Laboratory guidelines for the detection and quantification of plastics particles from freshwater environmental samples

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SUMMARY

The present document develops the laboratory protocols to analyse plastic pollution in freshwater environmental samples, although the methods followed can be extrapolated to other ecosystems. These protocols are developed in the framework of the PLASTICØPYR project, which aims to tackle plastic pollution associated with mountain tourism activities by developing transversal actions involving several stakeholders. These guidelines are developed in order to quantify plastic pollution in different river habitats, from large items (macro- and mesoplastics) to tiny plastic particles (microplastics). The protocols are developed for the analysis of microplastics (MicP) up to 63 µm, from samples from the water column, the fluvial sediments, the fluvial biota (i.e., brown trout and biofilm), as well as plastic particles present in the atmosphere (either wet or dry deposition). The MicP analysis is done following the Nile Red method, a fluorescent dye that facilitates the observation of small particles. Although Nile Red cannot provide chemical characterisation, it can be compatible with MicP identification techniques, such as FTIR (Fourier-transform infrared spectroscopy). The samples procedure includes: the extraction of MicP from the inorganic matrix by density separation (using ZnCl₂), a digestion step with hydrogen peroxide and catalysed with Fenton’s reagent to eliminate the organic remains and finally, the staining of the sample with Nile Red.


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**1. INTRODUCTION**

The present document gathers the laboratory procedures to characterise and describe MacP and MesP items and assess MicP concentrations from different environmental samples in water, sediments, and biota (Table 1). These protocols have been developed in the Laboratory of Plastics Iosune at the Centre for Advanced Studies of Blanes (CEAB-CSIC), in the framework of the Interreg Poctefa project: PLASTICØPYR “Strategies to reduce plastic pollution in mountain ecosystems”. The procedures presented in this document are supplementary to the “Guidelines of field-tested procedures and methods for monitoring plastic litter in Mountain riverine systems” by Margenat et al. (2021), which explains the field procedure to collect samples from different river habitats, such as the riparian zone, the water column and the benthos, for assessing plastic pollution. According to the classifications proposed by Emmerik et Schwarz, (2020) and Alimi et al. (2017) plastic debris can be classified in four size classes. According to the classifications proposed by Emmerik et Schwarz, (2020) and Alimi et al. (2017) plastic debris can be classified into four size classes:

- **Macroplastic (MacP):** > 5 cm
- **Mesoplastic (MesP):** 0.5 cm - ≤5 cm
- **Microplastic (MicP):** 0.1 µm - ≤5 mm
- **Nanoplastics:** 1 nm - ≤0.1µm (not considered for monitoring in these guidelines)

In addition to size, plastics can be categorised according to chemical composition, shape, colour, and even density (Shim et al., 2017; Lusher et al., 2020). These characteristics are highly relevant for determining the source of emission of these contaminants, especially for those fragmented items and MicP particles.

**Table 1.** Set of environmental samples taken to characterise plastic pollution in mountain rivers in the framework of the PLASTICØPYR project.

<table>
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<th>Riverside plastics</th>
<th>MacP and MesP</th>
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<td>● Manta net samples</td>
<td>MesP and MicP &gt;0.33 mm</td>
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<td>● Grab samples</td>
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<td>● Biofilm</td>
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<td><strong>Fish</strong></td>
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<td><strong>Atmospheric deposition</strong></td>
<td>MicP &gt; 0.63 µm</td>
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1.1. Common analytical procedure steps for MicP detection in environmental samples

It has been demonstrated that MicPs are widespread in all-natural environments (Horton and Dixon, 2018), even in remote areas (Zhang et al., 2020). Once in the environment, plastics interact with biota in different ways, for example, acting as a passive trap where animals get entangled, such as ghost fishing gears (e.g., nets) in the marine environment (Laist, 1997), or being swallowed by animals causing intestinal block (Isangedighi et al., 2020). Still, they can also be a new habitat to colonize, not just for microorganisms in the biofilm but also for macroinvertebrates, as shown in Figure 1.

For that reason, different environmental matrices are under the scope of the PLASTICØPYR project. Although these samples have a different nature, they share common steps to identify MicP. The analysis of MicP in environmental samples comprise two main steps: extraction and purification from the natural matrix, and secondly, the identification and quantification of MicP particles. It is important to recall that each technique has its limitations. For that reason, it is strongly recommended to be clear about the type of target particles or those that will be able to observe to adjust the technique chosen for the specific MicP analysis required.

EXTRACTION AND PURIFICATION TECHNIQUES

The first step in processing MicP samples is to isolate possible plastic particles from the sample matrix. Microplastics have low density (0.01-1.58 g/cm³), which allow them to float in hypersaline solutions (1.3-1.6 g/cm³). For this reason, this type of solution is used to extract microplastics from specific matrices, such as river, lake or ocean sediments or soil samples. On the other hand, in the analysis of microplastics, the organic matter remaining in the sample might interfere with MicP identification methods. For that reason, it is essential to remove the organic remains to obtain a sample as clean as possible. For this purpose, digestion of the organic matter is carried out. The most common digestion method used is wet digestion using hydrogen peroxide since it is the least aggressive digestion for plastic particles (Stock et al., 2019). Depending on the sample type, these two steps can be carried out in different order. In the following protocols, ZnCl₂ solution (density 1.5 g cm⁻³) is used to extract MicP from the environmental matrix, and a hydrogen peroxide digestion is applied to digest organic remains. After use, ZnCl₂ solution can be filtered through a GF/C filter (Sigma-Aldrich®, Whatman® glass...
microfiber filters, ø 47 mm, pore size ø 2.7 μm) to be re-used (it should last ~30-40 samples or until solution density changes). Digestion can be catalysed with Fenton’s reagent for those samples that present a high amount of organic remains.

