Standardized protocol for microplastic sampling and identification in pelagic faunal gastrointestinal tracts

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Table of Contents

1. Introduction

This protocol describes the extraction of microplastics (> 100 µm in Feret diameter) from pelagic faunal gastrointestinal tracts. While the protocol has been tested on deep-sea fishes of less than 20 cm in size, inhabiting pelagic depths down to approximately 1000 m, it is applicable to other fish types, cephalopods and crustaceans. For fish with higher fat content than deep-sea fishes, digestion times will be longer. The extraction methodology is based on the digestion of the digestive apparatus by sodium hydroxide (NaOH).

Microplastics identification is done visually by stereomicroscopy (Nile red staining), and confirmed by micro-Fourier transform infrared spectroscopy, µ-FTIR (Catarino et al., 2018; Bessa et al., 2019; Meyers et al., 2022, 2024a,b).

2. Materials & Reagents

2.1 Materials

- Polytetrafluoroethylene (PTFE) filters (10 µm, ⌀ 47 mm) or other suitable ones
- Borosilicate scintillation vials
- Beakers (≥250 ml)
- Eppendorf vials
- Glass Petri dishes
- Concave glass microscope slides
- Magnetic stirring bars
- Magnetic hotplate stirrer
- Ultrasonic bath
- Laminar flow hood cabinet
- Filtration set-up
    - - Filtration manifold set
    - - Glass funnel with dust cover per sample
    - - Fritted glass base with stopper per sample
    - - Aluminum clamp per sample
    - - Rubber stoppers
    - Büchner flask (1 L)
- Vacuum pump
- Vortex mixer
- Metal tweezers
- Aluminum foil
- Non-disintegrating natural fiber tissues
- Orange-colored cotton laboratory coats (color is optional)
- Latex gloves
- Fluorescent polyethylene microspheres (UVPMS-BG-1; 1.025 g/cm³; 63 – 75 µm)
- Fluorescent microscope including the following fluorescence filters: Blue filter: 450–490 nm, Green filter: 515–560 nm, UV filter: 340–380 nm

2.2 Reagents

- Milli-Q water (MPF-grade)
- Sodium hydroxide (NaOH, 1 mol / L)
- Sodium dodecyl sulfate (SDS, 10% solution)
- Saturated sodium chloride solution (NaCl; 1.8 g / cm³)
- Hydrogen peroxide (H_2O_2, 3%) – optional, for glassware cleaning
- Ethanol (70 – 100%, MPF-grade; optional for cleaning)

Note: Whenever possible, reagents should be filtered up to 0.22 µm, and stored in covered glass bottles or vials, to avoid microplastics contamination.

3. Procedures to mitigate background contamination

3.1 Microplastics-free water (MPF-water)

Use deionized, filtered Milli-Q water. It can be obtained from various filtering devices– blank samples should be analyzed to ensure they are free of microplastics (MPF) in the size range of interest.

3.2 Workspace decontamination and general recommendations

- Work surroundings should be as particle-depleted as possible
- It is best to use a reversed laminar flow cabinet equipped with an air particle filter, while processing samples in the lab
- Otherwise: use dedicated workspaces/fume hoods to MP sample treatment. Avoid working near open doors or windows
- Wear a cotton laboratory coat (100% orange-colored when possible)
    - Orange-colored laboratory coats allow for a better retrieval (in case of contamination) and to pinpoint its source.
    - Avoid using synthetic fibers underneath the laboratory coat, to minimize airborne particle contamination. If a synthetic garment is worn underneath the laboratory coat, register its color and processing date, to trace to potential operator contamination
- Latex gloves should be prioritized to avoid nitrile particles in the FTIR
- Avoid working in crowded laboratories and limit door opening to minimize airborne particle contamination

Prior to experiments:

- Clean all work surfaces with tissues made of non-disintegrating natural fibers and MPF-water or MPF-ethanol solutions (70 - 100%)
- Wash hands/latex gloves with MPF-water prior to and throughout processing, when needed
- Rinse the exterior of all containers that come from non-MPF conditions thoroughly with MPF-water
- Avoid using plastic items whenever possible. If you cannot avoid a plastic item (e.g., nitrile gloves), provide a sample for control
- Do not use Parafilm at any stage, as it can lead to sample contamination

3.3 Glassware decontamination

New glassware (e.g., scintillation vials) should be preferably used for stomach collection for digestion. Glassware that is used in the laboratory for purposes other than microplastics related work can be potentially compromised if not cleaned properly, and therefore should either be thoroughly washed and carefully air dried (inverted, under a reversed laminar fume

hood) or avoided. Keep the scintillation vials covered when not in use and ensure all are clearly labelled.

