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ANDROMEDA Portfolio of Microplastics Analyses Protocols

2024



ANDROMEDA Portfolio of Microplastics Analyses Protocols

February 2024

ANDROMEDA was a research project developing analysis techniques for quantifying nanoplastics and microplastics and their degradation in the marine environment. ANDROMEDA brings together a multidisciplinary consortium of 15 international partners to improve the quantification of nanoplastics and microplastics in our oceans and seas.

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ANDROMEDA WP5 Communication & Stakeholder Engagement: Task 5.6 and its associated Deliverable 5.5 was developed as part of ANDROMEDA's WP5 to support the development and collation of protocols that describe the analyses of microplastics particles. The ANDROMEDA portfolio of protocols aims to facilitate the production of comparable data outside of the project and beyond the lifetime of the project by other research groups. This work was undertaken in conjunction with the ANDROMEDA's technical WP.

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ABBREVIATIONS

CMTT - Cryo-milled tire tread
RE - Extraction procedure
FM - Fluorescence microscope
FSM - Fluorescence stereomicroscope
GITs - Gastrointestinal tracts
HDPE High density polyethylene
ICP-MS - Inductively coupled plasma mass spectrometry
LC - Liquid chromatography
ME - Matric effect
MP - Microplastic
MPs - Microplastics
MS - Mass spectrometer
NP - Nanoplastic
NPs - Nanoplastics
OOB - Out-of-bag
PCs - Principal Components
PCA - Principal component analysis
PDM - Plastic Detection Model
PE - Process efficiency (Protocol 2)
PE - Polyethylene (Protocol 8)
PET - Polyethylene terephthalate
PIM - Polymer Identification Model
PP - Polypropylene
PS - Polystyrene
PUR - Polyurethane
PVC - Polyvinyl chloride
RC - Regenerate cellulose
RE - Extraction procedure
RF - Random Forest
RGB - Red, Green, Blue
SMOTE - Synthetic Minority Oversampling Technique
SOPs - Standard operating procedures
TWPs - Tire wear particles
UPLC - Ultra high performance liquid chromatography
Zn - Zinc

Introduction

The analysis of microplastics in marine matrices can be divided into different analysis steps, from sampling and sample transport to sample preparation, sample analysis, data reporting, and quality control. The optimal choice for each step is dependent on different factors such as the size, shape, and type of microplastics to be analysed, the matrix (water, sediment, biota) or the amount and type of potential interferences (solid particles, organic material etc.). Additionally budget, available equipment, and the experience of personnel are important factors to consider in selecting the optimal analysis method.

Within the JPI Oceans ANDROMEDA project, it was acknowledged that there was a pressing need for a portfolio of cost-efficient and fit-for-purpose microplastic methodologies. These include advanced, high-end techniques which allow the identification of very small micro (<10 µm) or even nanoplastics (<1 µm) or challenging forms of microplastics such as tire wear particles or paint flakes, as well as so-called cost-effective techniques which use in-situ measurements, less expensive equipment, or increased automatization to analyse microplastics often >50, >10 or >300 µm.

Independent of the method, microplastic analysis should reach an acceptable level of accuracy and precision, ensuring the detectability of a large variety of polymers with a low risk of contamination and mismatches. Harmonization of protocols is therefore essential to increase the quality of microplastic measurements in the marine environment.

In addition to a wide range of microplastics analysis methods, there is also a need for methods to reproducibly mimic environmental degradation. Most plastics degrade very slowly, over decades, in the marine environment through a combination of photo-oxidation, bio-oxidation, and mechanical abrasion. Also here, harmonisation of accelerated degradation methods is key to study plastic degradation in an efficient way, mimicking natural fragmentation and additive chemical leaching.

This protocol bundle aims to facilitate knowledge transfer and method harmonization on a variety of methods by including 9 protocols - developed or optimised within the JPI Oceans ANDROMEDA project:

1. Microplastic sampling methods

- Sampling of microplastics in water by an automated microplastic sampling device

2. Microplastic analysis methods

- Chemical marker identification to study exposure of biota to tyre wear particles
- Chemical marker identification to study exposure of biota to common plastics
- Short wave infrared spectroscopy using snapscan hyperspectral sensor camera
- Optimised detection of microplastics by adapted Nile Red staining:
 - Extraction of microplastics from marine seawater samples followed by Nile red staining
 - Extraction of microplastics from marine sediment samples followed by Nile red staining
 - Extraction of microplastics from marine biota samples followed by Nile red staining
 - Automated microplastic analysis: Nile red staining and random forest modelling

3. Accelerated microplastic degradation

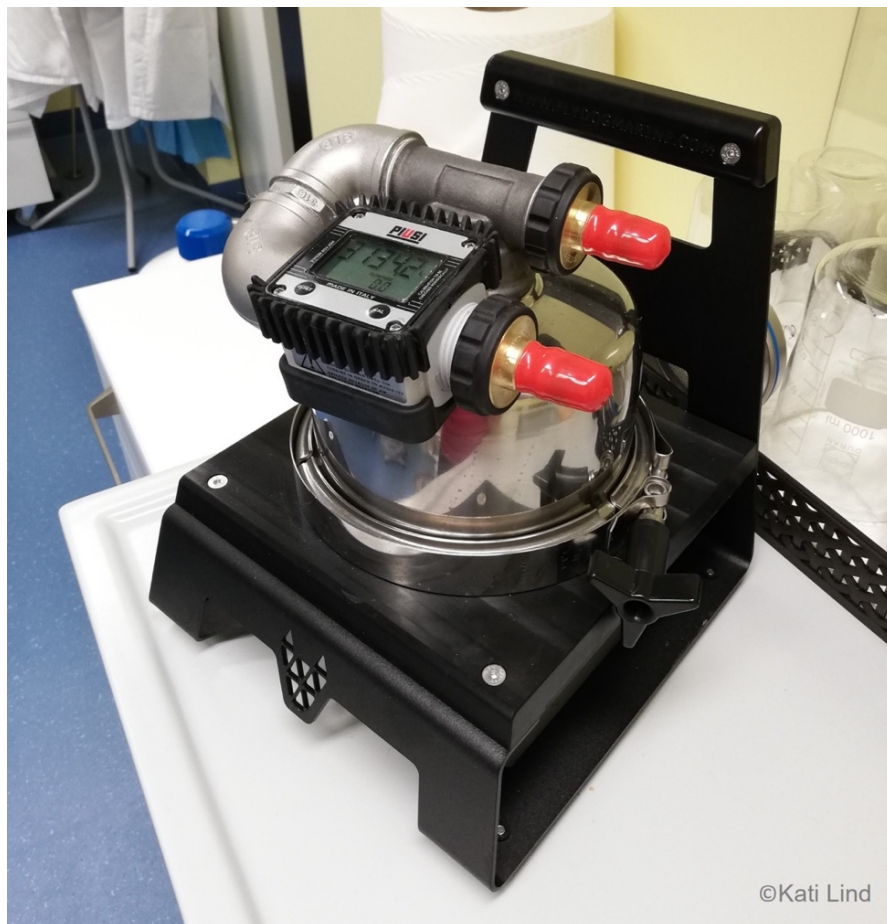
- Photo-Oxidation of Microplastics Materials in water

