the Fenton-based hot plate digestion provides more efficient degradation of organic matter and enhances MPs recovery, although the choice of digestion method may depend on the available laboratory equipment and safety considerations, as Fenton solution can be very reactive.

4. CONCLUSION

This study shows that MPs recovery from sediments is improved by combining the optimised steps. The use of CaCl_2 saturated solution with one drop of Triton X-100 enhanced the pre-density separation efficiency compared to NaCl saturated solution. Moreover, ultrasonication promotes the release of MPs from sediment aggregates.



Fenton-based hot plate digestion proved more effective than oven incubation for organic matter digestion, yielding higher MPs recovery and cleaner filters, which facilitates visual identification. To overcome less effective organic matter digestion more than one filter can be used to filter the digested solution to reduce the residual material. Another option is to carry out a density separation after the organic matter digestion, followed by the filtration step.

The high variability observed among replicates highlights the need for sufficient replication to ensure reliable results.

Overall, the optimised protocol provides a more efficient approach for MPs extraction from sediments, although methodological choices should also consider laboratory constraints and safety aspects.

5. REFERENCES

Angiolillo, M., G rigny, O., Valente, T., Fabri, M.-C., Tambut , E., Rouanet, E., Claro, F., Tunesi, L., Vissio, A., Daniel, B., & Galgani, F. (2021). Distribution of seafloor litter and its interaction with benthic organisms in deep waters of the Ligurian Sea (Northwestern Mediterranean). *Science of the Total Environment*, 788, 147745. <https://doi.org/10.1016/j.scitotenv.2021.147745>

Rivoira, L., Castiglioni, M., Rodrigues, S. M., Freitas, V., Bruzzoniti, M. C., Ramos, S., & Almeida, C. M. R. (2020). Microplastic in marine environment: reworking and optimisation of two analytical protocols for the extraction of MPs from sediments and oysters. *MethodsX*, 7, 101116. <https://doi.org/10.1016/j.mex.2020.101116>

Rodrigues, S. M., Almeida, C. M. R., & Ramos, S. (2019). Adaptation of a laboratory protocol to quantify MPs contamination in estuarine waters. *MethodsX*, 6, 740-749. <https://doi.org/10.1016/j.mex.2019.03.027>

Silva, D. M., Almeida, C. M. R., Guardiola, F., Rodrigues, S. M., & Ramos, S. (2022). Optimization of an Analytical Protocol for the Extraction of MPs from Seafood Samples with Different Levels of Fat. *Molecules*, 27(16), 5172. <https://doi.org/10.3390/molecules27165172>



6. TABLES

Table 1. Summary of microplastics (MPs) recovered using the reference method with adaptations. Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 10 replicates).

Sample	Total MPs	Morphology					Size (mm)			Colour						
		Fibre	Fragment	Film	Fibre bundles	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	red	green	grey	brown
1	22	13	8	0	0	1	9	8	5	5	9	2	0	2	3	1
2	19	16	3	0	0	0	7	5	7	5	6	4	1	1	1	1
3	18	17	1	0	0	0	5	7	6	6	6	3	2	0	1	0
4	12	9	1	0	0	2	4	7	1	4	3	1	1	2	1	0
5	27	6	3	1	0	0	6	16	5	15	5	2	0	2	2	1
6	31	28	1	0	1	1	7	16	8	20	2	6	0	1	2	0
7	36	27	3	0	6	0	6	27	3	24	9	1	1	0	1	0
8	18	16	2	0	0	0	4	10	4	6	4	4	1	0	3	0
9	18	16	2	0	0	0	6	8	4	10	1	3	0	2	2	0
10	7	5	2	0	0	0	3	2	2	3	2	1	0	1	0	0
Mean	21	15	3	0.1	0.7	0.4	6	11	5	10	5	3	0.6	1.1	1.6	0.3
SD	8	7	2	0.3	1.8	0.7	2	7	2	7	3	2	0.7	0.8	0.9	0.5



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Table 2. Summary of microplastics (MPs) recovered using the pre-density separation optimized method, with CaCl₂. Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 10 replicates).

Sample	Total MPs	Morphology					Size (mm)			Colour							
		Fibre	Fragment	Film	Fibre bundls	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	red	pink	green	grey	brown
1	38	34	3	0	0	1	14	19	5	11	7	6	2	0	1	10	1
2	41	40	1	0	0	0	11	24	6	26	3	4	1	1	0	6	0
3	66	61	4	1	0	0	30	25	11	26	16	12	0	1	0	11	0
4	31	23	8	0	0	0	17	8	6	4	12	4	0	1	0	9	1
5	24	22	2	0	0	0	13	5	6	6	8	1	1	0	1	7	0
6	24	24	0	0	0	0	5	18	1	13	4	2	0	0	0	5	0
7	24	24	0	0	0	0	5	18	1	12	9	1	1	0	0	1	0
8	25	21	3	1	0	0	7	15	3	8	5	2	3	0	1	6	0
9	16	14	2	0	0	0	5	9	2	7	4	2	0	1	2	0	0
10	39	36	2	0	1	0	9	20	10	19	7	2	0	1	0	10	0
Mean	33	30	3	0.2	0.1	0.1	12	16	5	13	8	4	0.8	0.5	0.5	7	0.2
SD	13	13	2	0.4	0.3	0.3	7	6	3	8	4	3	1.0	0.5	0.7	4	0.4