**QUANTIFICATION AND IDENTIFICATION TECHNIQUES**

MicPs can be identified either using visual techniques or chemical composition analysis. Visual techniques include how MicPs are visually recognised according to specific parameters, such as size, shape, or colour. Visual techniques can be either carried out with the naked eye (although it is not recommended for particles <100 μm) (Hanvey et al., 2017) or according to particle fluorescence, which can be helpful to recognise smaller particles. In this protocol, the identification of MicPs is carried out using the Nile Red staining fluorescence technique, which allows the identification of MicPs from 20 μm to 1 mm (Erni-Cassola et al., 2017). Our detection limit is 63 μm, so we only consider particles with a maximum Feret’s diameter larger than the mentioned size in the following protocols.

On the other hand, chemical composition analyses tell us the specific compound of a particular particle and can reach smaller particles. The most used techniques are the Fourier-transform infrared spectroscopy (FTIR) and Raman spectroscopy, which are the most reliable techniques since it can be confirmed that the observed particles are from plastic (Veerasingam et al., 2021). Still, they present a more costly and time-consuming process, which may be a challenge for low-resources projects. However, both visual and chemical techniques can be complementary. In fact, in the case of visual identification of MicP, it is recommended that 10-25% of the samples should be analysed chemically to have confident results. In our case, we followed a fluorescence quantification method with Nile Red staining, which is compatible with FTIR identification (but not with Raman). The steps of the microplastics observation procedure followed in this guideline is explained in the Annex - “Sample observation: device characteristics and procedure”.

1.2. Preparation of materials and cross-contamination control

1.2.1. Prevention measures to avoid samples contamination

The processing of MicP samples requires measures to prevent contamination that may occur in a laboratory. Some of the commonly used prevention measures are outlined below:

- Wear 100% cotton lab coats and avoid wearing clothing that contains synthetic fibres in its composition.
- Keep doors and windows closed, and non-essential staff should not enter the room.
- Previous to work, decontaminate the workbench surface with filtered denatured alcohol.
- Avoid the use of plastic materials, but in case of using it, note the type of material, colour, and if possible, the chemical composition.
● Cover the sample and material with aluminium foil when not manipulating it.
● Decontaminate all laboratory equipment to be used, especially glass materials (Table 2).

<table>
<thead>
<tr>
<th>Table 2. Decontamination procedures for materials used for MicP analysis</th>
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<tbody>
<tr>
<td><strong>Metal decontamination</strong></td>
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<tr>
<td><strong>Glass decontamination</strong></td>
</tr>
</tbody>
</table>

*Note: Please, avoid exposing wet material to drafty areas and place glass items upside down during material drying.*

### 1.2.2. Cross-contamination and recovery controls

Despite following all the prevention measures listed above, airborne MicPs are present in the environment. For that reason, the following measures should be considered during MicPs analysis in environmental samples:

● Control air pollution by strategically placing wet filters in open Petri dishes in different parts of the laboratory and checking daily for the presence of particles or fibres of synthetic origin. Run air controls per each set of samples analysed.
● Process procedural blanks in conjunction with samples by subjecting them to the same processes (recommend: three procedural blanks in each processing).
  ➢ *Specifically, for ZnCl₂ solution, run a blank control before processing any sample and after every 10-15 samples are processed.*
● Replicates or samples from the same study site should be analysed in sequence and share laboratory controls.

Finally, we suggest running tests or recovery controls before analysing real samples, especially for the MicP extraction and observation procedures. For example:

● To extract MicP from environmental matrices, add known MicP of different densities in the hypersaline solution used for the extraction, and calculate the recovery rate of microplastics.
● To observe MicP particles using the fluorescence method, calibrate the observation device (Nel et al., 2021).
2. LABORATORY PROCESSING

2.1. Riverside plastics (MacP and MesP)

Plastic litter on riverbanks is highly likely to end inside the river system (i.e., water column and benthos) during high rainfall due to surface runoff occurrence. Also, large plastic items can be fragmented due to environmental physicochemical forces and be sources of MicP particles. For that reason, it is vital to do a proper characterization about the riparian MacP and MesP litter, not just to tackle the source of pollution but also to figure out the level of MicP that can be found in the vicinity habitats or downstream locations. For example, aged MacPs are easily fragmented, which means they can be a great source of secondary MicP. The following section describes the shared procedure for MacP and MesP laboratory characterization. Field sampling strategy is described in Margenat et al., 2021.

MATERIAL (per procedure)

- Absorbent protector sheets or another cover to protect work surface
- Paper towel roll to clean plastic items
- Water spray bottle to clean plastic items
- (1) Ruler or meter to measure items
- (1) Precision balance (0.001 g) to weight items
- (1) Camera
- Aluminium or metal trays to dry wet items in the oven

SAMPLE PREPARATION

1. Once in the lab, remove organic and inorganic remains attached to the MacP/MesP item, and clean it as much as possible using a piece of paper. Let wet items dry in the oven (max. 60°C) until constant weight.
2. Classify plastic items according to the corresponding categories from Marine Debris Tracker App (PLASTICØPYR list):
   - MacP categories (see Annex “MARINE DEBRIS TRACKER PLASTICØPYR LIST CATEGORIES”).
   - MesP categories
     - Film
     - Beads
     - Pellet
     - Fragment (hard plastic)
     - Foam
     - Fiber
     - Filament/line
     - Other
3. Note area (cm²) and weight (g) of each item:
   - To calculate area (cm²), measure maximum length (cm) and maximum width (cm). Calculate area according to basic shape (rectangle, circle, triangle).
• Note weight in grams (g). Light MacP or MesP can be weighed together (precision 0.001 g).
4. Take a picture with scale and note SITE, date and, SAMPLING POINT for MesP (right or left, 0,3 or 6m) or number of items for MacP (see Margenat et al., 2021).
5. Calculate the number of items, the area (m²) occupied and the total weight (kg) per sampled area (m²).
6. If possible, check MacP and MesP chemical composition with FTIR. For FTIR analysis, items must be well-clean and dried, without any remains of organic or inorganic material.