1. Sampling of microplastics in water by an automated microplastic sampling device

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Deliverable 2.1 Analysis techniques for quantifying nano-and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023



1.1 Scope of Protocol

The objective of this protocol is to provide a comprehensive guide for efficient and cost-effective sampling of microplastics (MP) in water using an optimized device attached to flow-through systems on research vessels and ships-of-opportunity (*ferryboxes*). The developed device enables unattended sampling of MP within specific particle size ranges, ranging from 300/100 μm (comparable to Manta and Neuston nets) to 50/100 μm , which are considered environmentally more relevant fractions. By implementing cost-effective sampling and analysis methods, this protocol aims to detect and quantify MPs in environmental samples. The utilisation of *ferrybox* systems can provide the greatest value for money in plastic analysis, offering a promising approach for future monitoring strategies. The developed sampling instrument has the potential to support researchers and policy makers in making informed decisions regarding different MP workflows.

1.2 Materials and Equipment

- Flow-through system or *ferryboxes* on research vessels
- Automated sampling device for MPs
- Sieves with needed size of mesh (recommended 300, 100, 50 μm)
- Collection containers for water samples
- Blank filters to check contamination
- Chemicals for sample preservation
- Sampling recording system or notebook

1.3 Protocol

1.3.1 Construction

The device is a simple flow through add-on for *ferrybox* systems to collect MP samples with commercial sieves (see Figure 1). Its construction consists of outer case which contains inner container (both made from stainless steel) where are, on top of each other, three Retsch 100 mm diameter sieves (top to bottom) – 300 μm , 100 μm and 50 μm mesh. On top of the outer container, there are connectors for seawater inlet and outlet and valves for easy

detachment. Flowmeter is included and connected to the outlet - this can be replaced with different flowmeter if more precise measurement is required.

Connecting the device into existing *ferrybox* system can be done via $\frac{3}{4}$ " connectors and attaching between existing pipe or hose to the device. It should be connected via separate loop to avoid interfering with other measurements of the *ferrybox* system *i.e.* biological or other environmental measurements that can be affected by the sieves collecting the material before sensors and *vice versa*. The device comes with a flowmeter that must be turned on before the measurement starts as well marking the start and end time. Taking the initial reading and end reading gives the volume in liters passed the system during measurement, knowing the time duration from start to end of measurement time and material collected from the sieves can be then analyzed together with *ferrybox* data.

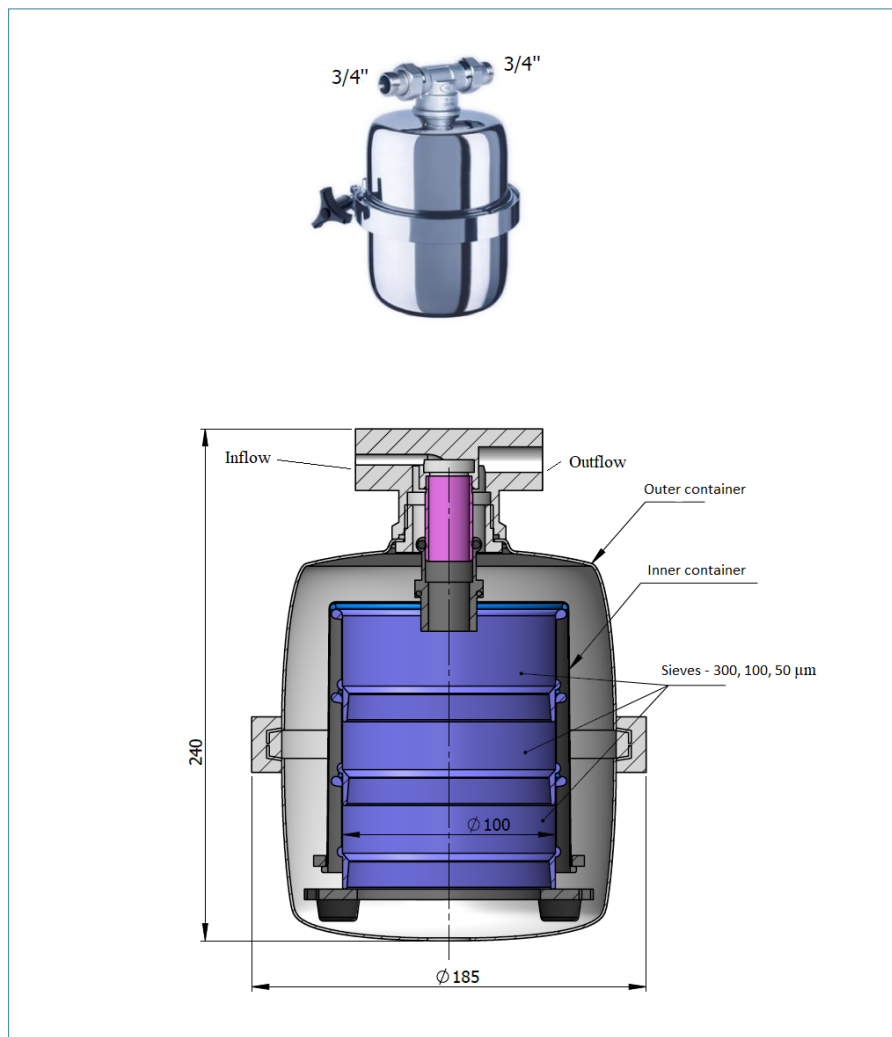


Figure 1: Microsampling device and design by Flydog Solutions LLC

1.3.2 Working Principal

The main aim of this device is to collect micro-litter samples with sieves placed on top of each other and collecting material according to the mesh size. After releasing water to the

device, it goes through a sieve with the biggest mesh size (300 μm), then medium size (100 μm) and finally the smallest mesh size (50 μm). After passing the final sieve, water goes through outflow and registries in flowmeter for volume (l) and speed (l/min).

1.3.3 Assembling the Device

Outer container is hold together with collar that can be tighten or open unscrewing the black thumbscrew (see Figure 2).



Figure 2: Microplastic sampling device outer container with black thumbscrew

After opening the outer container, you might notice some water coming out if additional valve does not have the functionality to extract excess water (depends on the use case of the device). This is expected as the outer container acts as a medium for outflow when water has passed the sieves. Inside the outer case inner container holding the sieves can be found (see Figure 3) by pulling it out.

The inner container is connected to the base with three Hex bolts (Size 4) after unscrewing them, three sieves can be found. Some attention must be paid to the rubber seal and ensuring it stays correctly on top of the sieve when inserting them into the container.



Figure 3: Inner container with three Retsch sieves

1.3.4 Transporting the Device

After collecting the device from *ferrybox* system, the inflow is be covered with aluminum foil to minimize the contamination. To prevent any potential presence of seawater, it is recommended to transport the device in a waterproof box, such as a thermal box.