Glassware cleaning:

- Fill the glassware with 3% MPF-H_2O_2 (if available) and leave it for 15 min
- Dispose of the MPF-H_2O_2 and rinse the glassware with MPF-water
- Fill the glassware with MPF-water and keep it in an ultrasonic bath (45 kW) for 10 min
- If H_2O_2 is not available, fill the glassware with MPF-water and keep the glassware in an ultrasonic bath (45 Kw) for 10 min
- Repeat ultrasonication with fresh MPF-water for overall three cycles

You can choose to use a protocol that deviates from the recommendation as long as they yield clean glassware, for example by using diluted Deacon or other products that can work as surfactants.

Always dry glassware facing down or covered by aluminum foil, preferably under a reversed laminar fume hood.

3.4 Filtration of reagents

Prior to use in experiments, filter all reagents and solutions (1.2 µm, ⌀ 47 mm). The pore size of the filters defines the theoretical lower size limit for particle retention; smaller pore sizes capture finer particles. Particles smaller than the filter's mesh size are not quantitatively retained and are not reliably represented in the sample. In addition, the effective detection limit is constrained by the stereomicroscope resolution.

4. Sample acquisition and digestion

The following steps are based on Ferreira et al. (2023):

1. Weigh each selected specimen
   If samples were stored in ethanol, a brief rehydration step is advisable prior to weighing to ensure accurate mass measurements. Those samples should be transferred to MPF-water in glass containers and left for a few hours for the ethanol to evaporate before recording wet weight.
2. Measure the maximum standard length, upper jaw length, lower jaw length, and eye diameter of each specimen of fish (Fig. 1a). For cephalopods and crustaceans, measure the eye diameter, mantle length (for cephalopods), and abdomen and carapace length (for crustaceans)
3. Extract the entire gut (from anus to esophagus) of each fish specimen. For crustaceans, extract the foregut, midgut and hindgut (and hepatopancreas, optional). For cephalopods, extract the gill, stomach, and intestine

4. Weigh each individual gut
5. Rinse the gut externally with Milli-Q water to remove any surface particles
6. Weigh the specimen again without the gut
7. Place each entire gut into a labelled, closed borosilicate scintillation vial
8. Digest the gut using sodium hydroxide (NaOH) at a final concentration of 1 mol / L, at 60 °C, for 24 hours, in a laboratory oven to increase the reaction rate. Sodium hydroxide is cost-effective and does not alter the integrity of any particles present in the gut (Catarino et al., 2017; Budimir et al., 2018)
9. Mix each sample with a clean glass rod twice during incubation to homogenize the solution

Note: Leave a blank filter exposed in the laminar flow cabinet during dissection to assess airborne contamination during sample processing.

5. Sample filtration

1. Perform the filtration step using a vacuum pump under a reversed laminar flow hood
2. Use sterilized metal tweezers to place 47-mm filter papers onto the filtration set-up
3. Pour the contents of the scintillation vials onto the filter papers
4. Rinse each vial with Milli-Q water three times to ensure all particles are transferred
5. Rinse the inner walls of the filter funnels with Milli-Q water
6. Wrap each filter paper in aluminium foil after filtration
   Alternatively, store each filter paper in individual glass Petri dishes with lid (about the same size as the filters)
7. Dry the wrapped filters in a laboratory oven for 6 hours. Prepare the filters this way to allow safe transportation for further analysis without particle loss