Figure 2. Schematic protocol illustrating the procedure to follow for the analysis of MacP and MesP items from the riparian area.
2.2. Water column samples

2.2.1. Manta net (MesP and MicP)

Pyrenean rivers present high discharge, especially after snowmelt or rainfall seasons (i.e., autumn and spring). When these rivers flow through forest areas, they can transport significant quantities of organic matter, from tiny seeds to large trunks. The presence of organic material may complicate the detection of MicP, especially for those particles below 0.5 mm. We suggest dividing the manta net sample into different fractions to facilitate MesP and MicP observation to process the samples. The pore of the manta net will determine the minimum size that will be considered, in this case, >0.33 mm.

Figure 3. Cigarette butt collected at the field by the manta net device. Source: PLASTICØPYR

MATERIAL

- Access to pressured tap-water
- (4) Stainless steel sieves with different mesh sizes (25 mm, 5 mm, 1 mm, and 0.3 mm mesh Ø)
- (1) Stainless steel big and (1) small tweezers, to collect large and small fractions
- (1) Stainless steel spatula
- (4) Aluminium trays (per manta sample)
- Aluminium foil, to cover samples and avoid air contamination
- (1) 250 ml glass jar, to digest smallest fraction
- 30% H₂O₂
- (1) Black and (1) white construction paper
- (4) Glass vials to keep MesP and MicP found in each fraction
- (1) Ruler
- (1) Camera
- (1) Stereomicroscope with reflected illumination
- Fume hood, orbital shaker, and oven
**SAMPLE PREPARATION**

1. Decontaminate the sieves following the procedure described in section 1.2.1. *Prevention measures to avoid samples contamination*, and clean them with pressured water before use them.

2. Place metal sieves from small mesh size (at the bottom) to large mesh size (upper part) (Ø 0.3 mm, 1 mm, 5 mm, 25 mm).
   - FRACTION 1: > 25 mm
   - FRACTION 2: 5-25 mm
   - FRACTION 3: 1-5 mm
   - FRACTION 4: 0.3-1 mm

3. Label 4 aluminium trays, one for each fraction, labelled as following: M-SITE-DATE-#fraction, M for Manta, SITE for sampling site (three letters), and DATE for date of the campaign (or number of campaign C#) (see Margenat et al., 2021). For example, for samples from Catllar:
   - M-CAT-20201015-1
   - M-CAT-20201015-2
   - M-CAT-20201015-3
   - M-CAT-20201015-4

4. Place the sample at the upper sieve (Ø 25 mm) and separate the sample into the other fractions with pressurised water.

5. Pick the collected material carefully with the help of tweezers and a spatula. Place the collected material into the four different decontaminated aluminium trays.

   - In case you find any plastic, place it into a glass vial and dry it at 60°C for 24-48h. Keep the plastics for further analysis (to obtain weight, chemical composition...).
   - To digest natural fibres to facilitate plastic observation, place material from *fraction 4* into a decontaminated 250 ml glass jar and add 20-50 ml of 30% H₂O₂ (depending on the amount of organic material). Cover the glass with aluminium foil, making some holes in the surface. Digestion must be carried out inside the fume hood (room T, 24h and orbital shaker at minimum power). After digestion, filter the sample through the 0.3 mm metal sieve, collect it into the labelled aluminium tray, and dry it at 60°C for 24-48h.

6. Cover the fractions 1, 2, 3 and digested 4, with aluminium foil, make some holes in the surface and place the samples into the oven. Dry them at 60°C for 24-48h (until constant weight)

**SAMPLE OBSERVATION**

1. When dried, observe the 1, 2 and 3 fractions with the naked eye. In case of detection of plastic particles, note:
   - MesP category and colour
     - Film
     - Beads
     - Pellet
     - Fragment (hard plastic)
     - Foam
     - Fibre
     - Filament/line
     - Other
   - If possible, type of polymer (FTIR)
2. For the tiniest fraction (4), transfer the sample from the aluminium tray to a glass petri dish (previously decontaminated). Observe the sample under the stereomicroscope. We suggest using different background colours (black and white). For each MicP, note:

- MicP category and colour:
  - Film
  - Microbeads
  - Pellet
  - Fragment (hard plastic)
  - Foam
  - Filament/line
  - Fibre

- If possible, type of polymer (FTIR)
- Take a scaled-picture
- Weight (g) (if possible, if a single plastic is not detected, weight all the MicP found in each fraction).

3. Keep the plastics found in each fraction in different glass vials (one vial per fraction, four vials for sample) for further analysis (ex: FTIR).

