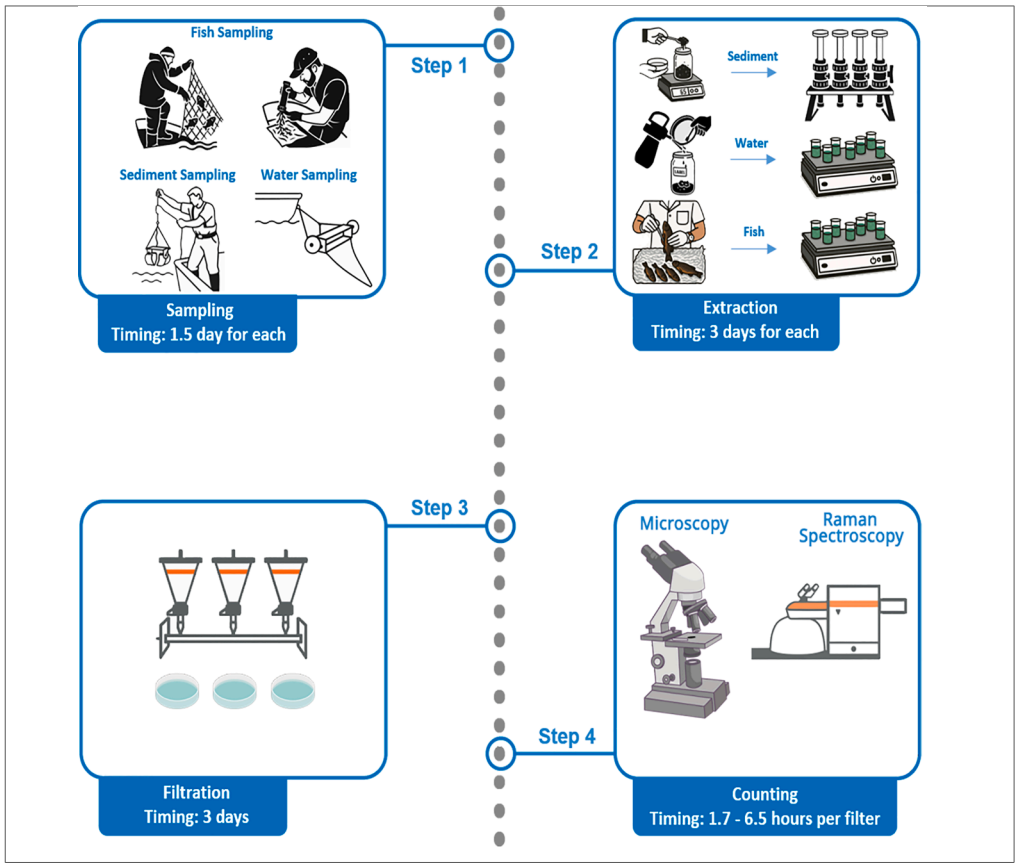
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# Protocol for extraction and analysis of microplastics in freshwater, sediment, and fish samples




Microplastic pollution threatens aquatic ecosystems and food webs. Here, we present a protocol for extracting microplastics from freshwater, sediment, and fish digestive system samples. We collect water using manta nets, sample sediment with benthic grabs, and dissect fish to isolate digestive organs. We perform chemical digestion, density separation, vacuum filtration, and microscopic enumeration, followed by polymer identification using μ-Raman spectroscopy. Quality control steps ensure high recovery and minimize contamination.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Instructions for collecting surface water, sediment, and fish samples

Steps for extracting MPs from sediment using density separation

Procedures for dissecting fish GIT and processing for MP recovery

Guidance on polymer identification of MPs using μ-Raman spectroscopy

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# Protocol for extraction and analysis of microplastics in freshwater, sediment, and fish samples

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## SUMMARY

**Microplastic pollution threatens aquatic ecosystems and food webs. Here, we present a protocol for extracting microplastics from freshwater, sediment, and fish digestive system samples. We collect water using manta nets, sample sediment with benthic grabs, and dissect fish to isolate digestive organs. We perform chemical digestion, density separation, vacuum filtration, and microscopic enumeration, followed by polymer identification using μ-Raman spectroscopy. Quality control steps ensure high recovery and minimize contamination.**

**For complete details on the use and execution of this protocol, please refer to Gündoğdu et al.[1,2]**

## BEFORE YOU BEGIN

This protocol includes detailed procedures for investigating the presence and diversity of microplastics (MPs) in freshwater samples collected from natural lakes using manta nets, benthic sediment samples obtained via Van Veen Grab, and fish digestive system samples collected using gillnets. The protocol is adaptable to various freshwater ecosystems, such as lakes, rivers, and reservoirs, providing essential baseline data for identifying MP sources and assessing ecosystem-based risks. Procedures specific to each sample type are outlined based on previously established techniques used widely in sampling practices[3–5] and adapted specifically for this study. Extraction steps vary among sample types (surface water samples[6–8]; sediment samples[9–11]; fish samples[12]). However, procedures from the filtration step onward, aimed at identifying and quantifying MPs, remain consistent across all sample groups.

The steadily increasing global production and consumption of plastics since the 1950s has led to significant environmental pollution in both aquatic and terrestrial ecosystems.[13,14] Initially recognized by the scientific community between the 1960s and 1970s, this issue was formally identified as a global environmental concern by the late 1990s.[15] Plastics, valued for their ease of processing, low cost, and durability, have seen a substantial increase in use particularly over the last 50 years.[16,17] However, their inherent properties allow them to fragment into smaller particles, facilitating their widespread distribution across distant regions via marine currents and winds, leading to escalating levels of pollution.[17] Plastic pollution, especially from single-use items,[18] constitutes one of the most serious environmental threats to aquatic ecosystems.[19,20] In aquatic environments, MPs enter food chains affecting both large organisms such as fish and microscopic organisms, posing risks to human food safety as well as causing economic losses.[21–23]


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⚠ CRITICAL: Due to the high risk of contamination inherent in microplastic analysis, strict quality assurance and contamination control measures are essential. These include filtration of all solutions, thorough cleaning of laboratory equipment, and the use of procedural blanks to monitor background contamination. Detailed QA/QC procedures, including contamination prevention during sampling, laboratory processing, and analysis, are described comprehensively in Section 6: quality assurance and quality control (QA/QC). Readers are strongly encouraged to review this section before initiating the protocol to ensure data reliability and methodological integrity.

*Note:* The effective size range of microplastics detectable using this protocol depends on the sample matrix and filtration method. For water samples collected via manta trawl (330 μm mesh), the lower particle size limit is ~330 μm. However, during sampling, partial clogging of the net mesh may enable occasional capture of smaller particles. For sediment and fish gastrointestinal tract (GIT) samples, filtration through 1.2 μm GF/C filters permits detection of microplastics ≥1.2 μm in size.

## Innovation

This protocol introduces an integrative approach for extracting and analyzing microplastics from three distinct freshwater matrices: surface water, benthic sediment, and fish gastrointestinal contents. While many previous protocols target a single sample type, this method harmonizes sample processing steps beyond initial extraction to allow standardized downstream analysis. It features matrix-specific optimizations for digestion efficiency (e.g., $H_2O_2$ and NaClO/KOH treatments), and addresses issues such as filter clogging, polymer degradation, and cross-contamination. The protocol enhances accuracy and reproducibility by including systematic procedural blanks and particle validation via μ-Raman spectroscopy. Furthermore, it offers modular adaptability for laboratories with different levels of instrumentation and field conditions, including alternatives for low water levels and minimal equipment scenarios. By streamlining methodology across sample types while preserving analytical rigor, this protocol supports comprehensive microplastic monitoring in freshwater ecosystems.

