7. Extraction of microplastics from marine biota samples followed by Nile red staining

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Deliverable 2.6 Analysis techniques for quantifying nano-and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023

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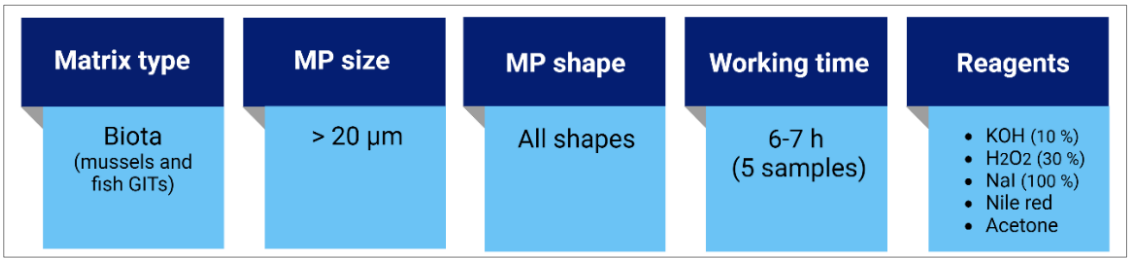
7.1 Scope of Protocol

MPs, defined as plastic particles sized 0.1µm – 5mm (Hartmann *et al.,* 2019), are an issue of concern because of their ubiquity in the marine environment. The omnipresence of these global contaminants (2008/56/EC Marine Strategy Framework Directive, Descriptor 10; United Nations Sustainable Development Goal 14 target 14.1.1) has been confirmed in numerous research studies, going from surface waters to the bottom of the ocean covering all depths, and from Arctic waters to Antarctic waters, including coastal waters, open waters and deep sea waters (Zhang, 2017; Pakhomova *et al*., 2022). They are readily accessible to a wide variety of marine organisms in the food chain because of their small size (e.g. Peng *et al*., 2018; Botterell *et al*., 2019; Pan *et al*., 2019; Wang et al., 2020). Moreover, increasing empirical evidence suggests potential adverse impacts on organisms (Franzellitti *et al*., 2019). To understand the effects of MPs on an individual, population and ecosystem level as well as on human health, risk assessment evaluations are necessary, which in turn require reliable monitoring data on MPs uptake by and accumulation in marine biota. Despite this, a limited understanding of MPs abundance, distribution, and fate in the marine environment, as well as their associated potential risk exists (Everaert *et al*., 2020), which emphasizes the need for standardized, representative, and feasible sampling, sample processing and sampling analysis protocols, to obtain accurate measures of MPs pollution in marine waters.

The JPI Oceans-funded ANDROMEDA project is a multidisciplinary collaboration of 15 international partners focused on improving the quantification of NPs and MPs in our oceans and seas. Within the project, new sampling and advanced analysis methodologies that focus on smaller MPs (< 10µm) and NP (< 0.2µm) particles have been developed, which will enable a more accurate assessment of risks associated with plastic pollution. Novel sampling techniques as well as cost-effective MPs measurements methods have been developed for a more efficient and effective MP monitoring. As a result, a series of protocols related to MPs extraction, analysis and degradation were developed and optimized, and used by project partners. They are now made available to the plastic research community as SOPs.

This particular protocol focuses on the efficient extraction of MPs > 20µm from marine biota samples, needed for the subsequent semi-automated MPs analysis based on machine learning and RGB colour quantification of Nile red stained fluorescent particles (Meyers *et al*., 2024a). This newly developed analysis method allows to detect MPs and identify their polymer types in a cost- and time-effective way. Special attention was given to the analytical quality control and quality assurance associated with the development of this extraction

protocol. The SOP was validated for the extraction of MPs from mussels (both commercial and non-commercial), as well as from gastrointestinal tracts (GITs) of various North Sea fish species (common dab, whiting, plaice, and European flounder).