1.3.5 Sample Collecting

When the device arrives in the laboratory, it is recommended to collect the MP samples as soon as possible to avoid the sieves inside the device from drying. If it is not possible, the collection device must be kept in a tightly sealed waterproof thermal box. Otherwise, when the device is completely dry, the sieves are very difficult to clean and most of the collected material will stick to the sieve.

To collect the samples, unscrew the device and remove the lid covering the sieves, do not remove the aluminum foil from inflow. Remove the rubber sealing on top of the first sieve, and make sure it does not fall into the sieve. Take out the top sieve (size class of 300 μm) and leave the other two (100 and 50 μm) inside the device. Cover the remaining sieves with the covering lid. Make sure they are covered properly so the contamination would be minimized.

Place the sieve (300 μm) upside down on a glass funnel that is on top of a glass jar and rinse it with ultrapure milli-Q water. Repeat the rinsing procedure with the other two sieves. Use

a separate jar for each sieve, and mark the jars with date, sampling event name, device no. and sieve no. For longer preservation time, fix the samples with 37% formaldehyde solution.

The smallest sieve (50 μm) may contain seawater due to a vacuum or clogging by organic material and sediments. Carefully remove the sieve on top of it (100 μm) and let the seawater filter through the 50 μm sieve. When the sieve is empty, repeat the rinsing procedure as described above.

1.3.6 Sample Processing

Before further processing the samples, let them settle to the bottom of the jar. Use a glass pipette to remove the water on top of the settled material and filter it through a glass fiber filter. Use a separate filter for each sieve. After filtering, place the filter in a glass petri dish for future analysis. All petri dishes should be marked the same as the glass jars, where the samples were pipetted from.

Dry the filters in the oven at 60 °C for 15 minutes without the lid of the petri dish. The filters should be placed in the oven while it is still heating up. After drying, cover the filters with the lid and analyze them under stereomicroscope.

Organic material and different types of sediments, such as sand and mud, can potentially influence the analysis of samples. MPs can be hidden or mistaken while identifying them under a microscope. In addition, the analyzing process can also be prolonged. If it is not clear, if the sample contains a lot of organic material or sediments, homogenize the sample by shaking it and filter a small amount of the sample through the glass fiber filter. Dry the sample and analyze it under a stereomicroscope.

If the sample contains a lot of organic material, use hydrogen peroxide (H_2O_2) to process the sample. Depending on the amount of organic material, add 1:1 H_2O_2 solution (~35%) into each sample. Let the samples process at least 7 days at room temperature, then filtrate the solution, dry in the oven and analyze under stereomicroscope.

If the sample contains a lot of sediments, use sodium iodide (NaI) to process the sample. Depending on the amount of sediment, add ~25-50 ml of NaI solution (1,8 g/cm³) to the settled material. Use orbital shaker to mix the samples at 200 rpm for 10 minutes. After mixing, let the samples settle. Settling time depends on the amount and type of sediment. If the material has settled, use an automatic pipette to collect the surface solution. Pipette the solution through the sieve with the same size class where it was initially taken from. Rinse the sieve similarly as described above, using a glass funnel and ultrapure milli-Q water.

Filtrate the solution through glass fiber filters, dry them in the oven at 60 °C for 15 minutes and analyze under stereomicroscope.

1.3.7 Cleaning the Collection Device

After collecting the samples, the device must be cleaned thoroughly. Wash the device cover, the sieves and the base of the device with tap water, use the sponge if necessary. Afterwards rinse with milli-Q water. Lastly, use the ultrasonic bath to maximize the cleaning process of sieves after every sample collection.

1.4 Quality Control Measures

The samples may be contaminated very easily throughout the transportation and preparation process. To minimize the contamination, the device inflow must be covered with aluminum foil throughout the time when the sieves with collected material are inside. To minimize the contamination from clothes, all the researchers preparing the samples must wear 100% cotton laboratory coats. The sample collecting, processing and analyzing must be carried out in secluded space with minimal traversal (none if possible) by other personnel.

Before opening the device, place one clean filter near the sample preparation as a dry blank sample. A dry blank sample will be used to assess potential contamination from the laboratory air during the sample preparation, processing, and analysis.

To check the contamination during filtering process and in the milli-Q water, filter 100 ml of ultrapure milli-Q water through one clean filter. This filter will be a wet blank sample and treated the same as regular samples.

Note: This protocol provides a general framework for water sampling of MPs using an optimized device. Specific adjustments and considerations may be required based on the study objectives, sampling environment, and available resources. It is crucial to consult relevant guidelines, literature, and expert advice when implementing this protocol to ensure best practices and adherence to specific research requirements.

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2. Chemical marker identification to study exposure of biota to tyre wear particles

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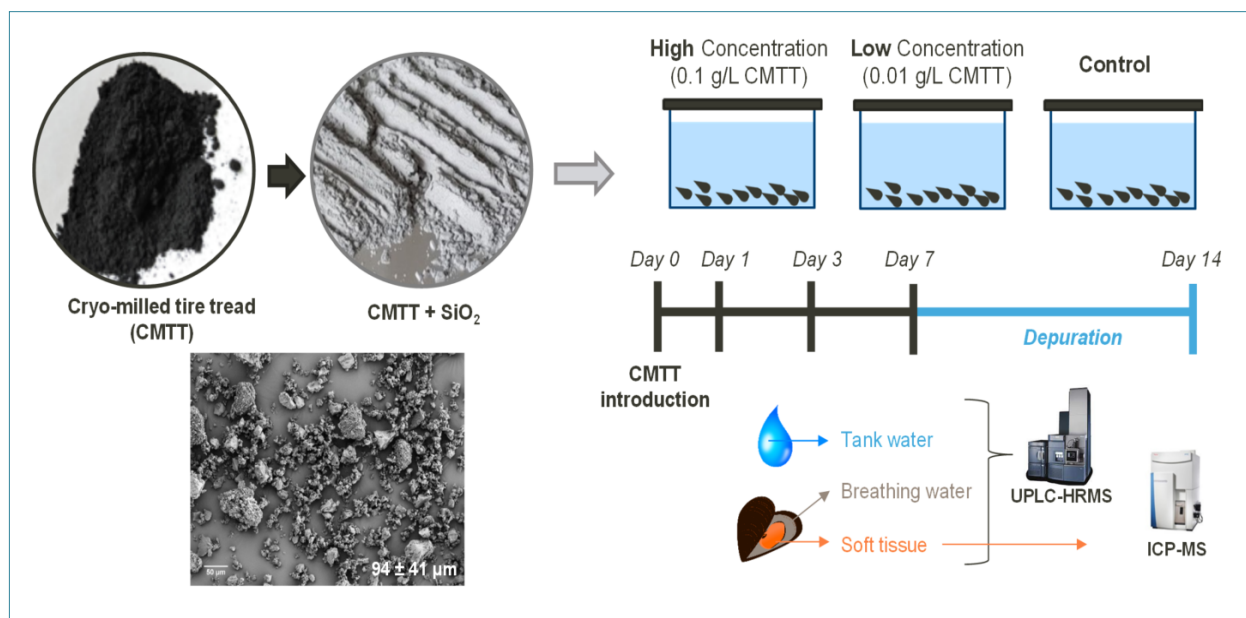
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Deliverable 2.5 Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023.