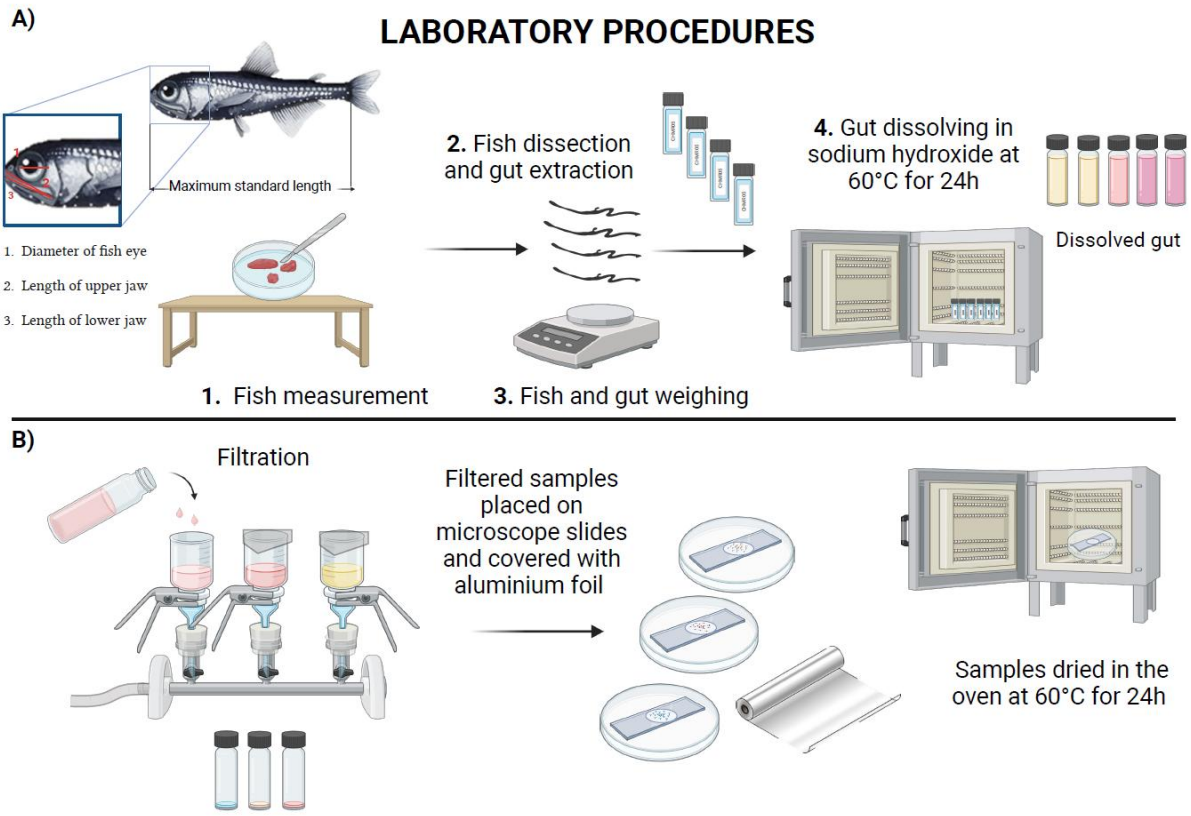

Fig. 1. Schematic diagram of the sampling and sample processing steps: (A) standard fish measurements and gut sampling, followed by sample digestion, (B) filtration and drying steps. Figure created with BioRender.com

6. Resuspension and recovery of particles (optional)

If unsuitable filters for µ-FTIR observations were used, microparticles can be transferred between filters. To do so, particles should be resuspended (various options available, see below), and then recovered by a second filtration. Three different types of resuspension procedures can be used (Fig. 2):

Optional Procedure 1: SDS resuspension

1. Prepare a solution of 10% SDS (sodium dodecyl sulphate) in 100 mL Milli-Q water, as SDS works as a surfactant which assists in dispersing particles
2. Place the filter paper into a clean beaker with a magnetic stirring bar
3. Add the SDS solution to the beaker
4. Cover the beaker with aluminum foil to prevent contamination
5. Place the beaker on a magnetic hotplate stirrer and stir continuously at 60 °C for 24 hours

Optional Procedure 2: Ultrasonic resuspension and separation

1. Add 100 mL Milli-Q water to a clean beaker and add the filter containing the particles
2. Transfer the beaker with its contents to an ultrasonic bath for 10 min.

Optional Procedure 3: NaCl density separation

1. Prepare a supra-saturated NaCl solution (1.8 g / cm^3), pre-filtered to avoid contamination
2. Place the filter paper and a magnetic stirring bar into a clean beaker
3. Pour the NaCl solution into the beaker
4. Cover the beaker with aluminum foil
5. Stir the contents on a magnetic hotplate stirrer at 60 °C for 24 hours

Post-Resuspension Filtration

After each resuspension procedure, filter each beaker content using a filtration set-up fitted with polytetrafluoroethylene (PTFE) filters (10 µm, ⌀ 47 mm). Handle filters carefully to avoid contamination or loss of particles.