7.2 Materials and Equipment

Glassware

- Glass beakers (600 mL)
- Closed petri dish per sample
- Large glass slide per sample

Filtration

Large filtration apparatus (for stainless steel filter):

- Glass funnel
- Fritted glass base (⌀ 106 mm)
- Aluminium clamp

Small filtration apparatus (for Whatman PTFE filters):

- (Filtration manifold set)
- Glass funnel with dust cover per sample
- Fritted glass base with stopper per sample
- Aluminium clamp per sample

Additional:

- Vacuum pump
- Rubber tubing
- Büchner flask (1 L)

Laboratory machinery

- Centrifuge
- Multi-position digital magnetic hotplate stirrer

Laboratory consumables

- Filters compatible for µ-FTIR analysis, e.g., PTFE membrane filters (10 µm, ⌀ 47 mm)
- Pasteur pipette with rubber stop
- Conical centrifuge tubes
- Aluminium foil
- Whatman glass microfibre filters (2.7 µm, ⌀ 47 mm)

Other laboratory equipment

- Metal sieve of ⌀ 5 cm and mesh size 20 µm.
- Stainless steel filter (20 µm, ring ⌀ 110 mm (fitting large glass base)
- A large and a small metal funnel
- Magnetic stirring rod (8 mm)
- Metal spatula
- Tweezers
- Cotton lab coat and nitrile protection gloves
- Milli-Q water
- Wash bottle

Reagents

- Potassium hydroxide (KOH - 10%)
- Hydrogen peroxide (H2O2 - 30-33%)
- Sodium Iodide (NaI - 100%)
- Acetone
- Nile red

Note:

- Prior to the sample processing, all solutions/liquids used (except Milli-Q water) should be filtered over a filter of a mesh size smaller than 10 µm using the filtration apparatus, to reduce potential contamination (e.g., Whatman glass microfibre filters - 2.7µm, ⌀ 47 mm).

- If not at hand, Milli-Q water can be replaced by filtered tap or distilled water, using filters of mesh size 2.7µm or smaller).
- Density separation steps using a saturated sodium iodide solution (NaI – 1.8 g/cm³; Enders *et al*. 2015) are necessary to extract all plastics polymers. Because of the high density of the salt solution, plastics with a density below 1.8 g/cm³ will float. Sodium tungstate dehydrate ($Na_2WO_4.2H_2O$ – 1,4 g/cm³) or even sodium chloride (NaCl - 1,2 g/cm³) solutions can be used as more economical alternatives, but plastics with relatively high densities such as polycarbonate (PC - 1.20-1.22 g/cm³), polyurethane (PU - 1.20-1.26 g/cm³), polyethylene terephthalate (PET - 1.38-1.41 g/cm³) and polyvinyl chloride (PVC - 1.38-1.41 g/cm³) will not be separated when using NaCl. If $Na_2WO_4.2H_2O$ is used, PVC particles will not be extracted either.