## Institutional permissions

This protocol involves sampling from natural freshwater ecosystems, including the collection of water, sediment, and fish specimens. In accordance with national regulations, sampling activities described herein were conducted under official authorization from the **Ministry of Agriculture and Forestry of the Republic of Türkiye**, which granted permission for specimen collection from natural lakes. Ethical approval for animal research was not required in this case, as the fish samples used were collected in accordance with local regulations for scientific sampling and were not subjected to experimental manipulation beyond dissection for gastrointestinal tract removal. Additionally, no ethical approval is required for sediment and water samples or for invertebrate remains, as these do not involve live animals or protected taxa. However, researchers intending to use this protocol are strongly advised to consult and comply with their local and institutional guidelines. In many countries, collecting vertebrates—especially fish—may require ethical authorization or sampling permits. Researchers must ensure that all necessary approvals are obtained **before** beginning fieldwork or laboratory analyses involving animal material. Under no circumstances should microplastic studies involving biotic sampling proceed without securing the appropriate institutional or governmental permissions where required.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Hydrogen peroxide 35% | ISOLAB, Germany | CAS# 932.122.2500 |
| Acetone ≥99.8% (not diluted) | CARLO ERBA, France | CAS# 67-64-1 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Potassium carbonate ≥99% | CARLO ERBA, France | CAS# 584-08-7 |
| Formaldehyde 37% | Merck, Germany | CAS# 923.012.2500 |
| Sodium hypochlorite (14% active chlorine) | Merck, Germany | CAS# 105614 |
| Potassium hydroxide pellet, hydration ≤ 1% | CARLO ERBA, France | CAS# 1310-58-3 |
| Other | | |
| Glass microfiber filter (GF/C; Ø 47 mm, 1.2 µm pore size) | Whatman, England | CAS# 1822-047 |
| U-PVC transparent pipe | Pimtaş, Türkiye | N/A |
| Pressurized hand pump | Gardena, Germany | N/A |
| Vacuum filtration system; vacuum pump, manifold, Nuche Erlen | Rocker, Taiwan; ISOLAB, Germany | Rocker 800, 043.05.101, 043.06.011, |
| Forceps, spatula | ISOLAB, Germany | N/A |
| Petri dishes (60 mm) | NA | N/A |
| Sample bottle (at least 1 liter volume, large-mouth) | NA | N/A |
| Stainless steel sieve (5 mm mesh size) | NA | N/A |
| Stainless steel funnel (compatible with sampling bottles) | NA | N/A |
| Marker/pencil (compatible for glass writing, water resistant) | NA | N/A |
| Flowmeter | Hydro-Bios, Germany | N/A |
| Manta net (60 × 25 cm net frame, 330 µm mesh size) | NA | N/A |
| Van Veen grab (20 × 30 × 60 cm) | Hydro-Bios, Germany | HYDR-437-330 |
| GPS or any device that can record coordinates | NA | N/A |
| Binocular microscope | Olympus, Japan | SZX16 |
| µ-Raman spectroscopy | Renishaw, UK | inVia Qontor |

## MATERIALS AND EQUIPMENT

⚠ CRITICAL: All of the chemical preparations and digestion process should be done in a fume hood.

**30% KOH: NaClO digestion solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| $ddH_2O$ (double-distilled) | N/A | 700 mL |
| KOH | 1120 g/L | 150 mL |
| NaClO (%14 active chlorine) | – | 150 mL |
| **Total** | **N/A** | **1000 mL** |

Store at room temperature during the analysis. For long term storage condition, follow the chemical brand instruction for storage conditions.

⚠ CRITICAL: NaClO should be added first, then water and finally concentrated KOH. Otherwise, chip formation may be observed. Since KOH solution is exothermic, it should be allowed to reach room temperature (20°C–22°C) before adding to the mixture. This solution should be filtered through GF/C filters before use.

**$K_2CO_3$ density separation solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| $ddH_2O$ | N/A | 312 mL |
| $K_2CO_3$ | 1120 g/L | 138.2 g |
| **Total** | **N/A** | **250 mL** |

Store at room temperature.

⚠ CRITICAL: Since $K_2CO_3$ solution is exothermic, it should be allowed to reach room temperature (20°C–22°C) before adding to the mixture. This solution should be filtered through GF/C filters before use.

## STEP-BY-STEP METHOD DETAILS

The following list outlines the specific steps to follow for MP extraction and analysis based on sample type: Water Samples: Step 1 to Step 5, Sediment Samples: Step 6 to Step 13, Fish Samples: Step 14 to Step 19, Enumeration of Microplastics Under Microscopy: Step 20 to 22, Polymer Identification Using μ-Raman Spectroscopy: Step 23 to 31.

### Water samples

Timing: 5–8 days total

Timing: 1 day for step 1

Timing: 1 h for step 2

Timing: 1 day for step 3

Timing: 1–3 days for step 4

Timing: 1–3 days for step 5

Here, we describe steps for extracting and analyzing microplastics from surface water samples collected using a manta trawl. The protocol begins with field collection at designated freshwater locations, where water is filtered through a 330 μm mesh net to retain plastic particles. Samples are then transferred to clean laboratory containers, followed by chemical digestion using hydrogen peroxide ($H_2O_2$) to remove organic matter. A density separation step using potassium carbonate ($K_2CO_3$) solution is applied to isolate MPs from remaining debris. The sample is then vacuum filtered onto glass microfiber (GF/C) filters for subsequent analysis. This sequence ensures effective recovery of microplastics ≥330 μm, with occasional retention of smaller particles due to mesh clogging during sampling.

⚠ CRITICAL: Before starting the study, please carefully read Section 6: quality assurance and quality control (QA/QC).

1. Field Preparation for Water Sampling.
   a. Prepare pre-cleaned glass sample bottles according to the number of water samples to be collected.
      i. Rinse the bottles first with distilled water and then with acetone, ensuring they are clean and free of contaminants.
      ii. Seal the bottles and label them appropriately.
   b. Prior to sampling, ensure that all necessary equipment is present on the boat.

   *Note:* Equipment includes manta nets, flowmeter, metal funnel, pressurized water pump, marker or pencil, formaldehyde, three plastic basins (tubs), aluminum foil, labeled glass sample bottles, forceps, spatula, large resealable plastic bags, 5 mm mesh sieve, field notebook, GPS, and a compartmentalized sample carrying case (Figure 1).

2. Collection of Surface Water Samples Using Manta Nets and Transfer to Labeled Glass Sample Bottles.



**Figure 1. Compartmentalized carrying case for transporting sample bottles**

a. Navigate the boat to a pre-determined sampling station using GPS or a suitable mobile application (e.g. Google Maps, Garmin Explore).
b. Attach the flowmeter securely to the manta net,

*Note:* Ensure that the propeller spins freely to measure the volume of water filtered.

c. Open the collector valve of the manta net, lower the net into the water from the side of the boat, and allow it to remain submerged for approximately one minute.
d. After one minute, carefully remove the manta net from the water and close the collector valve.
e. Verify that the flowmeter propeller has rotated and record the reading in the field notebook.
f. Deploy the manta net into the water.

*Note:* Ensure that it is securely positioned beside the boat and that wake turbulence does not enter the net (Figure 2).

g. Steer the boat at a slow speed (1–3 knots or 2–6 km/h) for 15 min.
h. After 15 min, stop the engine and briefly reverse the boat to prevent the flowmeter from over-rotating while retrieving the net.
i. Record the final reading of the flowmeter in the field notebook.
j. Calculate the volume of water sampled by subtracting the initial flowmeter reading from the final reading.
k. Record environmental parameters (water temperature, weather conditions, wind speed, and direction) for each station.