2.1 Scope of Protocol

The knowledge regarding suitable chemical indicators to assess the exposure of aquatic organisms to tire wear particles (TWP) is still very scarce; this is particularly true when it comes to marine organisms. The combination of possible different scenarios, such as the uptake of TWPs and/or leached chemicals, makes the determination of TWPs in biota very challenging. The detection of organic additives used during the tire production (such as vulcanization accelerators and antioxidants) and the zinc (Zn) content (known to be almost 1% in the tire) might be useful for this purpose. The aim of this protocol was to investigate suitable chemical tracers to assess to which extent the exposure of mussels to TWP is reflected by internal concentrations of tire-related chemicals. To do so mussels (*Mytilus edulis*) have been exposed to a mixture of 20 types of cryo-milled tire tread (CMTT) particles (< 100 µm). Mussels were exposed to CMTT particles for 7 days, followed by additional 7 days of depuration. At day 1, 3, 7 and 14, both water and tissues samples were collected and analysed by UPLC-HRMS and ICP-MS for the detection of organic tire-related chemicals and Zn content, respectively.

2.2 Materials and Equipment

Table 1: Equipment Used

Equipment	Name	Manufacturer
<ul style="list-style-type: none"> Analytical balance 	<ul style="list-style-type: none"> Excellence Plus BL 1500S 	<ul style="list-style-type: none"> Mettler Toledo Sartorius
<ul style="list-style-type: none"> Ultrasonic bath 	<ul style="list-style-type: none"> BANDELIN SONOREX 	<ul style="list-style-type: none"> BANDELIN Electronics
<ul style="list-style-type: none"> Microwave digestion 	<ul style="list-style-type: none"> MW PRO 	<ul style="list-style-type: none"> Anton Paar Multiwave PRO
<ul style="list-style-type: none"> Mini spin plus 	<ul style="list-style-type: none"> Eppendorf 	<ul style="list-style-type: none"> Eppendorf AG
<ul style="list-style-type: none"> Shaker 	<ul style="list-style-type: none"> Grant - bio shaker 	
<ul style="list-style-type: none"> Centrifuge 	<ul style="list-style-type: none"> Eppendorf Centrifuge 5804 	<ul style="list-style-type: none"> Eppendorf AG

<ul style="list-style-type: none"> • Mini centrifuge • Balance • Freeze Dryer • Turbovap Evaporator 	<ul style="list-style-type: none"> • Galaxy 14D • METTLER PM4800 DeltaRanna • Christ Alpha 1-4 LSC plus • Turbovap 	<ul style="list-style-type: none"> • VWR INTERNATIONAL • Mewes Wagalheunik • VACUBRAND • Biotage GB
<ul style="list-style-type: none"> • Mass Spectrometer (Q-OF) 	<ul style="list-style-type: none"> • Xevo G2-XS 	<ul style="list-style-type: none"> • Waters Corp., Milford, US
<ul style="list-style-type: none"> • UPLC 	<ul style="list-style-type: none"> • ACQUITY I-class UPLC system (FTN-sample manager, column manager and binary solvent manager) 	<ul style="list-style-type: none"> • Waters Corp., Milford, US

Table 2: Solvents and Substances Used

Substance	Manufacturer
<ul style="list-style-type: none"> • Methanol • Acetonitrile • Milli Q-Water • Formic acid • Pure Sand (SiO₂) • Nitric acid • HCL 37% • H₂O₂ 30% 	<ul style="list-style-type: none"> • Biosolve • Biosolve • Barnsted GenPure water purification system • Biosolve • Th.Geyer GmbH&Co.KG • Chemsolute • Merck KGaA • Merck KGaA

2.3 Protocol

2.3.1 Tire additives by RPLC-HRMS

To evaluate the internal and external concentration of tire related chemicals in mussel samples, the tank water, breathing water, and soft tissue were analysed. The tank water was

sampled together with the mussel samples. Once the breathing water was released by the mussels, the soft tissue was collected afterwards.

2.3.1.1 Sample Preparation

Soft Tissue

1. **Wet weighing:** Weigh and measure the whole mussel shell. Thereafter, remove the soft tissue and weigh it separately.
2. **Freeze-drying:** Insert the soft tissue into an Eppendorf tube (5 ml) and freeze dry it for 48 hours, ensuring that all the sample is dried afterwards.
3. **Ball-milling:** Insert inside the ball milling a suitable number of milling stainless-steel balls (between 10 and 15) according to their size (we adopted a combination between 2- and 3-mm diameter stainless-steel balls). Transfer the content of the Eppendorf tubes into the ball-mill and run it for 2 min at a frequency of 30 s^{-1} . Pour the contents of the stainless ball-milling boxes in a weighing paper and remove the stainless balls (the use of a magnet can be helpful during this procedure).
4. **Dry weighing:** Before pouring the content of the weighing paper into a falcon tube (15 ml), weigh the empty tubes and then weigh them again with the sample inside. The subtraction of both weights allows to obtain the dry weight of the sample.
5. **Sample Extraction:** Insert a defined volume of solvent (methanol) ensuring that all the sample is completely submerged. Place the samples in the ultrasonic bath for a total of 45 min, and vortex the samples at least 2 times in between ensuring that no larger agglomerates are formed.
6. **Centrifugation:** Centrifuge the samples for 10 min at $4193 \times g$. Afterwards, pour the supernatant into a separate vial, and store the falcon containing the precipitate for further analysis. Pipet out a certain volume of extract (e.g., $500 \mu\text{l}$) and place it into Eppendorf tubes (1.5 ml), and centrifuge them again at $7558 \times g$ for 10 min. These two centrifugation steps will ensure the removal of most of the suspended particulate matter from the extracts.
7. **Dilution:** Dilute the obtained extract 1:1 with ultrapure water and place the sample in the LC system for analysis.

Tank and Breathing Water

Filtration: the water samples were defrosted and filtrated by using a regenerate cellulose (RC) syringe filters ($0.45 \mu\text{m}$) into LC glass vials. Afterwards, place the sample in the LC system for analysis.

2.3.1.2 Analysis by UPLC-HRMS

The analysis was performed on an ACQUITY UPLC system coupled to a Xevo G2-XS mass spectrometer equipped with an electrospray ionization source (Waters Corp., Milford, USA). Chromatographic separation was performed using the ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm) with a flow rate of 0.45 mL / min and a column temperature of 45 °C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The gradient was as follows: 0 min 2% B, 12.25 min 99% B, 15.00 min 99% B; 15.10 min. 2% B, 17.00 min 2% B. The volume of injection was 10 μL. Full-scan spectra were collected from m/z 50-1200 in both positive and negative (centroid) modes.

Source conditions were set as follows: temperature 140°C, capillary voltage -0.8 kV, desolvation temperature 600°C, sampling cone voltage 20 V, and source offset 80 V. Nitrogen was used as a cone gas and argon as a collision gas. The flow of the cone gas was 50 L / h. The desolvation gas flow is 1000 L/h. In order to ensure accurate precision mass during the MS analysis, leucine enkephalin was infused as lock spray via the reference probe and a two-point calibration was applied.