7.3 Protocol

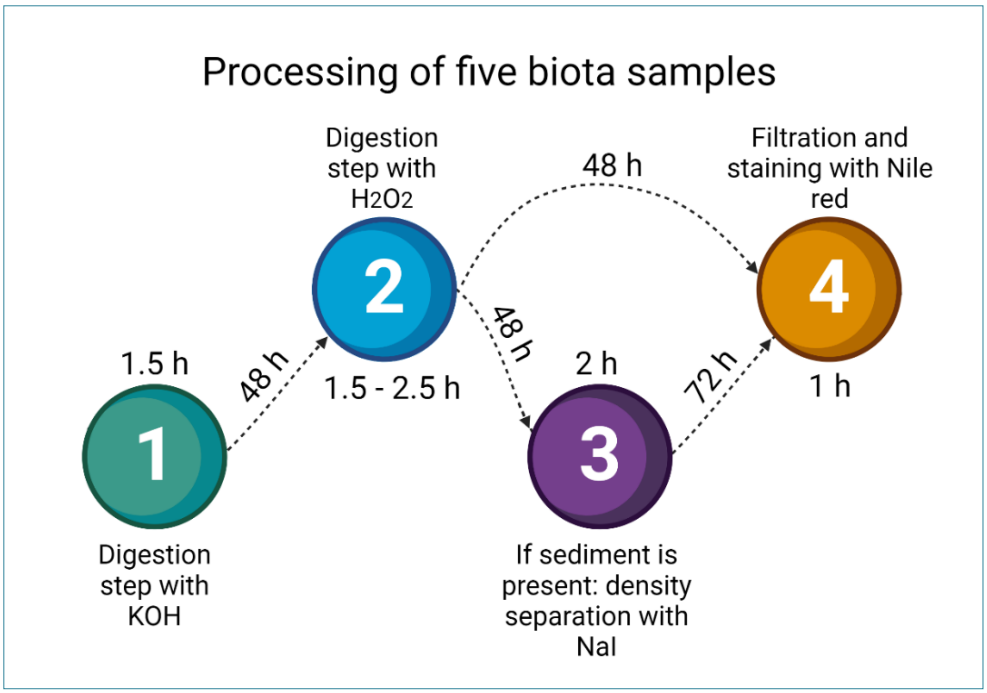


Figure 1: Scheme of all major steps within the protocol, as well as their durations

7.3.1 Digestion with KOH

A. Add 20 g biota (wet weight) to a glass beaker. First, the organic material needs to be digested (see figure 1). To efficiently remove animal tissue (Prata *et al*., 2019),

prepare a 200 mL 10%-KOH solution with Milli-Q water and add it to the beaker containing the sample, add a rinsed magnetic stirring rod, cover the beaker, and leave the sample to digest for 48h on a magnetic hotplate stirrer at 50 °C and 150 rpm. Always maintain a 1:10 ration of sample wet weight (g) to KOH volume (mL)

Note: If analyzing frozen mussels, they can be opened by wedging an oyster knife in the gap of the hinge ligament and breaking the hinge by sliding the knife down between the two shells.

B. In a later step, an additional digestion is performed to efficiently eliminate remaining plant material (Prata *et al*., 2019). Before this can be done, filtration of the semi-digested sample is needed. To do so, slowly filter the sample over the stainless-steel filter using the large filtration apparatus connected to a pump and Büchner flask.

Note: In case the stainless-steel filter clogs, it is advised to stop adding more of the sample, and to perform the filtration (and subsequent sonication step if applicable) in multiple steps where the sieved sample is washed off the filter (see next step) in multiple steps in between filtration steps.

7.3.1.1 Rinsing with Sonication

In case the stainless-steel filter contains a layer of organic material, which is often the case for GITS of larger fish, it is advised to do an additional sonication step to loosen the particles present on the filter. If this is not the case, skip this step and go straight to 'Rinsing without sonication'.

A. To sonicate the stainless-steel filter, carefully put the filter in a large beaker (2 L) so that the filter can lay flat on the bottom of the beaker, with the side containing the filtered particles facing upwards. Lower it slowly with a metal spatula if needed. Add 70 mL of Milli-Q water to the beaker to completely cover the filter. Next, place the beaker in an ultrasonic bath for 10 min. at a frequency of 40 KHz. Make sure the beaker is covered and cannot fall over in the bath, e.g. by using an ultrasonic beaker basket.

B. Place a metal funnel in a new glass beaker (600 mL). Carefully take the filter out of the large beaker using the metal spatula/spoon and vigorously rinse the filter with 50 mL Milli-Q water into a new beaker using a wash bottle. Rinse the funnel as well.

Ideally, stick to the minimum rinsing volume needed to effectively clean the filter, so that the volume of H_20_2 needed in the next step is kept low.

C. Pour the water (70 mL) from the beaker that contained the filter into this new beaker as well. Rinse the first beaker which was placed in the sonic bath thoroughly using a wash bottle filled with 50 mL Milli-Q water. In this way, the total sample volume in the new beaker should now be 200 mL.

7.3.1.2 Rinsing without Sonication

A. Place a metal funnel in a new glass beaker (600 mL). If the construction cannot be placed in the beaker in a stable way because of its size, the funnel can be held manually above the beaker by a second person, or a laboratory stand can be used. For the latter option, attach the funnel to the stand using a laboratory clamp/ring clamp, and place the glass beaker right underneath the funnel.