*Note:* These data are essential for interpreting the distribution of MPs.

⚠ CRITICAL: During net retrieval, one person should lift the frame of the manta net while another dips and lifts the net's filtering section, ensuring all retained materials flow into the collector.



**Figure 2. Deployment of the manta net alongside the boat**

l. Use a pressurized water pump to wash any particles remaining on the net into the collector.

⚠ CRITICAL: For particles stuck at the junction between the net and the collector, use the pressurized water pump to dislodge and transfer them to the collector.

m. If the collector contains minimal vegetation:
   i. Use the pressurized water pump to direct any adhered material towards the valve.
   ii. Attach a funnel to the mouth of a labeled glass sample bottle, carefully open the collector valve, and transfer the sample into the bottle using the pressurized water pump.

n. If the collector contains significant vegetation:
   i. Rinse a plastic basin (tub) with water from the sampling station.
   ii. Empty the vegetation and other particles from the collector into the rinsed basin.
   iii. Use the pressurized water pump to wash any adhered particles towards the valve.
   iv. Attach a funnel to the mouth of a labeled glass sample bottle, carefully open the collector valve, and transfer the liquid sample into the bottle using the pressurized water pump.
   v. Transfer the residual water in the basin containing the vegetation to the glass sample bottle.
   vi. Use a spatula or forceps to collect remaining plant materials, transfer them to aluminum foil, and properly fold the foil.
   vii. Label the aluminum foil with station information and place it in a large resealable plastic bag.
   viii. If particles remain in the basin, transfer them to the sample bottle using the pressurized water pump.

o. Add approximately 5–7 mL of formaldehyde to each glass sample bottle and seal it tightly.

*Note:* The primary purpose of adding formaldehyde is to prevent microbial decomposition of the sample and inhibit bacterial growth until processing.

p. Verify that the label on the glass sample bottle corresponds to the correct station, then place the bottles in the compartmentalized carrying case for safe transport to the laboratory.

q. Until analysis, store all collected samples in clean, acetone-rinsed glass containers with tightly sealed lids, in a dark environment at approximately 4°C to minimize contamination.

*Note:* For long-term storage, maintain samples at −20°C or lower.

⚠ CRITICAL: Avoid conducting water sampling during high wave or strong wind conditions, as these can compromise the accuracy and reproducibility of surface water MP measurements. Specifically, sampling should be avoided when wind speed exceeds 3 knots and wave height surpasses 25 cm. These conditions may alter surface water dynamics, cause

turbulence, or impair net stability, thereby affecting sample integrity and comparability across stations.

*Note:* The pressurized water pump system used for water sample processing operates using water directly sourced from the sampling location. To prevent external contamination during sample transfer and flushing, a mesh screen identical in pore size (330 μm) to the manta net is fitted to the pump inlet. This setup ensures consistency in sampling and eliminates contamination from external water sources.

*Note:* While at least two replicates per station are recommended to ensure statistical robustness, the number of water samples collected at each location may vary depending on available resources, time constraints, and logistical feasibility. In practice, several published studies have used a single manta tow per station due to such limitations. Researchers are encouraged to adjust sampling effort according to their specific project design and capacity.

3. Laboratory Preparation for Microplastic Extraction from Manta Samples.

*Note:* Ensure that the laboratory workspace is clean and restrict access to authorized personnel only.

a. Clean all equipment to be used (forceps, spatulas, funnels, filtration setup, glass jars, and glass Petri dishes) with water filtered through a 1.2 μm GF/C filter.
b. Prepare an excess of Petri dishes (3–5 more than the number of samples) by cleaning them with acetone and leaving them to dry in a closed environment.
c. Label the dried and closed Petri dishes according to the sampling stations.

*Note:* If multiple Petri dishes are required for a single sample, assign sequential numbers to each. If processing samples from multiple lakes or stations, ensure all equipment is rewashed with 1.2 μm GF/C filtered water between sample sets to prevent cross-contamination.

d. Flush the filtration system by pouring approximately 1 liter of 1.2 μm filtered (through GF/C filter) water through the vacuum filtration setup.

*Note:* Preparing a stock of 5 liters of distilled or properly filtered water beforehand can significantly improve workflow efficiency.

4. Digestion of Organic Matter.
   a. If organic matter is present in the filtered manta samples (in the glass sample bottle), add 100–150 mL of hydrogen peroxide ($H_2O_2$).
   b. Heat the sample on a hot plate at no more than 60°C for at least two days to ensure complete digestion of organic material.
   c. Before use, $H_2O_2$ must be filtered once using the vacuum filtration system. Troubleshooting 1.
   d. Once the organic matter is completely digested, pass the solution through a 200 μm metal sieve to remove residual $H_2O_2$.
   e. Place a 1.2 μm GF/C glass microfiber filter in the vacuum filtration system using forceps. Troubleshooting 2.
   f. Transfer the remaining material on the sieve to the filtration funnel using a pressurized water pump.
5. Digestion of Minimal Organic Matter (If Necessary).
   a. If the manta samples in the glass sample bottles contain minimal particulate or organic matter (or if organic matter has been digested as described above), filter the samples directly after passing them through a 200 μm metal sieve.
   b. Place the filter paper in the vacuum filtration system using forceps.

c. Use the pressurized water pump to transfer all particles from the sieve to the filtration funnel.
d. After filtration, wash the inner walls of the filtration funnel with the pressurized water pump to ensure that all particles are transferred to the filter.
e. Carefully transfer the filter paper containing the sample to pre-labeled Petri dishes using a spatula, minimizing handling time to avoid filter damage.

⚠ CRITICAL: To prevent cross-contamination, thoroughly clean all equipment (sieve, filtration funnel) with 20 μm filtered water before and after each use (for detailed explanation see section quality assurance and quality control (QA/QC).

*Note:* Always keep GF/C filter boxes closed when not in use.

*Note:* Petri dish lids should always be placed with the inner surface facing downward.

*Note:* The pressurized water pump system used for water sample processing operates using water directly sourced from the sampling location. To prevent external contamination during sample transfer and flushing, a mesh screen identical in pore size (330 μm) to the manta net is fitted to the pump inlet. This setup ensures consistency in sampling and eliminates contamination from external water sources.

### Sediment samples

Timing: 7 days total

Timing: 1 day for step 6

Timing: 2 h for step 7

Timing: 1 day for step 8

Timing: 1 day for step 9

Timing: 1 day for step 10

Timing: 1 day for step 11

Timing: 1 day for step 12

Timing: 1 day for step 13

Here, we describe steps for the extraction and isolation of MPs from benthic freshwater sediment samples. Sediment is collected using a Van Veen grab and stored in clean containers prior to laboratory processing. Dried sediment is subjected to density separation with potassium carbonate ($K_2CO_3$) to isolate MPs from mineral and organic fractions. The supernatant containing floated particles is filtered through GF/C filters. This procedure enables recovery of plastic particles down to 1.2 μm, depending on the filtration capacity. The sediment-specific steps are optimized to minimize organic interference and ensure efficient recovery across a range of polymer densities.

6. Field Preparation for Sediment Sampling.
   a. Follow the step 1 in water samples section.

   *Note:* Prior to sampling, ensure all necessary equipment is available on the boat, including a benthic grab sampler (Van Veen Grab).