**Figure 4.** Schematic protocol illustrating the procedure to follow for the analysis of MesP and MicP from water column-manta net samples.
2.2.2. Grab sampling (MicP)

Due to microplastics comprising a wide range of sizes (0.1 μm - 5 mm), these particles might interact in different ways with other components of the river environment. For example, small plastic particles could more easily enter the trophic network (Krause et al., 2021). For that reason, PLASTICØPYR field guidelines suggested adding grab sampling to catch tiny particles in water. The present section and the following ones for benthos, biota and atmospheric samples analysis describe the protocol for MicP detection. Although the procedure for MicP analysis (extraction, digestion, and staining steps) is shared between samples from different environmental compartments, each protocol is adapted to the type of sample to be analysed. According to PLASTICØPYR field guidelines (Margenat et al., 2021), label the sample as follow: number of campaign or date (C#), the sample type (GRAB for sampling) the sampling site acronym (i.e., CAT for the Catllar stream) and the replicate number (1, 2 or 3).

e.g.: “C1-GRAB-CAT-1”

MATERIAL (per sample)

- (1) 63 μm Ø mesh stainless steel sieve
- (1) Glass Pasteur pipette
- (1) Stainless steel tweezers
- Aluminium foil, to cover samples and avoid air contamination
- (1) 250 ml glass jar per sample
- (1) 50 ml glass essay tube or graduated cylinder
- (1) stainless steel stir bar
- Reagents: 30% H$_2$O$_2$, ZnCl$_2$ (density 1.5 g cm$^{-3}$), MilliQ H$_2$O and, Nile Red stock solution (1 mg/ml)
- (1) PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, ø 47 mm, pore size ø 1 μm)
- (1) Stereomicroscope with reflected fluorescence illumination (specific characteristics detailed in the Annex - “Sample observation: device characteristics and procedure”)
- (1) Glass vacuum filtration kit and (1) vacuum pump
- GF/C filter (Sigma-Aldrich®, Whatman® glass microfiber filters, ø 47 mm, pore size ø 2.7 μm)
- (1) Orbital shaker
- Fume hood and oven
SAMPLE PREPARATION

1. Pour the sample into the 63 µm metal sieve and recover the collected material (as dry as possible) inside a 250 ml glass jar.

2. Add 20 ml of 30% H$_2$O$_2$ and digest it at room temperature for 24 hours (*digestion must be carried out inside the fume hood*). We suggest using an orbital shaker at minimum power to increase digestion velocity.

3. After digestion, pour the sample again in the 63 µm metal sieve and recover collected material (as dry as possible) inside a 50 ml essay tube or graduated cylinder.

4. Add 40 ml of ZnCl$_2$ (density 1.5 g cm$^{-3}$). Stir the sample for 3-5 minutes and let it settle down.

5. Collect the supernatant (approx. 5 ml) and filter through the 63 µm metal sieve. Rinse with MilliQ.

6. Recover the sample and add 20 ml MilliQ H$_2$O and 100 µl of Nile Red sock solution (1 mg/ml). Shake the sample manually (every 10 min for 30 min). Keep in the dark 24h.

7. Filter the solution through a PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, ø 47 mm, pore size ø 1 µm) using a vacuum system.

8. Dry the filter 24h 60°C and observe it following the sample observation procedure specified in the Annex - “Sample observation: device characteristics and procedure”.

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**Figure 5.** Schematic protocol of the procedure to analyse water column-grab samples.
2.3. Benthic samples

2.3.1. Biofilm (MicP)

The present section describes the protocol for microplastics (MicP) counting in fluvial biofilms. Because biofilms are complex matrices that include organic and inorganic remains, the processing of the samples will involve two important steps: digestion of the organic matter and separation of MicP from the inorganic fraction. This protocol suggests applying two digestion steps based on 30% H$_2$O$_2$ to reduce organic material causing minimal damage on MicP particles. Following Margenat et. al (2021), sample codes refer to the field sampling number (i.e., C1 for the first one); the sample type (BIO for biofilm) the sampling site acronym (i.e., CAT for the Catllar stream) and the specific reach (i.e., 40 refers to biofilm samples taken from the reach located at 40 m).

\textit{e.g.: “C1-BIO-CAT-40m”}

In the laboratory, the pores of sieves or filters during the whole procedure will determine the minimum size that will be considered. A 63 μm-porous sieve was selected in our protocol due to methodological constraints concerning potential atmospheric contamination. However, smaller MicP may also occur in biofilm samples requiring further improvement of the methods used.

\textbf{MATERIAL (per sample)}

- (1) 63 μm Ø mesh stainless steel sieve
- (1) Glass Pasteur pipette
- (1) Stainless steel tweezers and (1) spatula
- Aluminium foil, to cover samples and avoid air contamination
- (1) 250 ml glass jar per sample
- (1) 50 ml glass essay tube or graduated cylinder
- (1) Stainless steel stir bar
- Reagents: 30% H$_2$O$_2$, Fenton’s reagent, ZnCl$_2$ (density 1.5 g cm$^{-3}$), MilliQ H$_2$O and, Nile Red sock solution (1 mg/ml)
- (1) PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, Ø 47 mm, pore size Ø 1 μm)
- Sigma-Aldrich®, Whatman® glass microfiber filters, Ø 47 mm, pore size Ø 2.7 μm
- (1) Stereomicroscope with reflected fluorescence illumination (specific characteristics detailed in the Annex - “Sample observation: device characteristics and procedure”)
- (1) Glass vacuum filtration kit and (1) vacuum pump
- GF/C filter (Sigma-Aldrich®, Whatman® glass microfiber filters, Ø 47 mm, pore size Ø 2.7 μm)
SAMPLE PREPARATION