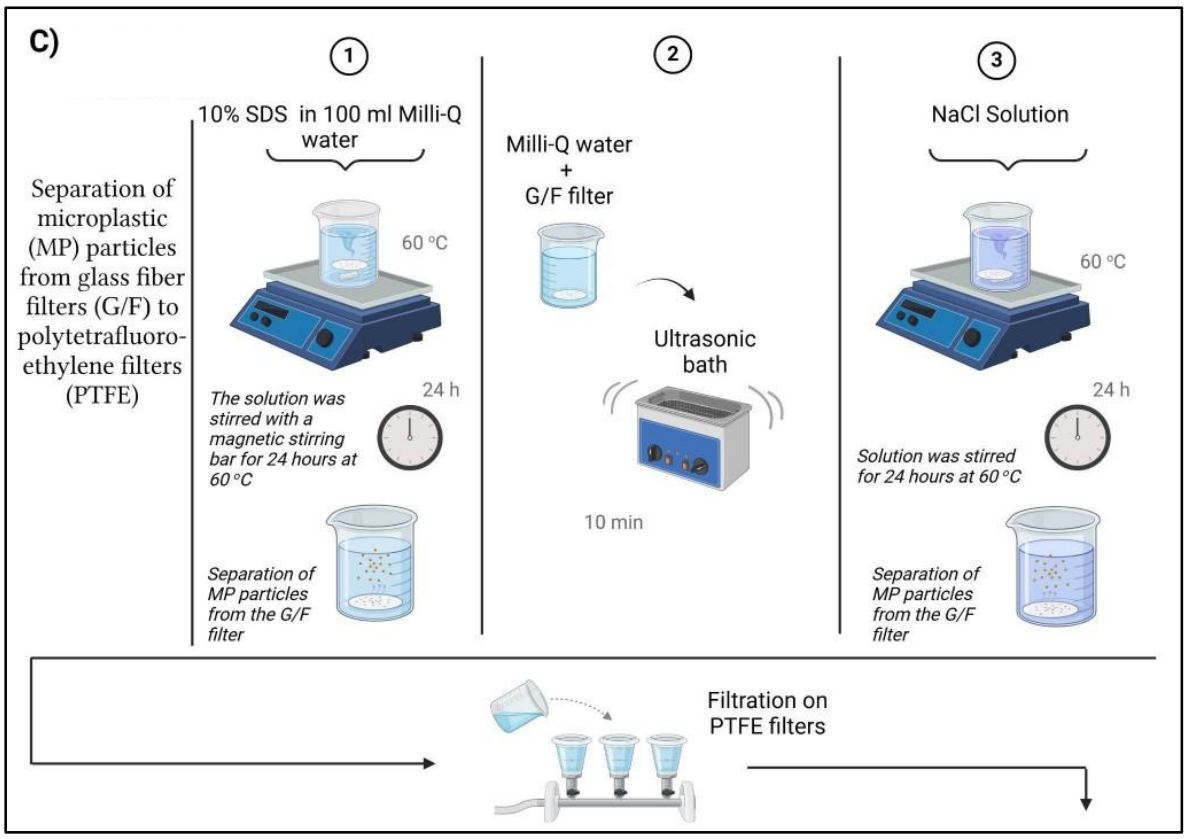

Fig. 2. Schematic diagram of the three optional resuspension procedures.

7. Nile red staining

1. Place the PTFE filters on a filtration set-up (preferably work in reversed flow cabinet)
2. Apply approximately 1 mL of Nile red solution (with a glass pipette) using a circular motion, moving from the edges toward the center of each filter
3. Cover the stained filters with aluminum foil to prevent airborne contamination
4. Allow the filters to stain for 15 minutes at room temperature
5. Turn on the filtration pump
6. Rinse each filter with a minimum of 50 mL Milli-Q water to remove the Nile red excess
7. Continue filtration to dry the filters (1 – 2 min)
8. Carefully transfer each PTFE filter onto a clean glass microscope slide (c.a. 7 x 5 cm)
9. Place the slides into individually labelled petri dishes
10. Store petri dishes in a dark environment at room temperature, for min 24h, until further analysis (microscopy and/or FTIR)
11. Retain the filters for future reference [Do not discard]

According to recommendations of Meyers et al. (2024b), samples stained with Nile red should be analyzed within one or two weeks after being stained (Fig. 3), as the fluorescence intensity fades over time.