Two MS data sets were collected in parallel using low collision energy (6 eV, effectively the accurate mass of the parent ions) and high collision energy (15-40 eV, fragment ions) in order to obtain the greatest extent of structural information on each suspect.

2.3.2 Zinc Content of Mussel Samples by ICP-MS

For this analysis, the stored precipitate samples obtained from the previous RPLC-HRMS sample preparation (at step 6) will be used.

1. **Solvent evaporation:** Perform a nitrogen evaporation on the stored precipitates if the sample looks completely dry.
2. **Freeze-drying:** Freeze-dry the obtained samples for an additional 24 hours to ensure that all the water content is removed.
3. **Dry weighing:** Weigh the dry samples and subtract the weights of the empty falcon tubes previously taken to calculate the amount of sample obtained.
4. **Acid digestion:** perform an acid digestion of the samples by adding a suitable volume of acids (HNO₃ and H₂O₂) according to the weight and placing them inside a microwave oven.

The corresponding parameters are reported in the table below:

Parameter	Condition
• P rate limit	• 0.5
• performance limit (Watt)	• 1500
• pressure limit (Bar)	• 40.0
• IR limit (°C)	• 210
• Inside temperature (°C)	• 240
• Number of vessels	• 16
• Weight of the sample (g)	• 0.300

5. **ICP-MS:** Zn analysis of the digestate was conducted by inductively coupled plasma mass spectrometry (ICP-MS) with the following instrument and operational conditions: Thermo Scientific iCAP Q S, nebulizer: PFA-ST MicroFlow nebulizer, spray chamber: MicroMist quartz cyclonic, RF power: 1548 W, nebulizer gas flow: 0.95 L/min, sample flow rate: 0.395 ml/min, collision gas flow: 5ml/min, cooling gas flow: 14 L/min, auxiliary gas flow: 0.8 L/min) and inductively coupled plasma optical emission spectroscopy (ICP-OES; Spectro ARCOS, nebulizer: cross flow nebulizer, spray chamber: Scott type glass chamber, RF power: 1400 W, nebulizer gas flow: 1.2 L/min, sample flow rate: 2 mL/min, cooling gas flow 14 L/min, auxiliary gas flow: 1.2 L/min).

2.4 Quality Control Measures

2.4.1 Recovery Experiment Performed for UPLC-HRMS

In order to assess the efficiency of the overall sample preparation procedure, a recovery test experiment was performed by using commercial mussels. The recovery test should include a proper number of samples in order to assess the recovery of the extraction procedure (RE), the matrix effect (ME) and the process efficiency (PE) (also known as apparent recovery).

1. **Standard mixture preparation:** Two spike solutions were prepared by mixing the standard compounds in order to obtain the standard mixtures with a concentration of 50 (Spike 1) and 15 ng/ml (Spike 2).
2. **Weighing, freeze-drying and milling:** The commercial mussels' samples were prepared by following the steps from 1 to 4 in Section 2.3.1.1 'Sample Preparation'.

In order to create a unique powder mixture all milled commercial mussels were mixed.

- 3. Recovery test procedure:** In order to test the overall recovery of the sample preparation (including the matrix effect), two different sample weights were selected: 500 and 50 mg. The spike solutions addition and the overall procedure of the experiment is showed in the scheme below:

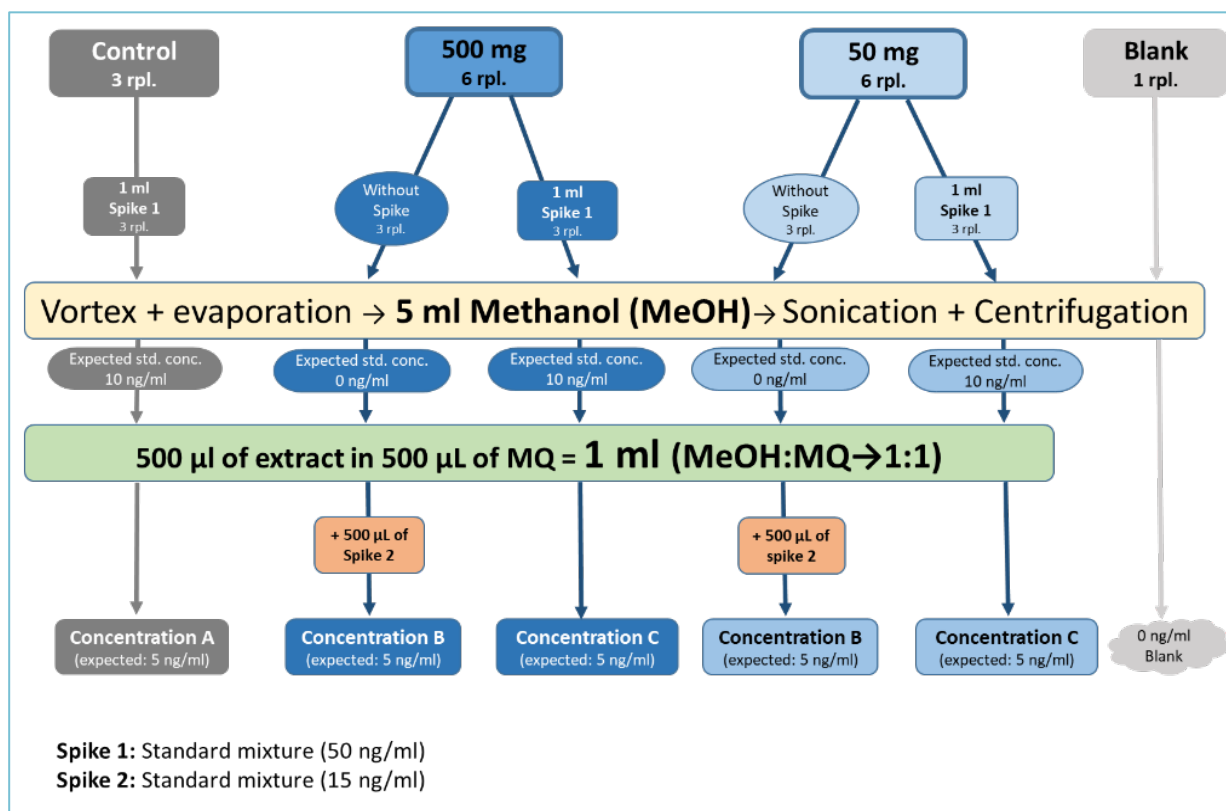


Figure 1: Recovery test experiment performed using commercial mussels, with addition of spike solution

- 4. Spiking of standard mixture before extraction:** spike a standard mixture of selected tire related compounds (known concentration) inside some of the samples.
- 5. Solvent evaporation:** perform a nitrogen evaporation on the samples, in order to remove the solvent of the spiking solution.
- 6. Sample extraction, centrifugation and RPLC-HRMS analysis:** follow the steps from 5 to 7 already explained in Section 2.3.1.1 'Sample Preparation'.
- 7. Recovery percentages calculations:** The related recovery and matrix effects percentages were calculated as reported by Matuszewski *et al.* (2003):

$$\text{ME (\%)} = (\text{Concentration B} / \text{Concentration A}) \times 100$$

$$\text{RE (\%)} = (\text{Concentration C} / \text{Concentration B}) \times 100$$

$$\text{PE (\%)} = (\text{ME} \times \text{RE}) / 100$$

Quality Control

To ensure the accuracy of the sample preparation procedure, two replicates of commercial mussel samples were analysed in parallel after spiking both the standard mixture of selected compounds (Spike 1) and two labelled internal standards, 6-PPDQ-d5 and DPG-d10, with initial concentrations of 2 mg/ml and 1 mg/ml, respectively.