B. Following this, wash the filtered sample present on the stainless-steel filter through the funnel into the glass beaker. Rinse the funnel as well. Ideally, transfer the sample using a volume of maximum 150 mL Milli-Q water.

7.3.2 Digestion with H_20_2

A. After rinsing with or without sonication step, add H_20_2 (30-33%) in a 1:1 ratio to the sample. In this case, add 150 ml of H_20_2 (200 mL if sonication was performed). Next, add a magnetic stirring rod to the sample, and leave the sample to digest again for 48 h on a magnetic hotplate stirrer at 50 °C and 150 rpm.

B. Slowly filter the semi-digested sample over the stainless-steel filter using the large filtration apparatus connected to a pump and Büchner flask, in the same way as done for the digestion step with KOH.

Note: If all organic matter is not digested after this step (pieces are visible), leave the solution to digest for another 24 h before filtration. If a lot of organic material remains undigested after this prolonged digestion, an additional digestion step using the same concentration of H_2O_2 can be added following the same procedure.

7.3.3 Density Separation

If the amount of sediment present on the digested sample is too high to allow for direct filtration, a density separation step prior to filtration is needed. If this is not the case, skip this step and go to Section 7.3.4 'Filtration'.

A. Prepare a saturated NaI solution with Milli-Q water (1793 g / L at room temperature). A total volume of 50 mL of saturated NaI solution is needed for each sample. Be aware that a large quantity of NaI needs to be dissolved. The best practice for a rapid dissolution process is to add NaI in small steps to a glass beaker filled with the appropriate volume of Milli-Q water, while placed on a magnetic plate at 50 °C and containing a stirring rod (200 rpm or more). Be cautious: once the solution is saturated, it will turn yellow and can leave very distinct yellow stains behind. Fill a wash bottle with NaI, and let it cool down.

B. **Density separation 1**: In the same manner as during first digestion step, rinse the filtered sample off the stainless-steel filter using the metal funnel, this time into a tall form beaker. To do so, replace the Milli-Q water with saturated NaI. The volume used depends on the volume needed to thoroughly clean the stainless-steel filter and should be minimum 70 mL to allow for an efficient density separation. Make sure to rinse the funnel as well.

C. Stir the solution containing the sample with a metal spatula so that MPs present in the sediment are mixed with the NaI. Using a wash bottle, gently wash any remaining material that is stuck to the sides of your glass beaker into the solution using the remaining +- 5 ml of NaI. Subsequently, leave the sediment to settle overnight for 24 h (first density separation). Because of the difference in density of the solution and the plastics present in the sample, most MPs will float and will in this way be separated from the sediment. Make sure the beaker is fully covered with aluminium foil (or with a cleaned petri dish). If not covered completely, you risk evaporation of the NaI in the sample, causing crystallization and a reduced volume, which complicates the density separation process.

D. After 24 h, the solution needs to be filtered to recover the MPs present. To do so, pour the supernatant through a small sieve (Ø 50 mm, 20 µm mesh or smaller). To avoid sediment particles from being filtered, pour slowly, so that floating material will flow into your beaker faster than the sediment. As a result, one beaker now contains the sediment pellet, one beaker contains the filtered NaI, and MPs as well as organic material are present on the 20 µm-sieve. Make sure both beakers and the sieve are covered with aluminium foil when not being handled.

E. Slowly pour 70 mL of the sieved NaI back onto the sediment pellet, and make sure all materials sticking to the sides of the beaker are washed back into the solution. Repeat the mixing step from the day before: slowly stir the solution with a metal spatula, wash the material stuck to the sides of the beaker back into the solution with the remaining 5 mL NaI, cover the beaker and leave it for another 24 h so that the sediment can settle a 2nd time.

F. Following this, take a new glass beaker, put the funnel in the beaker (or use a laboratory stand as mentioned above), place the sieve containing the sample upside down in the funnel and thoroughly wash the sieved sample into the beaker using Milli-Q in a wash bottle. Also rinse the funnel as MPs may stick to the sides. Use up to a third of the volume of the beaker used to do so (200 mL in the case of the 600 mL-beakers used here).