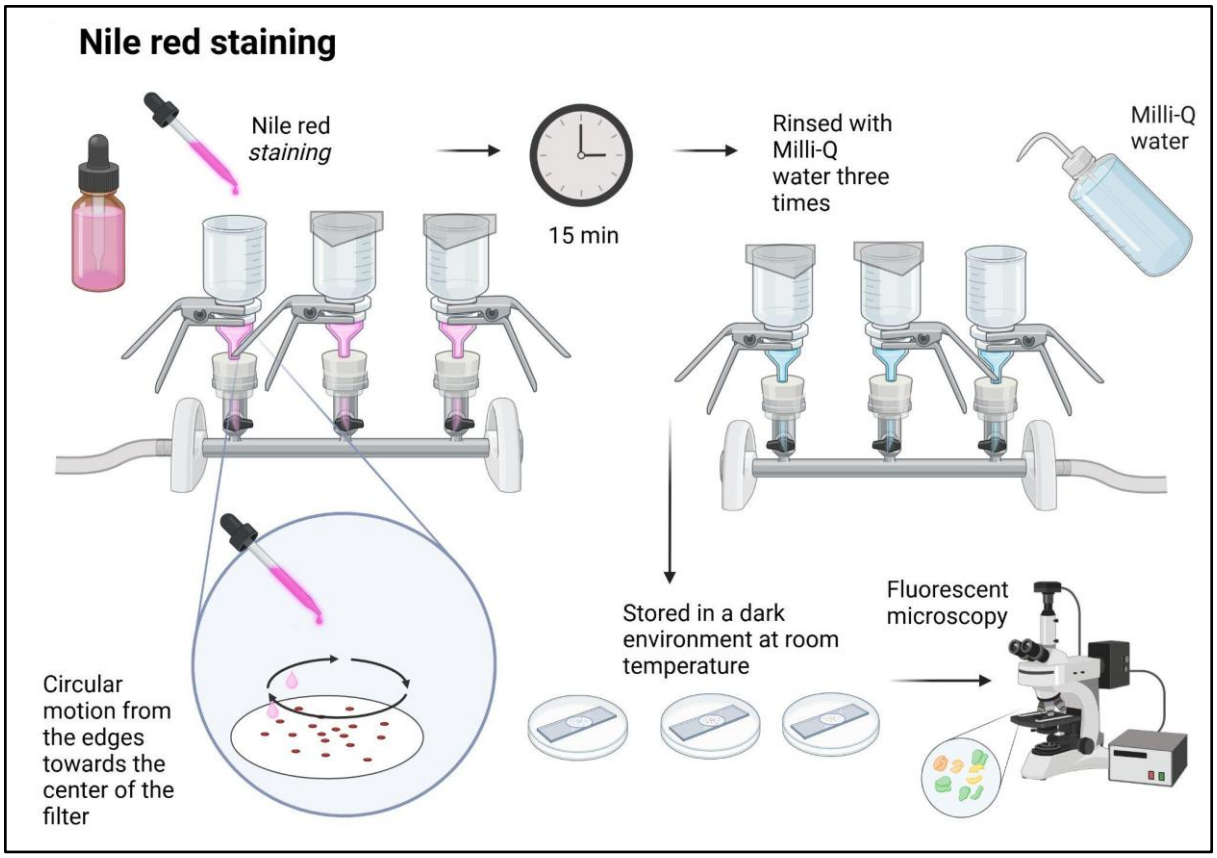

Fig. 3. Schematic diagram of the Nile red staining.

8. Recovery test

It is strongly advisable to perform a recovery test before processing samples, to verify the accuracy of the protocol and assess potential particle loss (Fig. 4). Each new operator should perform a recovery test, prior to processing samples. It is also advisable that two independent operators perform the recovery test independently, to assess repeatability. You can use the following procedure as an example, but this can be adjusted (e.g., you can use non-fluorescent particles, of different sizes).

