6. Extraction of microplastics from marine sediment 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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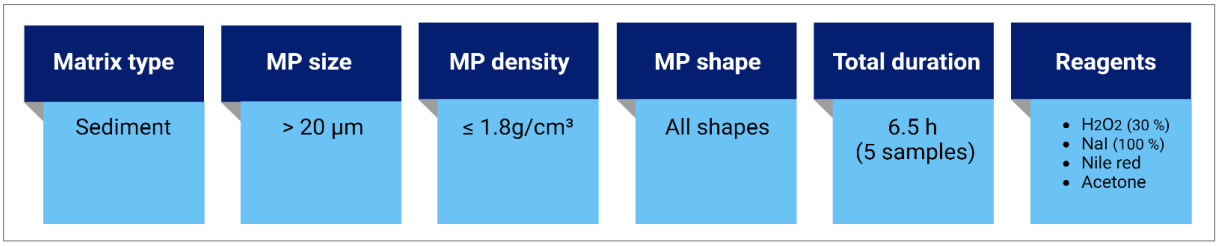
6.1 Scope of Protocol

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. These global contaminants (2008/56/EC Marine Strategy Framework Directive, Descriptor 10; United Nations Sustainable Development Goal 14 target 14.1.1) are known to accumulate in benthic sediments and beaches worldwide (Browne *et al*., 2011; Martellini *et al*., 2018; Kane *et al*., 2019) where they become accessible for ingestion to benthic species, including commercial fish and shellfish (Lusher *et al*., 2013; Rummel *et al*., 2016; Ding *et al*., 2020). A variety of polymer types has been reported in earlier studies (Lusher *et al*., 2015). The limited understanding of MPs accessibility and the associated potential risks MPs pose to benthic ecosystems emphasizes the need for standardised, representative, and feasible sampling, sample processing and sampling analysis protocols, so as to obtain accurate measures of MPs pollution levels in marine sediments.

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 (< 10 μm) and NP (<0.2 μm) 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 SOPs.

This particular protocol focuses on the efficient extraction of MPs > 20 µm from marine sediment 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*., 2024). 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 validation of the protocol for the extraction of MPs from marine sediment samples.



6.2 Materials and Equipment

Glassware

- Glass beakers (600 mL / tall form)
- Large petri dish (min. 100 mm)
- 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 consumables

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

- Filters to filer NaI, H_2O_2 and Nile red e.g. Whatman glass microfiber filters (2.7 µm, ⌀ 47 mm)

Other laboratory equipment

- Metal sieve of ⌀ 5 cm and mesh size 20 µm
- A small metal funnel
- Metal spatula
- Tweezers
- Cotton lab coat and nitrile protection gloves (H_2O_2)
- Milli-Q water

Reagents

- Hydrogen peroxide (H_2O_2 - 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 a filtration apparatus, to reduce potential contamination (e.g., Whatman glass microfibre filters - 2.7 µm, ⌀ 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.
- If subdivision of MPs into size classes is desired, stacked small sieves can be used, e.g. of 1 mm, 250 µm and 20 µm.