1. Homogenise the wet sample (25 ml for MicP analysis). Remove large organic debris using tweezers and rinse them with DI water to collect the MicP that may be adhered.
2. Dry the sample to constant weight (60°C ~48h). Sample must be well dried not to alter the concentration of the reagents used in the following steps. Temperature must be below 60°C to avoid damage on MicP.
3. Weight 5 g of the dried sample and place it into a 250 ml glass jar. We strongly recommend using wide/large jars because some samples may produce a strong effervescent reaction during digestion, leading to losing the sample.
4. Proceed with the 1st digestion - Add 40 ml of 30% H₂O₂ and place the jar on an orbital shaker at minimum speed for 24 h (room temperature).
5. Filter the sample through the 63 μm metal sieve. When pouring the sample, do it carefully on the edge of the sieve, in this manner, it will be easy to collect the sample later. During sample collection, be careful not to scratch the sieve with a metal lab spatula. Instead, it is better to collect the sample using a glass pipette Pasteur.
6. Collect the recovered material (as dry as possible) inside a graduated glass cylinder (or glass test tube).
7. Add 40 ml of ZnCl₂ solution (Sigma-Aldrich®, 1.5 g cm⁻³). Stir the sample for 3-5 minutes and let it settle down (it will take approx. 15-20 minutes, the ZnCl₂ solution should be transparent and without any particles in suspension).
8. Filter the supernatant (approx. 5 ml) through the 63 μm metal sieve.
9. 2nd digestion - Recover the sample (as dry as possible), transfer it into a glass jar, and add 20 ml 30% 
H₂O₂ and 20 ml of Fenton’s reagent (24h at room temperature).
10. Filter the sample through the 63 μm metal sieve.
11. Recover the sample and add 20 ml MilliQ H₂O and 100 μl of Nile Red stock solution (1 mg/ml). Shake the 
sample manually (every 10 min for 30 min). Keep in the dark 24h.
12. Filter the stained sample through a PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC 
circles, ø 47 mm, pore size ø 1 μm) and dry it at 50-60°C (24h).
13. Observe the filter following the sample observation procedure specified in the Annex - “Sample 
observation: device characteristics and procedure”.

**Figure 7.** Schematic protocol illustrating the extraction and 2nd digestion steps for the processing of biofilm 
samples.
2.3.2. Sediments (MicP)

It has been well reported that rivers act as a temporal sink for MicP depositions and that these particles can be remobilized during high flow events (Hurley et al., 2018). Regarding sediment samples processing, it can differ depending on the type of samples, as it may present a different type of sediment (sandy or silty) and amounts of organic matter. Therefore, the processing must be adjusted to each sample. In this case, the protocol explained below has been carried out on sandy sediment samples, which do not present large amounts of organic matter. We have carried out an extraction followed by digestion with hydrogen peroxide catalysed with Fenton’s reagent, in a ratio of 1:10.

On the other hand, if working with silty-high organic content sediments, we recommend applying the first digestion before extracting microplastics to reduce the amount of organic matter (since it has a lower density than the ZnCl₂ solution). After extraction, the second Fenton-catalysed digestion is applied, in a ratio of 1:1. Label the samples as follows (Margenat et al., 2021): the number of campaign or the date (C#), type of sample (Sed, referring to sediment), study site (three letters), reach (20, 40 or 60 m). For example:

\[ \text{e.g.: “C1-SED-CAT-40m”} \]

MATERIAL (per sample)

- (1) 63 µm Ø mesh stainless steel sieve
- (1) 1000 ml graduated glass vial
- (1) Stainless steel bar to stir (at least, 50 cm length)
- (1) Glass Pasteur pipette
- (1) Stainless steel tweezers and (1) spatula
- Aluminium foil, to cover samples and avoid air contamination
- (1) 250 ml glass jar per sample
- (1) 50 ml glass essay tube or graduated cylinder
- (1) Stainless steel stir bar (or magnetic stirrer and a glass flea)
- Reagents: 30% H₂O₂, Fenton’s reagent, ZnCl₂ (density 1.5 g cm⁻³), MilliQ H₂O and, Nile Red stock solution (1 mg/ml)
- (1) PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, Ø 47 mm, pore size Ø 1 µm)
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- Sigma-Aldrich®, Whatman® glass microfiber filters, ø 47 mm, pore size ø 2.7 μm
- (1) Stereomicroscope with reflected fluorescence illumination (specific characteristics detailed in the Annex – “Sample observation: device characteristics and procedure”)
- (1) Glass vacuum filtration kit and (1) vacuum pump
- GF/C filter (Sigma-Aldrich®, Whatman® glass microfiber filters, ø 47 mm, pore size ø 2.7 μm)
- (1) Orbital shaker
- Fume hood and oven