1. Place fluorescent polyethylene microspheres (e.g., UVPMS-BG-1 1.025g / cc -10 g 63 – 75 µm) into an Eppendorf vial containing Milli-Q water. Polyethylene microspheres are beads which emit bright green under UV light, therefore being detected using microscopy
2. Vortex the vial for 15 seconds to suspend the microspheres evenly
3. Pour the solution onto a concave microscope slide placed inside a Petri dish
4. Allow the Milli-Q water to evaporate completely
5. Count the microspheres under a microscope to establish the initial quantity
6. Add the counted microspheres to a scintillation vial containing the full digestive tract of a single species specimen used as reference and 1 mol / L NaOH
7. Repeat the process for two additional gut specimens (total of three trials)
8. Repeat all laboratory procedures including digestion, filtration, resuspension, and second filtration (optional, and only if applicable), on each spiked specimen

9. Compare the recovered number of microspheres to the initial count to assess accuracy and potential loss

10. If recovery rates are within 80 – 120%, the test is considered successful. If above this interval, please reassess and adjust as required (Meyers et al., 2024a,b).

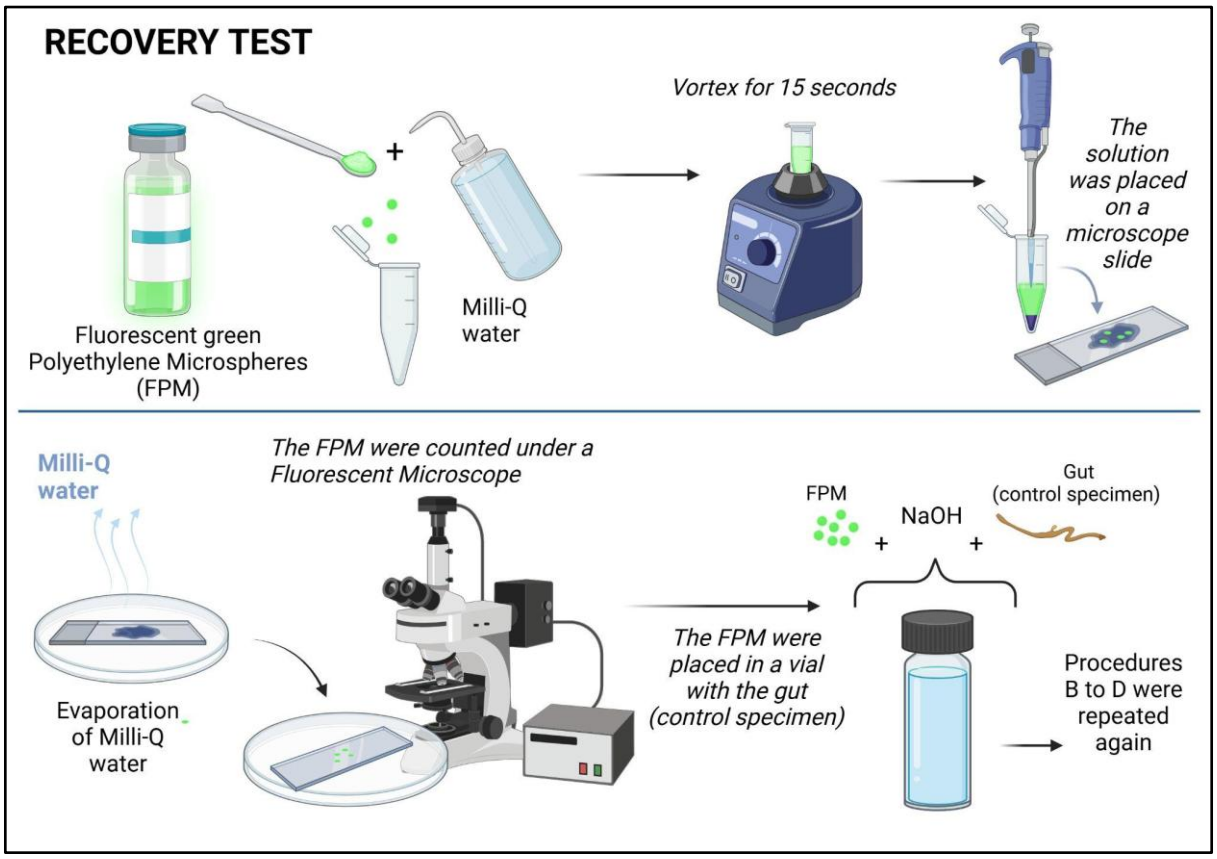

Fig. 4 Schematic diagram of the recovery test. Figure created with BioRender.com

9. Quality control

The quality control process includes the use of two types of controls, i.e., blank samples, to guarantee the control of possible contamination from the surrounding environment. The two different blanks are procedural blanks, and 'environmental/atmospheric' blanks. Manipulation control of the 'environmental/atmospheric' blanks is used to check for any potential contamination of the samples by particles that might be suspended in the ambient air.