**Figure 3. Deployment of the benthic grab sampler into the water**

7. Collection of Sediment Samples from Freshwater Ecosystems Using a Benthic Grab Sampler and Transfer to Labeled Pre-Cleaned Glass Containers.
   a. Navigate the boat to a pre-determined sampling station using GPS or an appropriate mobile application (e.g. Google Maps, Garmin Explore).
   b. Measure and record the depth of the station using the depth gauge in the field notebook.
   c. Adjust the benthic grab sampler and carefully lower it into the water until it reaches the bottom (Figure 3).
   d. Retrieve the sediment sample collected by the benthic grab and transfer the sediment into a clean basin (tub).
   e. Using a spatula, carefully collect the upper 5 cm layer of the sediment (surface layer) and transfer this portion into a labeled glass jar.
   f. Add 5–7 mL of formaldehyde to the glass jar and securely seal it.

   *Note:* The primary purpose of adding formaldehyde is to prevent decomposition of the sample and to inhibit any potential biological activity within the sediment.

   g. Verify that the label on the glass jar corresponds to the correct sampling station.
   h. Place the jar in the compartmentalized sample carrying case for safe transport to the laboratory.
   i. After use, rinse the benthic grab sampler thoroughly with water.
8. Laboratory Preparation for MP Extraction from Sediment Samples.

Follow the step 3 in water samples section troubleshooting 1.

9. Preparation of Potassium Carbonate ($K_2CO_3$) Solution for Density-Based Separation of Sediments.
   a. Prepare the solution by using chemicals provided the second table in the materials and equipment section.
   b. Stir the solution thoroughly to ensure complete dissolution and allow it to sit for one day.



**Figure 4. Weighing and preparation of sediment samples**

Conduct this process quickly and with at least two individuals to minimize contamination risks.

c. Given that the $K_2CO_3$ solution is exothermic, prepare the solution in a cold-water bath to prevent excessive heating.
d. Flush the filtration system with approximately 1 liter of 20 μm filtered water to ensure cleanliness.
e. Discard the filtered water collected in the receiving flask (Erlenmeyer flask).
f. Carefully place a 1.2 μm GF/C glass microfiber filter in the vacuum filtration system using forceps.
g. After one day, filter the $K_2CO_3$ solution using the vacuum filtration system, collecting the filtered solution in an Erlenmeyer flask.
h. Transfer the filtered $K_2CO_3$ solution to a pre-cleaned, acetone-rinsed glass jar or beaker and label it.
i. Store the filtered $K_2CO_3$ solution in a sealed container for use with sediment samples.

10. Preparation and Labeling of Sub-Samples for Each Station.
    a. For each station, prepare at least two pre-cleaned, acetone-rinsed, labeled glass jars with glass lids.
    b. Measure the tare weight of the jars and record the weight, ensuring the labels are clear and accurate.
    c. Mix the sediment for ensuring the homogeneity.
    d. Weigh and transfer 50 or 100 grams of sediment into each glass jar, sealing them with glass lids.

*Note:* The specific weight (50 g or 100 g) can be determined according to the research objectives.

⚠ CRITICAL: If a higher sample weight is chosen, consider that the amount of chemical reagents used in the subsequent extraction process will also increase (Figure 4).



**Figure 5. Separation setup for microplastic extraction from sediment samples**

11. Density-Based Separation of Sediments Using $K_2CO_3$ Solution.
    a. Add 150–200 mL of the prepared $K_2CO_3$ solution to each jar containing 50 or 100 grams of sediment.
    b. Stir the mixture gently using a glass or metal rod to break up any clumps and ensure homogeneity.
    c. Pour the homogenized sediment suspension into the upper section of the separation system (transparent tube) until two-thirds full (Figure 5).
    d. Briefly open and close the valve of the separation funnel once.

⚠ CRITICAL: If the liquid level in the transparent tube decreases below two-thirds, replenish with $K_2CO_3$ solution.

    e. Cover the open top of the separation system with a Petri dish, invert the system carefully, and secure it in a stand.
    f. After one hour, slightly open and close the valve again, then leave the system undisturbed for one day.

*Note:* This process helps relieve pressure and minimizes bubble formation at the phase boundary.

*Note:* To ensure complete separation, gently agitate the system once or twice within the first few hours to prevent sediment buildup in the upper phase.

12. Vacuum Filtration of Sediment Samples.
    a. After one day, if the upper phase of the separation system appears clear, close the valve. Troubleshooting 1.

b. For each station, prepare two separate 1.2 μm GF/C filters in the vacuum filtration system using forceps.
c. Carefully transfer the upper phase solution of the separation system into the filtration setup using a pressurized pump, minimizing water usage. Troubleshooting 11.
d. After filtration, wash any remaining particles adhering to the walls of the funnel using the pressurized water pump, ensuring they are transferred to the filter.
e. Transfer the filter papers with collected particles to pre-labeled Petri dishes, using a spatula, and minimize handling time to avoid damage.

13. Recovery of $K_2CO_3$ Solution.
a. After filtering the upper phase of the separation system, collect the remaining solution in the Erlenmeyer flask and transfer it into a clean glass jar for recovery.
b. Place a fresh 1.2 μm GF/C filter in the vacuum filtration system using forceps.
c. Filter the recovered $K_2CO_3$ solution through the system and collect it in an acetone-rinsed, labeled glass jar.
d. Transfer the lower phase of the separation system (sediment-free solution) into another clean glass jar for recovery.
e. Allow this solution to settle for one day, permitting any suspended particles to precipitate.
f. The following day, filter this solution through a fresh GF/C filter and collect it in a labeled glass jar.

*Note:* To prevent cross-contamination, thoroughly clean all equipment (sieve, filtration funnel) with 20 μm filtered water before and after each use.

*Note:* Always keep GF/C filter boxes closed when not in use.

*Note:* Petri dish lids should always be placed with the inner surface facing downward.

### Fish samples

**Timing: 10–11 days total**

**Timing: 2 days for step 14**

**Timing: 1 day for step 15**

**Timing: 1 day for step 16**

**Timing: 3 days for step 17**

**Timing: 3 days for step 18**

**Timing: 2–4 h for step 19**

Here, we describe steps for the dissection and chemical processing of fish gastrointestinal tract samples to isolate MPs. Fish specimens are humanely euthanized and dissected to remove the entire digestive tract. The tissues are subjected to chemical digestion using $H_2O_2$, with optional supplementation by NaClO/KOH in cases of high lipid or resistant organic content. The resulting solution is filtered through GF/C filters. This approach supports the detection of MPs $\geq$1.2 μm and is applicable across a range of freshwater fish species. All procedures are designed to prevent sample contamination and ensure reliable downstream analysis.

14. Field Preparation and Standard Sampling Methods for Fish Collection in Freshwater Ecosystems.

a. Follow the step 1 in water samples section.
b. Prior to sampling, ensure that all necessary equipment is present on the boat.

*Note:* Equipment includes all necessary permissions/ethics approvals (if necessary) and equipment is available on the boat prior to fish sampling, including a gillnet composed of 12 panels (each 25 meters in length) with varying mesh sizes (e.g. 9 × 9 mm, 16 × 16 mm, 20 × 20 mm, 24 × 24 mm, 30 × 30 mm, 35 × 35 mm, 40 × 40 mm, 45 × 45 mm, 50 × 50 mm, 55 × 55 mm, 60 × 60 mm, 70 × 70 mm).[24,25]

c. Navigate the boat to the designated sampling station or lake using GPS or a suitable mobile application (e.g. Google Maps, Garmin Explore).
d. Deploy the gillnet in the water and secure it with a visible buoy.
e. Return the following day to retrieve the net.
f. Randomly select a maximum of 5–7 fish specimens from each species, wrapping them in aluminum foil, and transport them to the laboratory in a cold container.
g. Release any live or excess fish back into the lake.