SAMPLE PREPARATION

1. In bulk sediment, homogenise it and take 50 g of dried sediment (60°C) for MicP analysis.
2. Transfer the sediment into a 1000 ml graduated glass cylinder and fill it with 250 ml of ZnCl₂ solution (1.6 g/ml), stirring the mixture with a wood stick to resuspend the particles.
3. Add ZnCl₂ solution until it reaches 750 ml. Stir the solution energetically for 5 minutes to resuspend the particles. To mix, a magnetic stirrer and a glass flea can be helpful.
4. When sediments settle down, filter supernatant through 63 μm sieve and rinse it with DI water.
   - **Settling time depends on each sample, but it takes 15-30 minutes for those with sandy sediments.**
5. Transfer collected material to a clean 250 ml glass jar with 20 ml 30% H₂O₂ and 2 ml of Fenton reagent, and place the jar on an orbital shaker at minimum speed for 24 h (room temperature)
6. After digestion, filter the solution through the 63 μm sieve and rinse with MilliQ water.
7. Transfer collected material to a clean glass jar with 20 ml MilliQ water and 100 μl Nile Red Solution (1 mg/ml).
8. Close the glass jar and shake every 10 minutes for 30-60 minutes. Keep in the dark 24h.
9. Filter the stained sample through a PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, ø 47 mm, pore size ø 1 μm) and dry it at 50-60°C for 24h.
10. Observe the filter following the sample observation procedure specified in the Annex – “Sample observation: device characteristics and procedure”.
**Figure 9.** Extraction and digestion steps for MicP analysis in sediment samples

**Figure 10.** Nile Red staining step for MicP detection in sediment samples
2.4. Fish sampling (MicP)

Several studies have detected plastics (from macro to micro) inside the intestinal tract of different animals in all environments. Regarding MicPs, the accidental consumption of these particles increases as they break up into smaller sizes (Critchell & Hoogenboom, 2018). The most-reported negative effect is gastrointestinal obstruction due to accidental plastic ingestion; still, other effects are being investigated, such as changes in growth and behaviour (da Costa Araújo & Malafaia, 2021). This guideline presents the protocol to quantify MicPs in the gastrointestinal tract of the brown trout fish (*Salmo trutta*), a piscivorous and benthic species. Because of that, any organic remains and inorganic particles, such as sediment grains, may be present in the sample. Therefore, for these samples processing, a 30% H$_2$O$_2$ digestion is applied, followed by a sediment extraction with ZnCl$_2$, and finally, a second digestion catalysed by Fenton’s reagent is applied. Following Margenat et al., (2021), label the sample as follows: type of sample (Fish), study site (three letters), and the number of individual (#). In case of doing different campaigns on the same site, indicate the number of the campaign (C#) or the date of sampling. For example:

e.g.: “FISH-CAT-1”

MATERIAL

- (1) 63 µm Ø mesh stainless steel sieve
- (1) Glass Pasteur pipette
- (1) Stainless steel tweezers
- Aluminium foil, to cover samples and avoid air contamination
- (1) 250 ml glass jar per sample
- (1) 50 ml glass essay tube or graduated cylinder
- (1) stainless steel stir bar
- Reagents: 30% H$_2$O$_2$, ZnCl$_2$ (density 1.5 g cm$^{-3}$), MilliQ H$_2$O and, Nile Red sock solution (1 mg/ml)
- (1) PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, Ø 47 mm, pore size Ø 1 μm)
- (1) Stereomicroscope with reflected fluorescence illumination (specific characteristics detailed in the Annex - “Sample observation: device characteristics and procedure”)
- (1) Glass vacuum filtration kit and (1) vacuum pump
- GF/C filter (Sigma-Aldrich®, Whatman® glass microfiber filters, Ø 47 mm, pore size Ø 2.7 μm)
- (1) Orbital shaker
- Fume hood and oven
SAMPLE PREPARATION

1. The individual is dissected. Remove the digestive tract and place it in a decontaminated glass vial, and disperse and homogenize it (using decontaminated material).

2. The homogenized sample is dried to constant weight (60°C).

3. Take 1 g of the dried sample (or the whole sample if it is small) and placed it inside a 250 ml glass jar.

4. Proceed with the 1st digestion, adding 20 ml 30% H₂O₂ and place the jar on an orbital shaker at minimum speed for 24 h (room temperature).

5. Filter the sample through the 63 μm metal sieve. Remember to pour the sample carefully on the edge of the sieve to facilitate sample collection.

   ➤ In case of presence of sediment grains, an extraction step is highly recommended. The procedure follows as:

   5a. Collect the recovered material (as dry as possible) inside a graduated glass cylinder (or glass test tube).

   5b. Add 40 ml of ZnCl₂ solution (Sigma-Aldrich®, 1.5 g cm⁻³). Stir the sample for 3-5 minutes and let it settle down (it will take approx. 15-20 minutes, the ZnCl₂ solution should be transparent and without any particles in suspension). After use, filter the ZnCl₂ solution through a GF/C filter (Sigma-Aldrich®, Whatman® glass microfiber filters, ø 47 mm, pore size ø 2.7 μm) to re-used (it should last ~30-40 samples).

   5c. Filter the supernatant (approx. 5 ml) through the metal sieve and follow step #6.

6. Proceed with the 2nd digestion. For that, recover the sample (as dry as possible) from the metal sieve, and transfer it into a glass jar (it can be the same glass jar as 1st digestion). Add 20 ml H₂O₂ and 20 ml of Fenton’s reagent (24h at room temperature).

7. After digestion, filter the sample through the 63 μm metal sieve.

8. Recover the sample and add 20 ml MilliQ H₂O and 100 μl of Nile Red sock solution (1 mg/ml). Shake the sample manually (every 10 min for 30 min). Keep in the dark 24h.

9. Filter the stained sample through a PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, ø 47 mm, pore size ø 1 μm) and dry it at 50-60°C (24h).