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Matuszewski, B. K., Constanzer, M. L. and Chavez-Eng C. M. (2003). Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS. *Analytical Chemistry* 75, pp.3019-3030. DOI: <https://pubs.acs.org/doi/10.1021/ac020361s>

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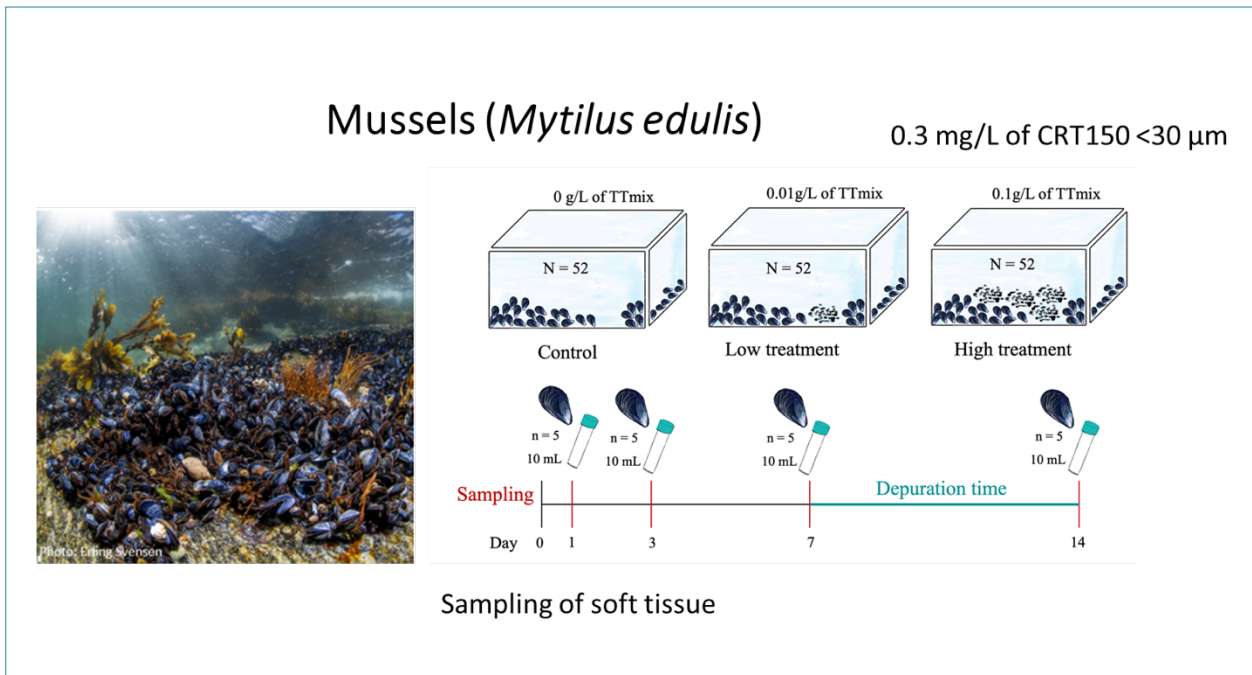
3. Chemical marker identification to study exposure of biota to common plastics

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Deliverable 2.5 Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023.



3.1 Scope of Protocol

Large knowledge gaps remain regarding the distribution and presence of MPs in the marine environment and their interaction with marine organisms. The determination of the occurrence of MPs of especially very small size ranges (<20 µm) in environmental samples as for instance biota is very challenging and time consuming. Chemical tracers of MP specific additives could be a tool to determine the presence of MP. Further, some plastic related additives have been recognized as persistent organic pollutants (UV 328), requiring further monitoring capabilities. The aim of this protocol is to investigate the occurrence and uptake of MP related additives into organisms in the field and under controlled laboratory conditions. To do so, we used blue mussels (*Mytilus edulis*) exposed to MPs. First, blue mussels (n = 60) were experimentally exposed for 7 days to low or high concentrations of a HDPE mix (CRT 150) (0.0003 g/L and 0.003 g/L, of 10-30 µm size range respectively), followed by a depuration period of 7 days. Individual mussels were collected on day 1, 3, 7, and 14 after initial exposure. Due to their presence in a broad variety of plastics, UV 320, 326, 327, 328, 329 were selected for analyses.

3.1.1 Sample Preparation

Frozen mussels are homogenised using a spatula and scalpel and tissue (1 gram) is transferred to glass centrifuge tubes (15 mL). Further, the samples are spiked with internal standard before adding ceramic beads, acetonitrile n-Hexane and Na₂SO₄. Homogenization is performed with a vortex shaker for 15-20 seconds before the samples are transferred to a sonication bath for 15 minutes (temperature: < 30°C). The vortex- and sonication steps are repeated once. Afterwards, the tubes are shaken in a horizontal shaker for 25 minutes, with a turning of the tubes halfway during the process (12.5 minutes), before centrifugation (1500 rpm, 10 minutes). The hexane- and acetonitrile layers are separated and stored in glass vials (2 mL) at -20°C (Galtung, 2023).

3.1.2 Determination of Chemicals

100 µL of hexane layer from extracted samples are transferred to GC/MS vials (0.3 mL) and spiked with recovery standard (13C6 - 6PPD-Quinone). Each vial is vortexed for 5 seconds before they are run on the gas chromatography mass spectroscopy (GC/MS, Orbitrap). The organic chemical extracts are analysed by a Q Exactive GC (Orbitrap GC/MS) at the NILU laboratory.

3.2 Quality Control Measures

To remove any contamination with the target compounds, Na_2SO_4 is burned at 600°C for 8 hours and all glass wear and ceramic beads are rinsed and burned at 450°C for 8 hours and covered with aluminium foil prior to laboratory work to avoid contamination. Ceramic beads are rinsed with tap water and an alkaline rinse before burning. Metal equipment is rinsed according to the standard of the laboratory (NILU), followed by an ultrasonic bath in n-hexane for 10 minutes. The 100 µL glass pipettes used are not burned due to the risk of changing their accuracy by shape changes caused by heat damage. No plastic equipment is used during sample handling.