*Note:* Researchers must obtain all relevant ethical approvals and collection permits for vertebrate sampling prior to initiating fieldwork, in accordance with local and national regulations. Fish should be euthanized using humane methods that comply with animal welfare laws specific to each country or institution. Following capture, specimens should be promptly wrapped in aluminum foil and stored in a cold container with ice or ice packs to preserve tissue integrity until laboratory processing.

15. Laboratory Preparation Prior to Microplastic Extraction from Fish Samples.
    a. Follow the step 1.3.
    b. Assign unique identification codes for each fish sample, including fish species, freshwater source, and sampling date. Example:
        i. *1. C. carpio* Eğirdir 14.04.2025.
        ii. *2. C. carpio* Eğirdir 14.04.2025.
    c. If the fish samples were stored at −20°C, allow them to thaw before proceeding.
    d. Record the fork length (or total length) and weight of each fish.
16. Dissection of Fish Digestive Organs (Stomach and Intestines) and Transfer to Pre-cleaned Glass Jars.
    a. Carefully place each fish on a clean, pre-disinfected dissection tray and use a scalpel or dissection scissors to make a ventral incision from the anus to the base of the gill cover.
    b. Using sterilized forceps, gently separate and remove the entire digestive tract (from the esophagus to the anus), taking care not to rupture the tract during removal.
    c. Transfer the entire digestive tract into a pre-labeled, acetone-rinsed glass jar and cover immediately with a clean glass Petri dish to prevent airborne contamination.

⚠ CRITICAL: Ensure the digestive tract is extracted in one intact piece to avoid the loss of gut contents. Confirm completeness by visually inspecting for continuity between the esophagus and anus and by observing whether any gut material remains inside the abdominal cavity or is spilled during dissection.

⚠ CRITICAL: If the digestive tract is ruptured and gut content visibly lost during dissection, discard the sample or record the deviation clearly to account for potential underestimation in downstream analysis.

*Note:* Visually note the fullness of the digestive tract (e.g., empty, partially full, fully distended), as this can influence digestion time and potential MP load.

*Note:* For large-bodied fish, avoid including additional internal organs such as liver or gonads to minimize reagent consumption and reduce digestion time.

17. Digestion of Digestive Tract Tissues Using Hydrogen Peroxide ($H_2O_2$).
    a. Add approximately 150–200 mL of $H_2O_2$ solution to each jar containing fish digestive tissues, and cover the jar with a glass Petri dish.
    b. Place the jars on a hot plate at a temperature not exceeding 60°C and maintain this temperature for at least two days to ensure complete digestion. Troubleshooting 4, 5, and 7.

⚠ CRITICAL: During daily checks, if excessive foaming occurs, gently swirl the solution or add a small amount of distilled water.

18. Secondary Digestion Using KOH + NaClO Solution for Undigested Samples.
    a. If any undigested material remains after $H_2O_2$ treatment, pass the remaining contents through a 20 μm stainless steel sieve.
    b. Transfer any residual material on the sieve into a pre-cleaned glass jar.
    c. Add a maximum of 250 mL of KOH + NaClO solution, prepared as provided in the first table in Material Setup section.
    d. When preparing the KOH solution, perform the dissolution in a cold-water bath due to the exothermic nature of the reaction.
    e. Place the jars containing the KOH + NaClO-treated samples on a hot plate at no more than 60°C for an additional two days.
19. Vacuum Filtration of Fish Samples.
    a. For each fish sample, carefully place a filter paper in the vacuum filtration system using forceps.

*Note:* Repeat this process for each sample.

    b. Once organic matter is completely digested, transfer the solution (either the $H_2O_2$ solution or KOH + NaClO solution, depending on the treatment used) through a 20 μm metal sieve.
    c. Use a pressurized water pump to transfer any remaining particles on the sieve into the filtration funnel. Troubleshooting 6, 8, and 9.
    d. After filtration, use the pressurized water pump to rinse any particles adhering to the walls of the funnel, ensuring they are directed onto the filter paper.
    e. Carefully transfer the filter paper containing the sample to pre-labeled Petri dishes using a spatula. Minimize handling time to avoid filter damage. Troubleshooting 8 and 10.

*Note:* To avoid cross-contamination between samples, clean all equipment (sieve, filtration funnel) thoroughly with 20 μm filtered water before and after each use. GF/C filter paper containers should always be kept sealed to prevent contamination. Petri dish lids should always be placed with the inner surface facing downward.

*Note:* For a fully digested set of 10 fish samples, vacuum filtration typically requires ~10 min per sample, assuming minimal residual organic matter and no clogging. An additional 10 min per sample is allocated for system decontamination to prevent cross-contamination between samples (e.g., rinsing filter funnels and sieves with 20 μm filtered water). Therefore, the total filtration process for 10 samples may take approximately 2 to 4 h, depending on the cleanliness of each digest and workflow efficiency. If digestion is incomplete or if lipidic residues are present, the filtration time may increase.



**Figure 6. Plexiglass enclosure containing the microscope for microplastic analysis**

### Enumeration of microplastics under microscopy

Timing: 7 days

Timing: 1.7 h per filter (for 100 particles)

Here, we describe steps for the microscopic identification and quantification of microplastics based on their physical characteristics. Following sample-specific extraction and filtration, particles retained on GF/C filters are examined under a binocular microscope. Suspected microplastics are classified by shape (fiber, fragment, film, sphere), color, and size. At least two trained analysts perform the enumeration to ensure accuracy and reproducibility. For samples with >100 particles, semi-quantitative grouping and categorization by size ranges are employed to improve efficiency. This step provides the foundation for downstream spectroscopic confirmation and enables robust data collection across all sample types.[26] Troubleshooting 15.

20. Place the Petri dishes containing the filter papers with retained particles under a binocular microscope.
21. Examine various magnifications to identify suspected MP particles.

⚠ CRITICAL: Microscopic analysis should be conducted by at least two trained individuals, with each sample being examined at least twice to ensure consistency and accuracy. One person is responsible for identifying and counting the particles, while the other records observations and enters data into a data sheet.

⚠ CRITICAL: The microscope used for this analysis must be placed within a closed enclosure, such as a plexiglass cabinet, to minimize contamination (Figure 6).

*Note:* Observations should begin with the lowest magnification, progressing through higher magnifications.

*Note:* Particles characterization should be based on their shapes (fragment, fiber, film, and sphere) and colors, and their sizes are measured (Figures 7 and 8).

*Note:* If the number of MP particles on the filter paper exceeds 100 (n > 100), direct counting of each particle may become impractical. In such cases:

a. Particles of similar color and shape can be grouped and counted collectively.

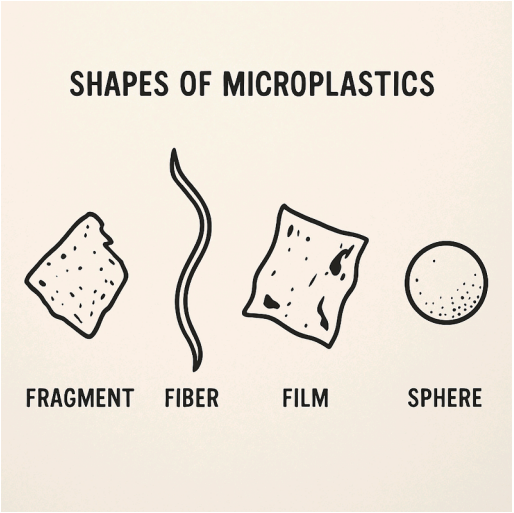



**Figure 7. Classification of particle types under the microscope**

b. Instead of measuring each particle individually, size ranges can be recorded (e.g., <0.05 mm, 0.05–0.1 mm, 0.1–0.5 mm, 0.5–1 mm, 1–3 mm, and 3–5 mm).

