5. Extraction of microplastics from marine seawater samples followed by Nile red staining

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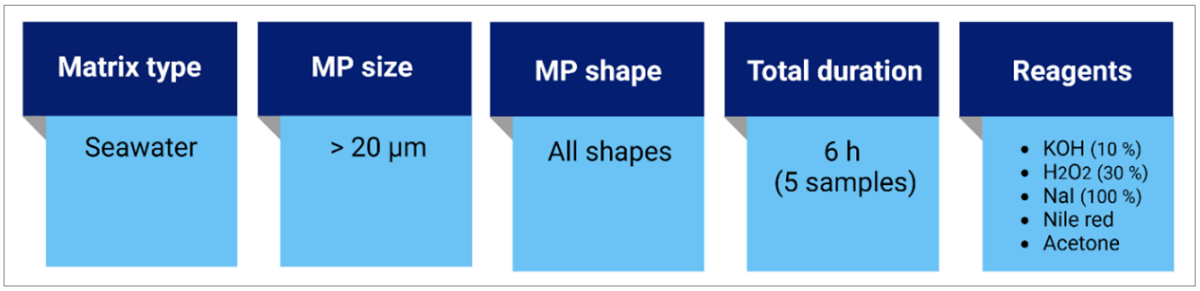
Deliverable 2.6 Analysis techniques for quantifying nano-and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023

5.1 Scope of Protocol

MPs, defined as plastic particles sized 0.1 µm – 5 mm (Hartmann *et al.*, 2019), are an issue of concern because of their ubiquity in the marine environment. The worldwide distribution 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 demonstrated 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;). Their small size makes them accessible to a wide variety of marine organisms in the food chain (e.g., Peng et al., 2018; Botterell *et al*., 2019; Pan et al., 2019; Wang et al., 2020), and consequently research investigating the ecological effects of MPs in marine ecosystems are of great significance (Koelmans *et al*. 2022). 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 highlights the need for standardised, 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 nanoplastics (NPs) and MPs in our oceans and seas. Within the project, new sampling and advanced analysis methodologies that focus on smaller MPs (< 10µm) and NPs (<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 MPs 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 standard operating procedures (SOPs).

This protocol focuses on the efficient extraction of MPs > 20 µm from marine seawater samples, needed for the subsequent semi-automated MPs analysis based on machine learning and Red, Green, and Blue (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 protocol for the extraction of MPs from marine seawater samples.



5.2 Materials and Equipment

Glassware

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

Filtration

Filtration apparatus or filtration manifold set (for Whatman 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 (minimum RCF of 101 x g)
- Multi-position digital magnetic hotplate stirrer

Laboratory consumables

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

- Aluminium foil
- Whatman glass microfiber filters (2.7 µm, ⌀ 47 mm)

Other laboratory equipment

- Metal sieve of ⌀ 5 cm and mesh size 20 µm
- A large and a small metal funnel
- Lab support stand with 2 laboratory clamps (1 can be a ring clamp)
- Magnetic stirring rod (8 mm)
- Metal spatula
- Tweezers
- Cotton lab coat and nitrile protection gloves
- Milli-Q water

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 10µm using a 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 2.7 µm 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.

5.3 Protocol

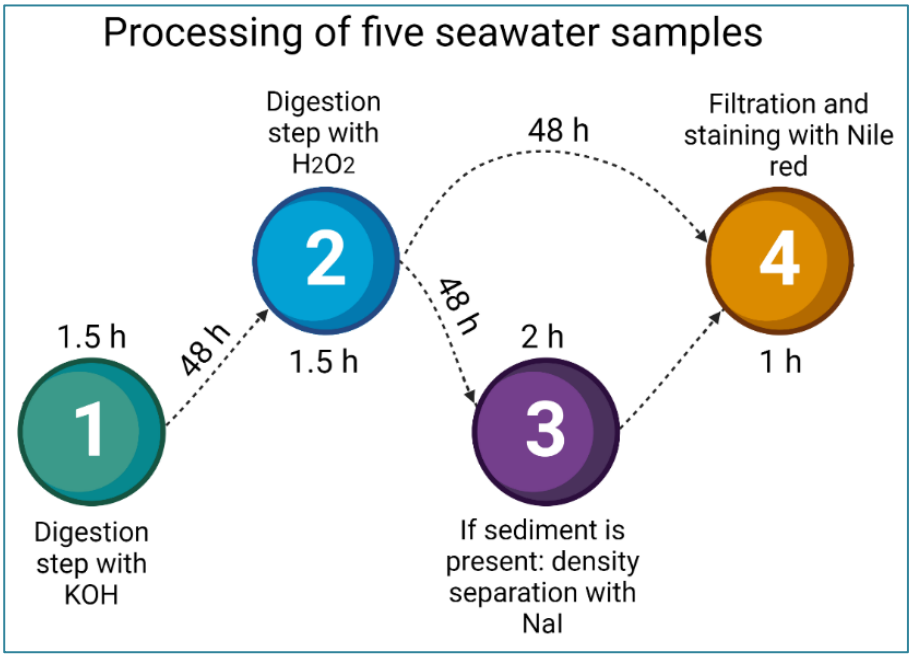


Figure 1: Scheme of all major steps within the protocol, as well as their durations

5.3.1 Digestion of Organic Material

Prior to starting the sample processing, make sure to have read Section 5.4 'Quality Control Measures'. As a first step, organic material present in the sample needs to be removed by performing a double digestion (see Figure 1), consisting of a first digestion step using KOH to remove traces of animal tissue present in the sample, and a second digestion step with H_2O_2 to remove plant material (Prata *et al*., 2019).