To ensure extraction of both polar and non-polar organic chemicals from the tissues, solvents such as acetonitrile (non-polar) and n-hexane (polar) are utilized. An internal standard is added to the samples to detect and quantify organic chemicals related to MP, combined with a recovery standard prior to GC/MS analyses, to control potential losses of target compounds during the laboratory procedure.

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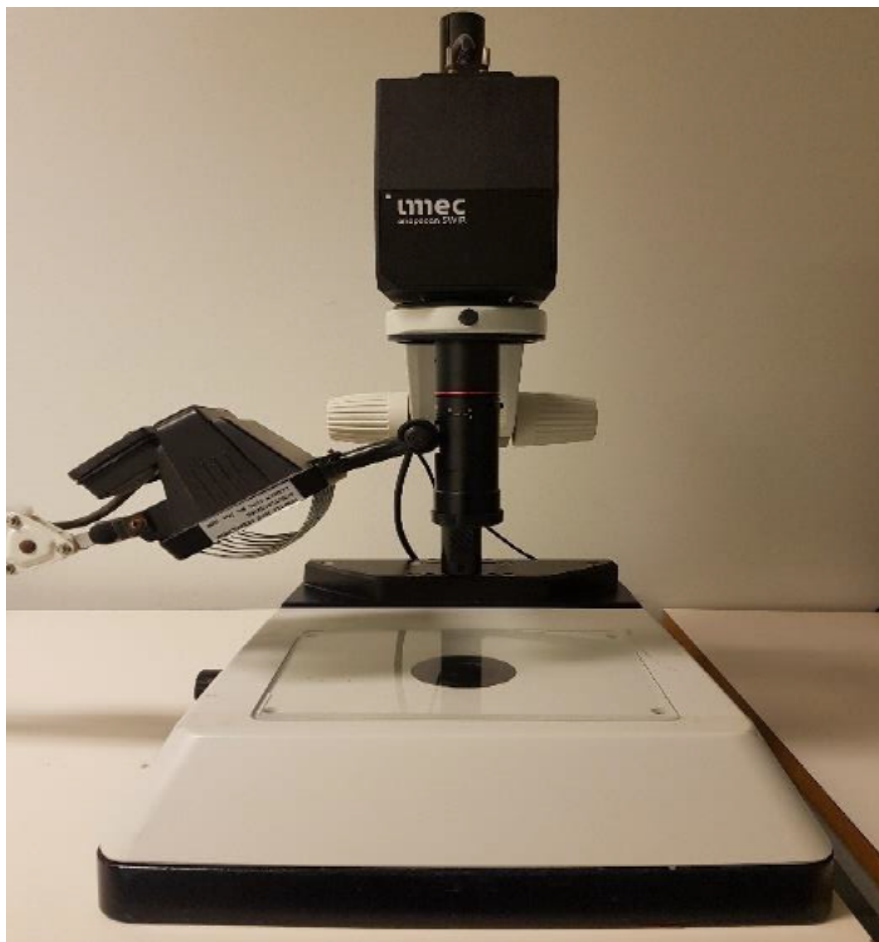
Cover photo credit: Erling Svensen, Akvaplan Niva

4. Short wave infrared spectroscopy using snapscan hyperspectral sensor camera

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Deliverable 2.5 Analysis techniques for quantifying nano-and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023



4.1 Scope of Protocol

There is a large need for cost effective and reliable MP analysis methods for high environmental monitoring where MPs could be distinguished from natural particles and comprehensive enough discrimination of different types of polymers. The focus here is to build up an evaluation platform for a novel snapscan type of hyperspectral sensors covering the short-wave infrared wavelength (1100-1700 nm). The target size range is down to 100 μm routinely and ideally down to 10 μm .

The setup could be either designed in a simple benchtop stand-alone system with lens and illumination and stage control, or more advanced integrated in a microscope setup.

4.2 Materials and Equipment

For both the benchtop and microscope integration setup, images were obtained using the Snap Scan SWIR hyperspectral imaging camera (IMEC, Belgium) with a spectral range of 1100-1700 nm. The spatial resolution is up to 1206 x 640 pixel with a sensor pixel size of 15 μm .

4.2.1 Stand-alone Benchtop Setup

The camera was operated in the "Benchtop Mode" as can be seen in Figure 1. The camera is attached to a stative and placed above the sample. A 2x telecentric lens FTV20C-110SW-S (Myutron, Japan), specifically made for SWIR applications was mounted, where a spatial resolution of the system was obtained to approximately 7 $\mu\text{m}/\text{px}$.

For the illumination a halogen floodlight was used which was placed sideways to the sample as it was recommended in the literature (Zhu *et al.*, 2020). This reduces light to be reflected from the substrate and gives a better contrast and increased signal of the particles of interest, rather than the underlying filter material.

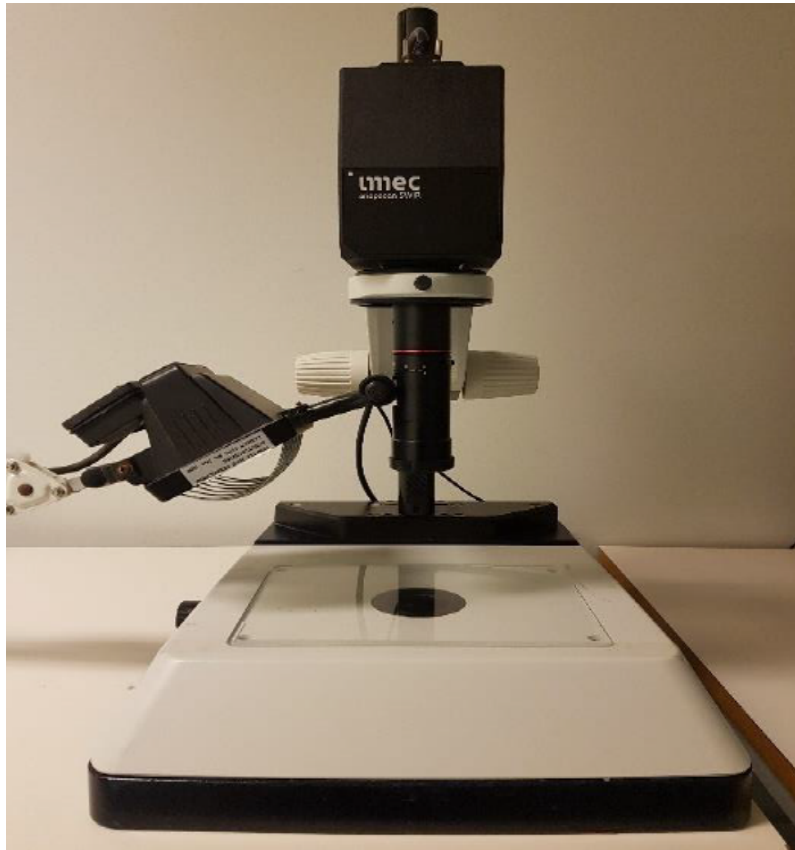


Figure 1: Experimental setup of stand-alone benchtop hyperspectral imaging sensor camera, lens, mounting stage and light source.

4.2.2 HSI Classifier as a Multifunctional Tool for Microplastic Classification

The HSI Classifier is a custom-made python software and was used in this project for data visualization and handling.