1. Prepare a procedural blank by filling a scintillation vial with 50 mL of 1 mol / L NaOH, without adding gastrointestinal tracks
2. Prepare an additional procedural blank using distilled water instead of NaOH, without adding gastrointestinal tracks
3. Verify 'environmental/atmospheric' contamination by placing a clean, uncovered beaker in the laboratory workspace, during both dissections and sample processing
4. After exposure, rinse the beaker three times with Milli-Q water

5. Fill the beaker with Milli-Q water
6. Filter the contents through a filter paper
7. Analyze all blank filters using the same procedures applied to the gut sample filters (Steps 5 to 7)

10. Microplastic identification and enumeration

A. Stereomicroscope

1. If plastic particles are present, examine them under a stereomicroscope coupled to a camera device, under the highest magnification available, with a detection limit of 0.02 mm
2. Photograph plastic particles, making sure that you calibrated the measurements using a scale (e.g., using a microscope stage calibration slide) at each used magnification
3. You can process photos following the machine learning model developed by Meyers et al. (2022)
4. Particles suspected to be made of plastic should be enumerated and characterized (using the Feret diameter, i.e., length of the longest axis – please see Niu et al. (2024) for an automated procedure to assess the Feret diameter using the freeware ImageJ (Schneider et al 2012)
   Categorize the particles into the following the EMODnet micro-litter types vocabulary (British Oceanographic Data Centre, 2025, http://vocab.nerc.ac.uk/collection/H01/current) and the EMODnet micro-litter shapes (http://vocab.nerc.ac.uk/collection/H02/current)
5. Categorize the particles into the following EMODnet micro-litter color classes vocabulary (http://vocab.nerc.ac.uk/collection/H04/current) [only when possible – colors may be altered after Nile red staining]

Note: When recording your data, you can use the following dataset template (adjust if necessary), which considers standardized (meta)data fields: Catarino, A.I. et al (2026). Dataset template for microlitter and mesolitter data collection in riverine and transitional waters, and coastal environments. Marine Data Archive. https://doi.org/10.14284/795

B. Identification of polymers by µ-FTIR

1. Please ensure the filter and particles are completely dry, as water and humidity are known to alter the polymer spectra readings
2. Carefully remove the filter from the microscope glass using tweezers and place it on the µ-FTIR plate
3. Use a black plastic ring or plexiglass plate to support the filter if it bends during transfer
4. Set the µ-FTIR to accumulate 32 scans with a 100 × 100 µm aperture. Make sure you establish the background readings prior to the particle polymer assessment

5. Adjust the aperture size and increase scan accumulations to 64 if particles are smaller
6. Proceed with particle identification using available libraries on the instrument's associated software, or available via open access sources (e.g., Open Specy, Cowger et al. 2021). It is recommended to use polymer matches of 70% or above, but a lower limit of 60% could be acceptable for environmental plastics. Polymers should be reported using the EMODnet micro-litter material or polymer type vocabulary (http://vocab.nerc.ac.uk/collection/H05/current)

C. Identification using ATR-FTIR spectroscopy

1. Use Attenuated Total Reflectance (ATR)-FTIR spectroscopy to identify particles larger than 0.5 mm
2. Analyze polymer particles using a Perkin Elmer Frontier FTIR spectrometer
3. Visualize and process spectra using Spectrum IR software (v. 10.7.2.1630)
4. Set the spectral range to 4000 – 380 cm^{-1} with a resolution of 4.0 cm^{-1}
5. Perform each measurement with either 8 or 25 scans
6. Compare measured spectra to reference spectra using the built-in libraries in Spectrum IR (or using open access libraries, see above)
7. Accept polymer identifications only if spectral similarity exceeds 70% (or 60%, see above), with polymer types reported as indicated above

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