10. Observe the filter following the sample observation procedure specified in the Annex - “Sample observation: device characteristics and procedure”.
Fish: lab procedure (extraction and digestions)

1. Dissect and remove the digestive tract
2. Disperse and homogenize it
3. Dry it at 60°C

1 g of the dried sample
add 20 ml 30% H₂O₂
agitation, 24 h, room T

40 ml ZnCl₂
(1.5 g cm⁻³)

Supernatant
(MicP and organic remains)

20 ml 30% H₂O₂
+ 20 ml Fenton’s reagent
agitation, 24 h, room T

Filter through PC filter
Dry at 60°C
24 h

20 ml MilliQ H₂O
+ 100 µl Nile Red (1 mg/ml)
24 h in dark

Figure 11. Schematic protocol illustrating the extraction and 2nd digestion steps for the processing of biofilm samples.
2.5. Atmospheric deposition (MicP)

Because of their size, MPs can travel easily by air and reach different ecosystems, from urban to remote areas and from the sea back to terrestrial ecosystems. Atmospheric samples also contain remains of organic and inorganic materials, so sample purification is needed in order to degrade organic matter and separate microplastics from inorganic particles. Although protocols for the analysis of water, sediment and biotic samples were focused on particles above 63 µm, the protocol for atmospheric particles aims to reach smaller particles (up to 10 µm). Label the sample as follows (Margenat et al., 2021): type of sample (ATM, referring to atmospheric deposition), the sub-type (wet or dry or bulk), study site (three letters), and date (year/month/day) or number of the campaign (C#). For example:

e.g.: “ATM-WET-CAT-20201027”

MATERIAL

- (1) Stainless steel tweezers
- Aluminium foil, to cover samples and avoid air contamination
- (1) 250 ml glass jar per sample
- (1) 50 ml glass essay tube or graduated cylinder
- (1) stainless steel stir bar
- Reagents: 30% H$_2$O$_2$, ZnCl$_2$ (density 1.5 g cm$^{-3}$), MilliQ H$_2$O and, Nile Red sock solution (1 mg/ml)
- (4) PC filter (Sigma-Aldrich®, Whatman®, Cyclopore® cyclopore PC circles, ø 47 mm, pore size ø 1 µm)
- (1) Stereomicroscope with reflected fluorescence illumination (specific characteristics detailed in the Annex – “Sample observation: device characteristics and procedure”)
- (1) Glass vacuum filtration kit and (1) vacuum pump
- GF/C filter (Sigma-Aldrich®, Whatman® glass microfiber filters, ø 47 mm, pore size ø 2.7 µm)
- (1) Orbital shaker
- Fume hood and oven

SAMPLE PREPARATION

1. At the lab, filter the atmospheric bulk sample through a PC filter. Either if the sample come from wet or dry deposition, MilliQ water can be used to rinse the container for particles collection.
2. Rinse the filter into a 250 ml glass jar with 20 ml of 30% H$_2$O$_2$ and place it in the orbital shaker at room T for 24h. Discard the PC filter.
3. After the digestion, filter the solution through a new PC filter, rinse it with 250 ml MilliQ water and dry it with filtered ethanol (96 vol%).
4. Rinse the material into a 50 ml glass essay tube and add 40 ml of ZnCl\textsubscript{2} solution (density 1.6 g cm\textsuperscript{-3}). Stir the sample for 3-5 minutes and let it settle down (it will take approx. 15-20 minutes, the ZnCl\textsubscript{2} solution should be transparent and without any particles in suspension).

5. After the microplastic extraction, filter the supernatant (approx. 5 ml) through a new PC filter, rinse it with 250 ml MilliQ water and dry it with filtered ethanol (96 vol%).

6. Transfer the collected material from the filter into a 250 ml glass jar, and add 20 ml MilliQ H\textsubscript{2}O and 100 µl of Nile Red sock solution (1 mg/ml). Shake manual the sample (every 10 min for 30 min). Keep in the dark 24h.

7. Filter the stained solution through a PC filter and dry it at max. 60°C (24h).

8. Observe the filter following the sample observation procedure specified in the Annex - “Sample observation: device characteristics and procedure”.

---

**Atmospheric samples: lab procedure**

- **Atm. bulk sample**
  - 20 ml 30% H\textsubscript{2}O\textsubscript{2}
  - Room T, 24 h

- **Filter through PC filter**

- **Dry it with ethanol 96%**

- **Filter through PC filter**

- **Supernatant (~5 ml)**

- **Dry with ethanol (96 vol%)**

- **Filter through PC filter**

- **Rinse using 250 ml MilliQ water**

- **Dry at 60°C 24 h**

**Figure 12. Schematic protocol for the processing of atmospheric samples (dry and wet deposition).**
3. References


Erni-Cassola G, Gibson M I, Thompson R C and Christie-Oleza J A 2017 Lost, but found with Nile red: a novel method for detecting and quantifying small microplastics (1 mm to 20 μm) in environmental samples Environ. Sci. Technol. 51 13641-8

Erni-Cassola G, Gibson M I, Thompson R C and Christie-Oleza J A 2017 Lost, but found with Nile red: a novel method for detecting and quantifying small microplastics (1 mm to 20 μm) in environmental samples Environ. Sci. Technol. 51 13641-8


Krause S et al 2021 Gathering at the top? Environmental controls of microplastic uptake and biomagnification in freshwater food webs Environ. Pollut. 268 115750


NOAA 2015 Laboratory Methods for the Analysis of Microplastics in the Marine Environment: Recommendations for Quantifying Synthetic Particles in Waters and Sediments (National Oceanic and Atmospheric Administration; U.S. Department of Commerce)


Tamminga M 2017 Nile red staining as a subsidiary method for microplastic quantification: a comparison of three solvents and factors influencing application reliability SDRP J. Earth Sci. Environ. Stud. 2 165-72