22. Record all of the suspected particles to a data sheet.

*Note:* The data sheet should include a comment column for each particle or particle group, allowing the addition of any relevant observations that may aid in subsequent polymer identification.

*Note:* Microscopic enumeration can be performed using a standard binocular microscope or, if polymer characterization is also required, using a spectroscopic instrument equipped with a microscope attachment. This combined approach may extend the analysis time. In such cases, particles are first counted, and then a subset of randomly selected particles (e.g., 30% of the total counted particles) is subjected to polymer identification.

⚠ CRITICAL: MP particles smaller than 100 μm, especially those that are transparent or have irregular shapes, can be challenging to identify accurately. These particles are particularly susceptible to misclassification, leading to potential contamination. To minimize this risk, the microscope should always be enclosed within a cabinet.

⚠ CRITICAL: Distinguishing between fragments and films for particles smaller than 100 μm can be highly challenging. For consistency, it is recommended to categorize particles primarily into fragment, fiber, and sphere categories.

*Note:* Some particles may visually resemble plastic but may not be true MPs. Prior to measurement or counting, these particles can be tested using the hot needle test.[27] For this test, a heated needle (attached to an inoculation loop and heated with a heat source) is carefully brought near the particle. Particles that melt or exhibit shrinking behavior upon contact with the hot needle are likely to be plastic and can be counted as such. However, it should be noted that this test might cause damage to the sample. Consequently, it is advised that this test not be used unless absolutely necessary.

*Note:* For the purposes of this protocol, MPs are defined as synthetic polymer particles ranging in size from approximately 1.2 μm to 5 mm. Particles are morphologically categorized



**Figure 8. The appearance of microplastics under microscope**

as fibers, fragments, films, or spheres based on their shape under microscopy. Due to methodological constraints, the lower detection limit varies by sample matrix (e.g., 330 μm for water samples via manta trawl; ~1.2 μm for fish tissue and sediment samples filtered through GF/C filters).

*Note:* Each suspected MP particle is visually inspected and categorized by shape, color, and size. On average, one particle takes approximately 1 min to fully analyze under the microscope. For an average sample with 100 particles, the total time required for enumeration is approximately: 100 particles × 1 min = 100 min (~1.7 h) per filter/sample. This includes repositioning of the filter, documentation, and verification. The time may be extended if the number of particles exceeds 100 or if complex morphological features require further scrutiny.

### Polymer identification using μ-Raman spectroscopy

Timing: 6.5 h per filter

Timing: 1–3 days for step 24

Here, we describe steps for the spectroscopic confirmation of MP particles using μ-Raman spectroscopy. This stage is critical for verifying the polymeric nature of visually identified particles and for preventing misclassification of non-plastic materials such as organic debris or mineral fragments. The filter paper containing counted particles is placed directly on the stage of a Raman microscope, and particles are selected based on morphology and randomly screened for spectral analysis. Using a 785 nm laser with appropriate settings, characteristic Raman spectra are obtained and compared against commercial or in-house polymer libraries. This step ensures precise polymer identification and enhances the robustness of MP data. Troubleshooting 14.

*Note:* Ensure that the Petri dishes are at least 10 mm wider in diameter than the filter paper to prevent the microscope objective from contacting the edges of the dish during focusing. This prevents displacement of particles on the filter paper due to contact with the objective and allows for full-surface scanning of the filter.

Once the filter paper is positioned under the Raman microscope, close the microscope's cabinet cover. Using the remote control mouse for the microscope stage, randomly select particles suspected to be MPs based on their shape, color, and size, and proceed with polymer identification (Figure 9).

*Note:* The selection of particles for Raman analysis must be based on a representative sampling approach: For example, if a total of 100 particles have been counted across 10 samples, a minimum of 30% of the counted particles (i.e., 30 particles) should be analyzed for polymer identification. This means that for each sample, at least 3 particles should be subjected to Raman analysis.

*Note:* Each particle selected for μ-Raman analysis undergoes three spectral acquisitions with an exposure of 10 s each, plus additional time for: Automated spectrum searching and library comparison (~10 min per particle); Focusing and stage adjustments (~2 min per particle). For a standard analysis of 30 particles per filter (representing ~30% of the total), the estimated total time is: (3 × 10 s) + 2 min (searching/focusing) + 10 min (spectral analysis) ≈ 13.5 min/particle; 30 particles × 13.5 min = ~405 min (~6.75 h per filter). This includes changing filters and background calibration. If 100% of particles are analyzed by Raman, the total time would increase proportionally.

23. Raman Spectroscopy Settings and Parameters.
    a. Optimize the laser wavelength and other settings based on the instrument configuration.

*Note:* For this protocol, the following settings were applied: Laser wavelength: 785 nm; Grating: 600/1200; Accumulation: 3; Exposure time: 10 s; Laser intensity: 0.5%–1% (adjustable)

For improved signal strength, the laser intensity can be increased to 5% or 10%, and the number of accumulations can be raised. However, caution is advised.

    b. Higher laser intensities may cause saturation problems, making it difficult to obtain clear spectra.
    c. Laser intensity should only be increased in cases of excessive noise or weak signal strength.
24. Polymer Identification and Spectrum Comparison.

*Note:* The acquired spectra are compared with reference spectra of known polymers to confirm the polymer type. This can be achieved in two ways:

*Note:* Comparing the obtained spectra with spectra of commercially available polymer standards analyzed under identical conditions.

*Note:* Utilizing commercial polymer libraries. In this study, both the ST-Japan commercial library and pre-obtained spectra of 12 standard polymers were used for reference. These standard polymers include: High-density polyethylene (HDPE): Low-density polyethylene (LDPE): Polypropylene (PP): Polyethylene terephthalate (PET): Polystyrene (PS); Polycarbonate (PC): Polyvinyl chloride (PVC): Polymethyl methacrylate (PMMA): Polyoxymethylene (POM): Polylactic acid (PLA): Polyamide-6 (PA-6/Nylon-6): Acrylonitrile butadiene styrene (ABS)

⚠ CRITICAL: If the acquired spectra do not perfectly match (less than %70) the reference spectra of standard polymers, this may be due to the presence of additives or chemical modifications within the MP particles. In such cases, focus on the specific characteristic peaks of the target polymer for accurate identification.

*Note:* In addition to commercial libraries, open-access libraries such as OpenSpecy (https://www.openanalysis.org/openspecy/) can be utilized for spectrum comparison.