Table 3. Summary of microplastics (MPs) recovered using the alternative H₂O₂ digestion procedure, in the oven. Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 5 replicates).

Sample	Total MPs	Morphology			Size (mm)			Colour						
		Fibre	Fragment	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	pink	green	grey	brown
1	12	11	1	0	5	7	0	5	4	1	0	1	1	0
2	1	1	0	0	0	0	1	0	0	0	0	0	1	0
3	6	4	0	2	4	2	0	1	16	12	1	0	11	0
4	9	6	3	0	4	5	0	1	5	0	0	0	2	1
5	4	1	2	1	4	0	0	1	1	0	0	2	0	0
Mean	6	5	1	0.6	3	3	0.2	2	5	3	0.2	0.6	3	0.2
SD	4	4	1	0.8	2	3	0.4	2	6	5	0.4	0.8	4	0.4



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Table 4. Summary of microplastics (MPs) recovered using the protocol without sonication before pre-density separation. Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 5 replicates).

Sample	Total MPs	Morphology				Size (mm)			Colour				
		Fibre	Fragment	Foam	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	green	grey
1	5	2	2	0	1	4	1	0	0	1	1	2	1
2	2	1	1	0	0	1	1	0	0	0	1	1	0
3	7	4	1	1	1	3	2	2	2	0	0	4	1
4	2	2	0	0	0	0	1	1	0	1	1	0	0
5	8	6	2	0	0	6	1	1	3	1	2	2	0
Mean	5	3	1.2	0.2	0.4	3	1.2	0.8	1	0.6	1.0	2	0.4
SD	2	2	0.7	0.4	0.5	2	0.4	0.7	1	0.5	0.6	1	0.5

Table 5. Summary of microplastics (MPs) recovered using the protocol with sonication before pre-density separation. Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 5 replicates).

Sample	Total MPs	Morphology					Size (mm)			Colour						
		Fibre	Fragment	Film	Foam	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	red	green	yellow	grey
1	5	3	2	0	0	0	2	3	0	3	1	0	0	1	0	0
2	8	4	3	0	1	0	4	4	0	3	3	0	0	2	0	0
3	20	11	0	2	0	7	13	6	1	6	0	4	0	8	1	1
4	2	1	0	0	0	1	2	0	0	1	0	0	0	1	0	0
5	8	2	4	0	0	2	7	1	0	1	2	0	1	3	0	0
Mean	9	4	2	0.4	0.2	2	6	3	0.2	3	1	0.8	0.2	3	0.2	0.2
SD	6	4	2	0.8	0.4	3	4	2	0.4	2	1	1.6	0.4	3	0.4	0.4



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Table 6. Summary of microplastics (MPs) recovered using the protocol optimised with digestion of organic matter in hot plate (Fenton based). Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 3 replicates).

Sample	Total MPs	Morphology			Size (mm)			Colour							
		Fibre	Fragment	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	red	pink	green	grey	brown
1	54	43	11	0	30	18	6	13	21	11	3	1	1	4	0
2	31	25	6	0	18	10	3	8	11	9	1	0	0	1	1
3	40	35	4	1	16	20	4	13	12	4	2	0	2	7	0
Mean	42	34	7	0.3	21	16	4	11	15	8	2.0	0.3	1.0	4	0.3
SD	9	7	3	0.5	6	4	1	2	4	3	0.8	0.5	0.8	2	0.5

Table 7. Summary of microplastics (MPs) recovered using the protocol optimised with digestion of organic matter in the oven. Results are expressed by total abundance, morphology, size (mm) and colour (mean and standard deviation (SD), n = 3 replicates).

Sample	Total MPs	Morphology			Size (mm)			Colour					
		Fibre	Fragment	Spheres	≤0.5	0.5<n<2	≥2	colourless	blue	black	pink	green	grey
1	18	16	1	1	14	4	0	2	5	7	1	1	2
2	10	8	2	0	6	2	2	3	4	2	0	0	0
3	20	17	3	0	10	7	3	5	6	5	0	2	2
Mean	16	14	2.0	0.3	10	4	2	3	5.0	5	0.3	1.0	1.3
SD	4	4	0.8	0.5	3	2	1	1	0.8	2	0.5	0.8	0.9