## 4. Annexes

### MARINE DEBRIS TRACKER PLASTICØPYR LIST CATEGORIES

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<th>PLASTIC CATEGORIES</th>
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<th>NON-PLASTIC CATEGORIES</th>
<th>SUB-CATEGORIES (MDT app)</th>
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<td>11.2. Aerosol cans</td>
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<td>1.3. Plastic cup</td>
<td>11.3. Metal bottle caps or tab</td>
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<td>2.2. Toothpaste tube</td>
<td>13.2. Paper bags</td>
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<td>2.3. Lotion bottle</td>
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<td>2.4. Shampoo / conditioner bottle</td>
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<td>13.5. Paper cup / plates / utensils</td>
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<td>2.6. Cotton swab</td>
<td>13.6. Paper and cardboard</td>
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<td>13.8. Receipt</td>
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<td>Category</td>
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<td>2.9. Other personal care items</td>
<td>13.9. Lumber / building materials</td>
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<tr>
<td>3. Fragments</td>
<td>3.1. Hard plastic fragments</td>
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<td>3.2. Thin-soft plastic fragments</td>
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<td>3.3. Thick-soft plastic fragments</td>
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<td>3.4. Line / thread plastic fragments</td>
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<td>3.5. Other fragments</td>
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<td>4. Other</td>
<td>4.1. Building or traffic items</td>
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<td>4.2. Other plastic items</td>
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<td>5. Rubber</td>
<td>5.1. Latex balloons</td>
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<td>5.2. Flip-flops</td>
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<td>5.3. Rubber gloves</td>
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<td>5.4. Tires</td>
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<td>5.5. Rubber fragments</td>
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<td>5.6. Rubber band</td>
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<td>5.7. Other rubber items</td>
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<td>6. Cloth</td>
<td>6.1. Fabric piece</td>
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<td>6.2. Glove</td>
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<td>6.3. Hat</td>
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<td>6.4. Towel / rag</td>
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<td>6.5. Rope / thread</td>
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<td>6.6. Clothes</td>
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<td>6.7. Shoe</td>
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<td>6.8. Other cloth items (i.e.: accessories)</td>
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<td>7. Styrofoam</td>
<td>7.1. Styrofoam / foam container</td>
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<td></td>
<td>7.2. Styrofoam cup / plate</td>
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<td></td>
<td>7.3. Styrofoam / foam fragment</td>
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</table>
## Fishing gear

<table>
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<tr>
<th>8.1. Buoys and floats</th>
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<tbody>
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<td>8.2. Hooks / lures</td>
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<td>8.3. Fishing line</td>
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<tr>
<td>8.4. Nets</td>
</tr>
<tr>
<td>8.5. Other fishing gears items</td>
</tr>
</tbody>
</table>

## Tobacco

| 9.1. Cigarette butt / cigar tip |
| 9.2. Cigarette box            |
| 9.3. Cigarette packet         |

## PPE (Personal Protective Equipment)

| 10.1. Disposable gloves       |
| 10.2. Face masks              |
| 10.3. Associated PPE packaging|
| 10.4. Other PPE items         |

### MESOPLASTICS (MesP) CATEGORIES

- Fibers
- Filaments/Line
- Film
- Fragment (hard plastic)
- Foam
- Beads
- Pellet
- Other
SAMPLE OBSERVATION: DEVICE CHARACTERISTICS AND PROCEDURE

Stereomicroscope characteristics (Plastic-free, provided by Winkoms Open Microscopy)

- 2x objective lens
- 20-megapixels camera (SONY IMX183 MONOCROM) binned to 5-megapixels
- GFP-B filter set (excitation filter bandwidth of 475, emission filter bandwidth of 542/20; beam splitter dichroic of 500 LP)
- Fluorescence light source (LED 475nm 3W), adjusted as follow:
  - Shuttle opened and maximum intensity (3W)

Particles analysed with FIJI Image-J:

- **Scale the image.** The first step is converting the scale of the image from pixels (pix) to micrometres (µm). For the P-free stereomicroscope, the relation is 2.72 µm/pix.
- **Thresholding.** Next, a threshold is applied to convert our image to a binary picture. During this process, each pixel in an image is replaced with a black pixel if the image intensity is less than the minimum threshold value fixed or a white pixel if it is greater than the minimum value. All values are indicated in arbitrary units (a.u.) For PLASTICØPYR samples, the threshold value was determinate from different reference plastics (PP, LDPE, HDPE, EPS) and for organic remains (i.e., plant and arthropods debris) stained with Nile Red (1 mg/ml; NR). For the thresholding process, a minimum value of 2750 a.u. was determined to detect PP, LDPE, HDPE; but not for EPS, to avoid false-positive readings of organic matter, since not always is completely removed during digestion, especially chitin and lignin substances. Thus, all particles with an intensity above 2750 a.u. are considered plastic particles*.

* Although particles present a fluorescence above the threshold, the observer must consider other particles’ characteristics, such as shape and possible organic structures, to discern those plastic particles from the organic remains.

* Fibres may not be detected using the Nile Red method due to their low fluorescence. We suggest quantifying visually without fluorescence mode. Consider the fibres from lab air control in order to have accurate results.

- **Analyse particles.** Before analysing particles, select the desired measurements that will be displayed (Analyse > Set measurements). Feret’s diameter must be selected. To analyse particles, Analyse > Analyse particles. Check Exclude on edges and Include holes for the analysis. Those particles <63 µm Feret’s diameter must not be considered.
Project co-funded by the European Regional Development Fund (ERDF)