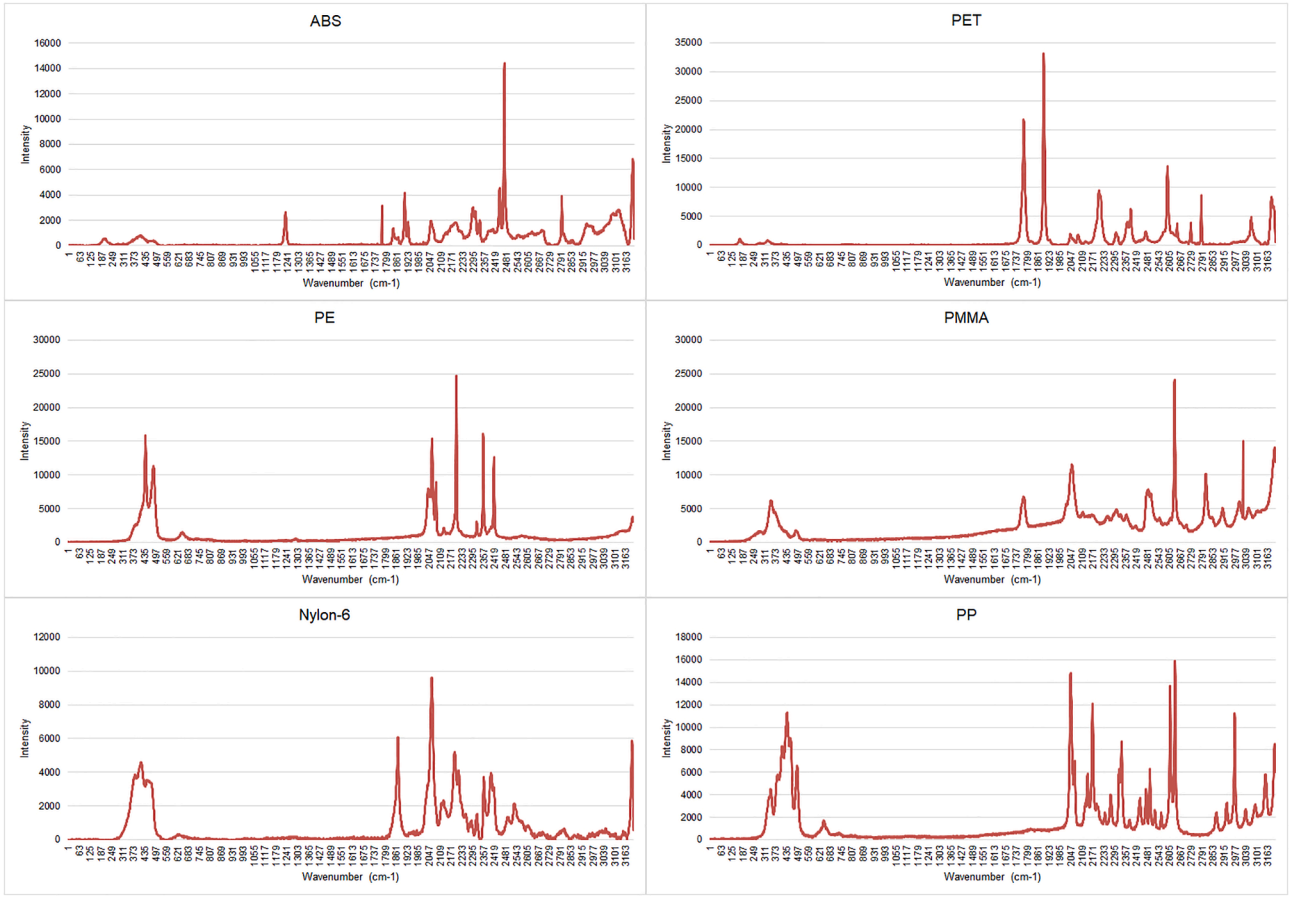



**Figure 9. Representative μ-Raman spectrums of some of the polymers**

25. Calculating Polymer Confirmation Rate.

*Note:* The proportion of particles identified as polymers among the total particles analyzed by Raman spectroscopy is termed the polymer confirmation rate. For example, if 100 particles were counted under the microscope, 30 particles were analyzed using Raman spectroscopy, and 27 of these were confirmed as polymers, then the polymer confirmation rate is calculated as:

$$Polymer\ Confirmation\ Rate = \frac{27}{30} \times 100 = 90\%$$

This rate indicates that 90% of the counted particles are confirmed as MPs. Consequently, for proportion calculations, this adjusted count should be used (e.g., 90 particles in 100).

## Quality assurance and quality control

*Note:* Ensuring data integrity in MP studies requires strict control over contamination, which can significantly affect the accuracy of results. Contamination may arise from two primary sources: Environmental contamination during sampling and sample processing and laboratory contamination introduced during sample handling and analysis.

This section provides detailed guidelines for minimizing contamination and ensuring high-quality, reliable results.

26. Preventing Contamination during Sampling.
    a. Use non-plastic equipment for sampling. This includes glass or metal containers for sample collection, sieves, funnels, sediment collection containers, and water sampling containers.
    b. Prior to field sampling, all equipment should be cleaned using an acetone rinse followed by rinsing with filtered water. Store equipment in sealed, sterile containers or wrapped in aluminum foil.
    c. Similarly, sample bottles should be rinsed first with acetone and then with filtered water.
    d. To prevent contamination from clothing, field personnel should wear cellulose-based clothing of a distinct color (e.g., pink color). This allows for the detection of any contamination originating from clothing fibers during sample analysis.
    e. To prevent contamination from the pressurized water pump system used for water sample processing using water directly sourced from the sampling location is crucial.

*Note:* To prevent external contamination during sample transfer and flushing, a mesh screen identical in pore size (330 μm) to the manta net is fitted to the pump inlet. This setup ensures consistency in sampling and eliminates contamination from external water sources.

27. Preparation of the Laboratory Workspace.
    a. Cleanroom Environment: Ensure that the workspace is regularly cleaned and, if possible, conduct analysis in a laminar flow cabinet or an area equipped with HEPA filters.
    b. Surface Cleaning: Clean all work surfaces with acetone using non-plastic materials.
    c. Air Control: Minimize airflow within the analysis area and restrict access to the laboratory during analysis to prevent airborne contamination.
28. Personal Protective Equipment (PPE).
    a. Laboratory personnel should wear cotton laboratory coats, natural fiber clothing, and a hair cover.
    b. Avoid synthetic clothing. Instead, use natural fiber garments with distinct colors to easily identify any contamination.
    c. Before starting the analysis, clean clothing with a lint roller to remove any loose fibers, reducing the risk of contamination from clothing.
29. Contamination Control for materials and equipment.
    a. Use only glass or metal equipment for all analysis processes, including pipettes, jars, spatulas, and other utensils.
    b. Ensure that all solutions and liquids used are either pre-filtered or verified to be free of MP contamination.
    c. Wrap cleaned equipment in aluminum foil and store it in a sealed container until use.
30. Blank (Negative) Controls.
    a. To monitor contamination from both the environment and analytical procedures, prepare and maintain negative control (blank) samples throughout the study.
    b. Blank samples should be exposed to the laboratory environment or processed through all analytical steps without a sample to assess potential contamination.
    c. Results from blank samples should be used to correct the final MP counts in the samples.
31. Positive Control Setup.

⚠ CRITICAL: Positive controls are essential for validating the efficiency of the MP extraction, filtration, and analysis methods described in this protocol.[1,2]

    a. Prepare standard reference MP particles.

*Note:* Standards includes PE, PET, PP, or PS in various sizes and shapes (fibers, fragments, and spheres). These can be either laboratory-prepared or obtained from certified suppliers.

*Note:* Carefully document the properties of the prepared particles, including size range, density, and quantity.

b. For each sample type (water, sediment, and fish tissues), prepare positive control samples by adding a known quantity of reference MP particles to the corresponding extraction solutions:
   i. Water Samples: Add the reference particles to 500 mL of filtered, deionized water.
   ii. Sediment Samples: Mix the particles into 50 g of clean, sterilized sand that has been tested for the absence of MP contamination.
   iii. Fish Samples: Simulate the fish digestive environment by adding the reference particles to a gelatin or agar-based, sterilized medium.

⚠ CRITICAL: Ensure that the mixtures are well homogenized before proceeding.

c. Process positive control samples through all steps of the protocol, including extraction, filtration, and analysis, alongside the actual samples.
d. At the end of the process, compare the recovered MP particles with the initial reference quantity and type to calculate the recovery rate.

*Note:* The expected recovery rate should be at least 80%.

*Note:* If the recovery rate is below 80%, review the protocol steps, identify potential contamination sources or process losses, and optimize the method as necessary.