6.3 Protocol

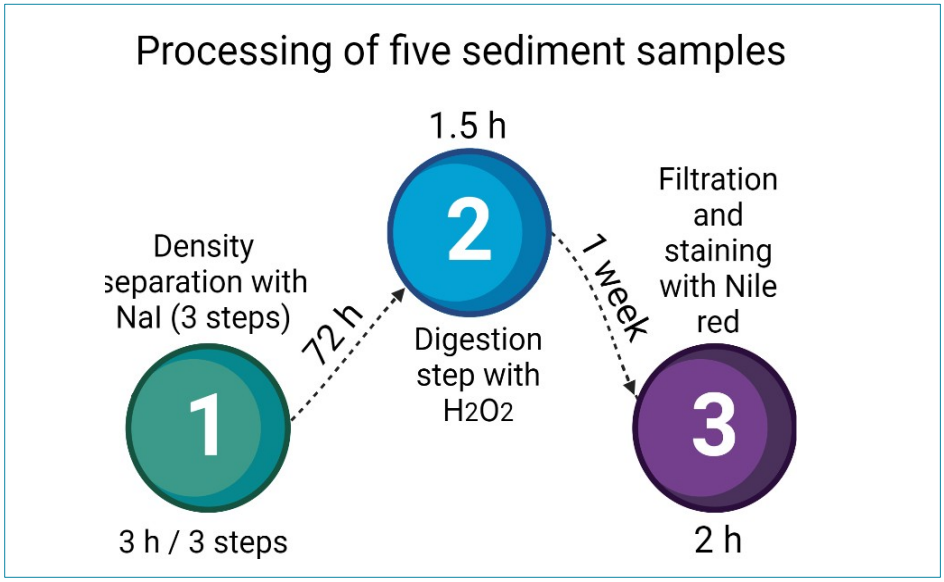


Figure 1: Scheme of all major steps within the protocol, as well as their durations

6.3.1 Sample Preparation

Prior to starting the sample processing, make sure to have read Section 6.4 'Quality Control Measures'. A density separation using a saturated NaI solution needs to be performed as a first step (see Figure 1). 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.

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.

B. Following this, and once the filtered NaI has cooled down to room temperature, add 70 mL of the dense salt to the sediment sample. Stir the solution 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 I. Subsequently, leave the sediment to settle overnight for 24 h (first density separation). The beaker must be fully covered with aluminium foil, or with a cleaned petri dish, to avoid evaporation of the NaI in the sample, which could induce crystallization and a reduced volume, creating complexity during the density separation process.

C. Density separation 1:

After 24 h, the solution will 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 the supernatant slowly, so that floating material will flow into the 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 will be present on the 20 µm-sieve. Make sure both beakers and the sieve are covered with aluminium foil when not being handled.

D. 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 previous day: 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 second time.

E. Take a new glass beaker, put the funnel and sieve on the beaker and pour Milli-Q water abundantly over the sieve containing the sample to remove any traces of the NaI (minimum 100 mL). It is very important to clean the sample as the smallest trace of NaI can cause an exothermic reaction with a lot of effervescence when added to H_20_2 in the next step. This needs to be avoided as it may lead to loss of the sample. The beaker used to capture diluted traces of NaI can be used for all replicates/samples being processed.

F. Once clean, place the funnel with sieve in a new glass beaker. Turn the sieve around in the funnel and wash the sieve with Milli-Q water, so that the sample is captured in the glass beaker. Next, turn around the sieve and wash the edges of the sieve above the funnel to make sure all traces of the sample are washed into the glass beaker. Rinse the funnel as MPs may stick to the sides. Accomplish the transfer of the sample into the glass beaker using 50 mL of Milli-Q water. Cover the glass beaker with aluminium foil.

G. Density separation 2 and 3:

Repeat this procedure two more times: 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, wash remaining traces of NaI from the sieve, 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 using a volume of 50 mL of Milli-Q water.

Once three consecutive density separations have been carried out, after 72 h, a resulting 150 mL of Milli-Q water containing MPs from three different density separations from the same sample should be obtained.

H. When 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.

6.3.2 Digestion of Organic Material

A. Before adding H_20_2, have a large beaker filled with cold water (10-15 °C) ready, in case it needs to be used to slow down the exothermic reaction.

B. Organic material needs to be removed from the sample. To do so, add H_2O_2, (30-33 %) to the sample at a 2:1 volume sample:solution ratio, so that a final concentration of 10 % H_2O_2 is obtained. For a sample of 150 mL, you add a volume of 75 mL H_20_2 (30 %). H_20_2 should be added in small amounts to avoid a strong exothermic reaction from taking place. Effervescence will be visible, and the sample can turn yellow, which is normal. If the beaker becomes hot to touch (> 50 °C), and foam in the beaker rises rapidly, place the sample in the beaker filled with cold water using protective equipment (e.g. thermal gloves). The cold water will slow down the exothermic reaction. Ensure that you remain in close proximity to the samples for a minimum of thirty minutes and consistently monitor them until the exothermic reaction has decelerated. Following this, leave the samples to digest for a week, covered with aluminium foil.

6.3.3 Filtration

A. Prepare a filtered 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 the filtration apparatus. Rinse abundantly with Milli-Q water (a minimum of 50 mL). 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 all particles stuck to the lower side of the glass funnel are washed onto the filter.

C. Leave the Nile red to soak for 15 minutes, then rinse abundantly with Milli-Q water. 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*., 2024).

6.4 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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