A. Place the small metal sieve in the metal funnel and place the construction in a glass beaker. Following this, slowly pour the sample through the small metal sieve, and rinse the sample bottle multiple times. If the construction cannot be placed in the beaker in a stable way because of its size, the funnel with sieve can be held manually by a second person when sieving the sample, 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. In case a lot of material is present, it is advised to sieve the sample in multiple steps, and to wash the sieved sample in a new beaker in between steps (see next step).

B. Carefully transfer the sieved material present on the sieve into a new beaker by placing the sieve upside down in the funnel, which is either placed on the beaker, held manually, or attached to the laboratory stand with the beaker placed underneath. To transfer the sample, wash the underside of the sieve thoroughly with Milli-Q water using a wash bottle. Next, turn around the sieve and thoroughly wash sample traces present on the edges of the sieve above the funnel into the glass beaker. Rinse the funnel as MPs may stick to the sides. Transfer the sample into the glass beaker using maximum 100 mL. Cover the glass beaker with aluminium foil.

C. Following this, the sample will be digested for 48 h using 200 mL of KOH (10%). To obtain the 10% v / v KOH concentration, prepare 100 mL of 20%-KOH using Milli-Q water and add it to beaker containing the sample and 100 mL of Milli-Q water. If the sample volume is different from 100 mL, make sure a final concentration of 10% and volume of 200 mL is obtained. Next, add a magnetic stirring rod, cover the beaker with aluminium foil, and leave the sample to digest on a magnetic hotplate stirrer at 50°C and 150 rpm.

D. After 48h, sieve the digested solution following the procedure and transfer the digested sample to a new beaker using 100 mL of Milli-Q water.

Following this, add H_2O_2, (30-33 % solution) to the sample at a 1:1 volume sample:solution ratio (= 100 mL in this case), so that a final concentration of +- 16% H_2O_2 is obtained. Finally, add a magnetic stirring rod, cover the beaker with aluminium foil and leave the sample to digest for another 48 h on a magnetic hotplate stirrer at 50°C and 150 rpm.

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

5.3.2 Density Separation

If the amount of sediment present in 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 5.3.3 '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. Again, sieve the digested sample over the small metal sieve. Once sieved, wash the sample abundantly on the sieve using Milli-Q water, to remove any trace of H_2O_2. Next, use the laboratory stand and two clamps to mount the funnel just above the falcon tube, and place the sieve with sample upside down in the funnel. Wash the sample into the falcon tube using the wash bottle with NaI and rinse the funnel as MPs may stick to the sides. Limit the volume NaI to wash the sieve to 45 mL (almost the complete volume of the falcon tube). Use a metal spatula to mix the sediment and NaI. Use the remaining 5 mL NaI to wash off the spatula into the falcon tube.

C. **Density separation 1:** Place the falcon tubes with NaI and the sieved material in a centrifuge for 5 min. at 1000 rpm (RCF of 101 x g or more).

Note: if a centrifuge is unavailable in the lab, the NaI-solution with sample material can also be left overnight in a glass beaker to let the sediment settle (three times, see developed sediment protocol in Meyers *et al.*, 2024b).

5.3.3. Filtration

A. Prepare a filtered Nile red solution dissolved in acetone (10 µg / mL). 1 mL is needed per PTFE filter. Slowly and carefully filter the NaI containing the sample material in the falcon tube on a PTFE filter using the filtration apparatus. Ensure that only a minimal amount of sediment, if any, reaches the filter: only the supernatant fraction should be filtered, while the sediment pellet should remain in the falcon tube.

B. Transfer the captured saturated NaI in the Büchner flask back to the sediment pellet, while washing the sides of the falcon tube, to ensure that any remaining particles sticking to the walls of the tube are washed back into the solution. Use a metal spatula to mix the resuspended sediment and NaI. Use the remaining NaI to wash off the spatula into the falcon tube.

C. **Density separation 2 and 3:** Repeat the density separation step using the centrifuge, filter the supernatant fraction onto the same PTFE filter from the first density

separation, which should still be present on the filtration apparatus. Repeat once more, so that NaI from three different density separations is filtered on the same PTFE filter.

Note: In case the filter clogs and the sample is no longer being filtrated in an efficient way, the sample will need to be filtrated over multiple PTFE filters.

D. Before rinsing off remaining traces of NaI with Milli-Q water, transfer the captured saturated NaI into a glass bottle. The salt solution should be filtered over a filter of small mesh size (e.g., Whatman glass microfibre filters - 2.7 µm, ⌀ 47 mm) afterwards, so that it is ready to be used during later experiments.

E. Rinse the PTFE filter abundantly with Milli-Q water (minimum 20 mL), to remove most traces of NaI. Following this, add 1 mL of the filtered 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.

F. 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).

5.4 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 minimize 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 always labelled 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) 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 are 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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