## EXPECTED OUTCOMES

This protocol enables the quantitative and qualitative analysis of MPs in water, sediment, and fish digestive system samples. MPs are classified through microscopic analysis based on their physical characteristics, including color, shape (fiber, fragment, film, or sphere), and size. For polymer identification, μ-Raman spectroscopy is employed, with a target polymer confirmation rate of at least 70%, achieved by analyzing a minimum of 30 particles per sample. The protocol ensures that sample material is evenly distributed on GF/C filters, which facilitates accurate and efficient microscopic counting. Moreover, it is designed to maintain an inter-sample contamination rate of less than 5%, thereby ensuring high data integrity and reliability throughout the analytical process.

## LIMITATIONS

MP detection in this protocol relies heavily on optical techniques and the expertise of the analyst, making it particularly challenging to accurately characterize particles smaller than 100 μm. The digestion of organic material using hydrogen peroxide ($H_2O_2$) and sodium hypochlorite/potassium hydroxide (NaClO/KOH) may be insufficient for some biological tissues, potentially leading to gel formation or foaming, which can delay the analysis process. In samples with a high particle load, filter clogging can occur, significantly extending the analysis time. Microscopic analysis can also lead to misclassification, particularly for transparent and white MPs, such as films, increasing the risk of false identifications. Furthermore, polymer confirmation using Raman spectroscopy may be affected by spectral ambiguities caused by the presence of additive chemicals within plastic particles, complicating accurate identification. Finally, due to the inherent environmental heterogeneity in freshwater ecosystems, the distribution of MPs between samples may exhibit high variability, necessitating careful interpretation of the results.

## TROUBLESHOOTING

### Problem 1: Incomplete digestion of macrophytes or large particles in water or sediment samples

Large organic materials, such as macrophytes or other bulky particles, may not completely degrade in the $H_2O_2$ solution, causing them to accumulate on the filter paper. This can obstruct the identification and analysis of MPs (related to water and sediment samples, Step 4, 17).

### Potential solution

If large organic matter is present, use sodium hypochlorite (NaClO) / potassium hydroxide (KOH) solution for secondary digestion. Heat the solution at a maximum of 60°C. Alternatively, manually remove these particles with forceps (time consuming), and use a pressurized water pump to wash them, ensuring that any adhering MPs are transferred to the filter.

### Problem 2: Leakage in the vacuum filtration system

A leakage in the vacuum filtration system may cause MPs in the solution phase to escape, compromising data reliability (related to sediment, water and fish samples, Step 12, 4, 19).

### Potential solution

Replace or adjust the gasket of the manifold system. Ensure that the gasket is appropriately sized to provide a tight, leak-proof seal.

### Problem 3: Fungal growth in Petri dishes due to moisture

Moist or wet filter papers left in Petri dishes can promote fungal growth, contaminating the samples (related to water, sediment and fish samples, Step 5, 12, 19).

### Potential solution

Ensure that filter papers are completely dry before placing them in Petri dishes. Verify that Petri dishes are clean and dry after washing and acetone treatment. Store Petri dishes under dry, sterile conditions.

### Problem 4: Incomplete lipid digestion in fish gut samples

Lipids in fish tissues may not dissolve completely in the $H_2O_2$ solution, slowing down the filtration process (related to fish samples, Step 17).

### Potential solution

Add an additional volume of $H_2O_2$ to the digestion solution and incubate for an additional 48 h at a maximum of 60°C.

### Problem 5: Gel formation due to prolonged lipid digestion in fish samples

Extended incubation of fish tissues in $H_2O_2$ may cause gel formation, which can adhere to the manifold walls and trap MP particles (related to fish samples, Step 17).

### Potential solution

Minimize the time the solution remains in the manifold or filtration funnel. Ensure rapid transfer and filtration of the solution.

### Problem 6: Filter clogging during filtration

Excessive particle accumulation can block the filter, significantly slowing down the filtration process (related to fish samples, Step 19).

### Potential solution

Gradually transfer the dissolved solution into the filtration funnel, considering its viscosity and particle density. For highly concentrated solutions, split the sample into 2–3 portions for filtration.

### Problem 7: Foaming and overflow during heating on hot plate

Solution may foam and overflow during heating, leading to the loss of MP particles (related to fish samples, Step 17).

### Potential solution

Monitor the solutions on the hot plate at regular intervals (e.g., hourly). Gently swirl the solution to maintain homogeneity, or add distilled water to maintain volume.

### Problem 8: Filter paper damage during filtration

Prolonged exposure of the filter paper to the solution can cause it to lose structural integrity, leading to tearing (related to fish samples, Step 19).

### Potential solution

Complete the filtration process using the minimum necessary volume of solution and in the shortest possible time.

### Problem 9: Uneven distribution of particles on filter paper

Excessive particle accumulation on the filter can prevent even distribution and hinder microscopic analysis (related to fish samples, Step 19).

### Potential solution

Gradually add the dissolved solution to the filtration funnel, adjusting for solution viscosity and density. If necessary, divide the sample into 2–3 portions and filter each separately.

### Problem 10: Spillage of Petri dishes containing filter papers during transfer

Petri dishes containing MP particles may tip over during transfer, causing particle loss (related to Step 20, 21, 22).

### Potential solution

Store Petri dishes with the lids facing downward in a stable container. Use a secure, compartmentalized storage box for transport.

### Problem 11: Crystallization of $K_2CO_3$ on filter paper

$K_2CO_3$ may crystallize on the filter paper, obscuring MP identification during microscopic analysis (related to sediment samples, Step 9).

### Potential solution

Ensure the $K_2CO_3$ solution is well-mixed before use. After filtering the sample, rinse the filter with a small amount of filtered water to minimize crystallization.

### Problem 12: Low water levels preventing manta sampling

The water level at the lake or station is too low for effective manta net sampling (related to water samples, Step 1).

### Potential solution

Use an alternative method by filtering 20 liters of water through the manta collector to complete the sampling process.

### Problem 13: Flowmeter malfunction during water sampling with manta net

If the flowmeter fails during sampling, the volume of filtered water cannot be directly measured (related to water samples, Step 2).

### Potential solution

Record the GPS coordinates and use a timer to measure the duration of the sampling. Calculate the distance traveled using the boat speed and duration. Estimate the volume of water filtered using the technical specifications of the manta net.

### Problem 14: Spectral noise in μ-Raman spectroscopy

Spectral interference or excessive noise prevents accurate polymer identification (related to polymer identification, Step 24).

### Potential solution

Ensure that the μ-Raman instrument is properly calibrated before use. Gradually increase the laser power (e.g., from 1% to 5%) to improve signal quality. Apply background subtraction and spectrum smoothing algorithms. Cross-check spectra using stronger reference libraries or open-access databases.

### Problem 15: Time-consuming microscopic counting of numerous particles

Counting more than 100 particles individually under the microscope is time-consuming and prone to errors (related to microscopic enumeration, Step 20, 21, 23).

### Potential solution

Use a semi-quantitative counting approach by grouping particles based on color and shape. For polymer confirmation, randomly select 30% of the counted particles for μ-Raman analysis.[28]

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sedat Gündoğdu (sgundogdu@cu.edu.tr).

### Technical contact

Further information and requests for methods, removals, and technical should be directed to and will be fulfilled by the technical contact, Tuncay Vural (tuncay.vural@tarimorman.gov.tr).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate any codes or data.

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## AUTHOR CONTRIBUTIONS

S.G. contributed to the experiments and drafted the protocol. T.V., V.Y., S.Ş., and S.Ç. contributed to the experiments and helped draft the protocol. T.V. and S.G. conceptualized, drafted, and reviewed the protocol and guided the project.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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