G. **Density separation 2 and 3:** Repeat this procedure a 2nd time: pour the supernatant of the settled NaI-solution over the sieve so that the sediment is separated from the NaI containing the MPs, add the sieved NaI back to the sediment pellet and leave to settle for another 24 h after mixing, place the sieve upside down in the metal funnel, and finally thoroughly wash the particles present into the same glass beaker as the one used for the first density separation, again using a volume of up to 200 mL Milli-Q water (and repeat this workflow once more).

H. Once three consecutive density separations have been carried out, after 72 h, a resulting maximum 600 mL of Milli-Q water containing MPs from three different density separations from the same sample should be obtained.

I. Once the NaI solution from all processed samples has been recovered, filter the whole solution over a 2.7 µm-filter using the filtration apparatus. Next, collect the filtered NaI in a glass bottle and store it for later use.

Note: If preferred, the density separation steps can be sped up by making use of a centrifuge and falcon tubes instead of letting the sediment settle overnight, as is done in the developed standard operation procedure for MPs extraction from seawater samples (Meyers *et al.*, 2024b). In that case, the stainless-steel filter containing the sample should be washed into a falcon tube with no more than 50 mL of NaI. This step is followed by centrifugation of the sample, filtration of the supernatant over a PTFE filter (see Section 7.3.4 'Filtration'), adding the filtered NaI in the Büchner flask back to the pellet in the falcon tube, and repeating the whole process two more times, so that a total of three density separation steps are

performed and the NaI is filtered three times over the same PTFE filter before staining.

7.3.4 Filtration

A. Prepare a filtrated Nile red solution dissolved in acetone (10 µg/mL). 1 mL is needed per PTFE filter.

B. Filter the sample over a PTFE filter using a filtration apparatus. Rinse abundantly with Milli-Q water. Following this, add 1 mL of the filtrated Nile red homogeneously to the filter using a glass Pasteur pipette with rubber stop. When doing so, make sure that all particles sticking to the lower side of the glass funnel are washed onto the filter.

C. Leave the Nile red to soak for 15 min, then rinse abundantly with Milli-Q water (minimum 50 mL). Transfer the filter to a labelled glass slide inside a petri dish using tweezers and leave it to dry in a dark environment for at least 24 h before photographing the filter under a fluorescence (stereo) microscope using the appropriate protocol (Meyers *et al.*, 2024a).

7.4 Quality Control Measures

A. Pre-clean all glassware before use. To do so, wash with soap and rinse thoroughly with tap water (three times), followed by Milli-Q water (another three times). Ideally, leave the glassware to dry upside down on a metal rack or on a cotton towel to avoid airborne contamination. Always clean the equipment before using it for another sample when switching between samples.

B. Always wear a 100% cotton lab coat and avoid wearing synthetic clothes underneath as much as possible. Write down the colours of the clothes you are wearing while processing the samples.

C. Sample processing should be performed in a laminar flow hood to minimise contamination. Thoroughly clean the laboratory workspace (around 3 m² needed) prior to starting, e.g., using cotton or lint free paper towels.

D. Always cover the samples with aluminium foil while not being handled, and make sure all beakers are properly labelled at all times to prevent the loss of samples.

E. To avoid airborne contamination, control air movement in the laboratory by closing all windows while working and prevent the passage of other lab users in the area where samples are being processed. Airborne contamination can be assessed by placing a PTFE filter in a labelled open petri dish in the sample processing area, from which particles will be quantified afterwards.

F. Run procedural blanks (ideally n = 3) in alongside the actual sample processing. To do so, follow the exact same steps as mentioned in this protocol, but using a Milli-Q water matrix with no added plastic particles. This should be done for every batch of samples that is being processed.

G. Run a positive control to determine the recovery efficiency. To achieve this, spike a known number of MPs of known size and polymer type into clean sediment (made MP-free by heating to 400°C for 2 h), and execute the same extraction procedure.

H. Safely dispose of chemical waste in the appropriate and secure containers until collected for safe disposal.

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