The software is available as open source at <https://github.com/Brandt-J/HSI-Evaluator> and offers loading, reviewing, and processing of HSI samples. This software was developed because the original HSI software delivered with the IMEC camera was missing some key features that were necessary for accurate analysis of HSI samples:

- The opportunity to process multiple samples at the same time and arrange these samples freely in a flexible workspace.
- An automatic particle detection based on brightness thresholding and watershed segmentation.
- Per-particle evaluation of spectra results: Particle detection gives clear boundaries of each particle which allows to pre-process all spectra within that boundary as a mini-spectra-batch. For example, spectra within these boundaries

can be binned to increase the signal-to-noise ratio. For the final particle classification, a majority vote on all individual spectra classification results can be cast.

- The connection to a SQL database for uploading and downloading spectral data that can be used for training the classifier.

4.2.3 Machine Learning Classifications

A good training dataset is essential for the machine learning classification. The final database consisted of 10 different polymer types and an additional sediment class (in total 298673 spectra), including both pristine and environmental particles but a bias on pristine.

A good pre-processing is essential to prepare data for further analysis such as classification. In this project, the PLS Toolbox from Eigenvector was used as a pre-sorting tool to find a good pre-processing method so that this method could then be implemented into the HSI Classifier. Therefore, principal component analysis (PCA) was used. PCA is a procedure which can be used to summarize information in a large data set by a smaller set of so-called Principal Components (PCs). With this method, the summarized data can be more easily visualized and analysed.

The investigation of a well working pre-processing method was a trial-and-error procedure, as no final method has been found yet. In addition, the pre-processing method depends on the quality of the data set. The hdr-files from the hyperspectral imaging camera could be loaded into the PLS Toolbox for analysing. Different pre-processing methods were investigated and PCA plots were generated and compared. The pre-processing method of the 1st derivative in combination with Savitzky-Golay smoothing and length normalization was found the most suitable for this project.

4.2.4 The Neural Network Classifier

During the project, a classifier for MP detection was developed (Fumo, 2017; Zhou, 2019; Nduati, 2021). The final classifier consisted of a Neural Network with an input layer of 102 neurons and three hidden layers with 200, 100, and 50 neurons. The reLu function served as the activation function for the hidden layers and a soft-max activation function was used to produce a probability distribution in the output layer which consisted of a number of neurons equivalent to the number of polymer classes.

Furthermore, 50 epochs were used for the final classifier and Synthetic Minority Oversampling Technique (SMOTE) was applied to balance the data. In addition, a particle binning of 10 was applied to the samples.

4.3 Protocol

4.3.1 Calibration

Before data acquisition, the camera was calibrated with a white reference (reflectance over entire spectral range = 1) using a white calibration tile. The dark calibration is done automatically by closing the shutter of the camera.

4.3.2 Acquisition

After calibration, measurements could be done by using the HSI Snap Scan software. The software has two main windows: the acquisition and the cube display. Acquisition is for scanning samples and having a live image of the sample. After the sample is scanned, the cube display provides a more contrasted image of the sample for further evaluations. Then the image (hypercube) can be exported as a hdr-file, which can then be loaded into the written HSI Classifier program, which allows more opportunities for data analysis.

4.3.3 Comparison between the Support Vector Machine and the Neural Network

As the current gold standard seems to be a Support Vector Machine (Pradhan, 2012; Paolo, 2021), this method was also considered here. Later, a Neural Network was implemented into the HSI Classifier allowing a comparison between these two classification models. Both models were trained with the same set ups and increasing number of spectra to investigate the performance but also the duration of the training and validation process. It can be seen that there is no big difference in performance of these two classifiers concerning the accuracy, but it should be noticed that the Support Vector Machine needs more time for training and validation. To have a better overview about this, a comparison between the Support Vector Machine and the Neural Network regarding the time needed for training and validation. Overall, the Neural Network was found more suitable for this purpose.

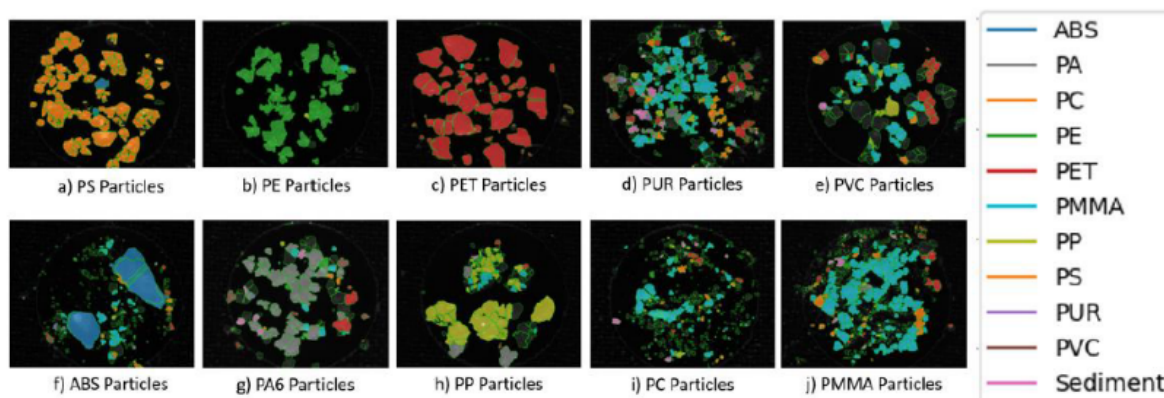


Figure 2: Classification results of pristine particles

4.4 Quality Control Measures

Validation showed that the classification model was able to separate large pristine plastic particles. However, the classifier shows a noticeable drop in accuracy when it comes to weathered particles. Therefore, a good training dataset is important, also for future research. Both, pristine and weathered particles must be included into the training.

The wavelength range needs further investigation. Most applications used a wavelength range of 1000-2500 nm while in this project a wavelength range of only 1100-1700 nm could be used. It has been demonstrated that the range of 1700 to 2500 nm provides important information about different polymers. A direct comparison could be useful to evaluate whether the range above 1700 nm is necessary. If a classification model can be developed excluding the upper wavelength range, the method would be more cost-effective as SWIR cameras are less expensive than NIR cameras.

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Cover photo credit: Josef Brandt

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 (< 10 μm) and NPs (<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 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.

Matrix type	MP size	MP shape	Total duration	Reagents
Seawater	> 20 μm	All shapes	6 h (5 samples)	<ul style="list-style-type: none"> • KOH (10 %) • H₂O₂ (30 %) • NaI (100 %) • Nile red • Acetone

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 , \varnothing 47 mm)
- Glass Pasteur pipette with rubber stop
- Conical centrifuge tubes

- Aluminium foil
- [Whatman glass microfiber filters](#) (2.7 µm, ø 47 mm)

Other laboratory equipment

- [Metal sieve](#) of ø 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, ø 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 (Na₂WO₄·2H₂O - 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 Na₂WO₄·2H₂O is used, PVC particles will not be extracted either.

5.3 Protocol

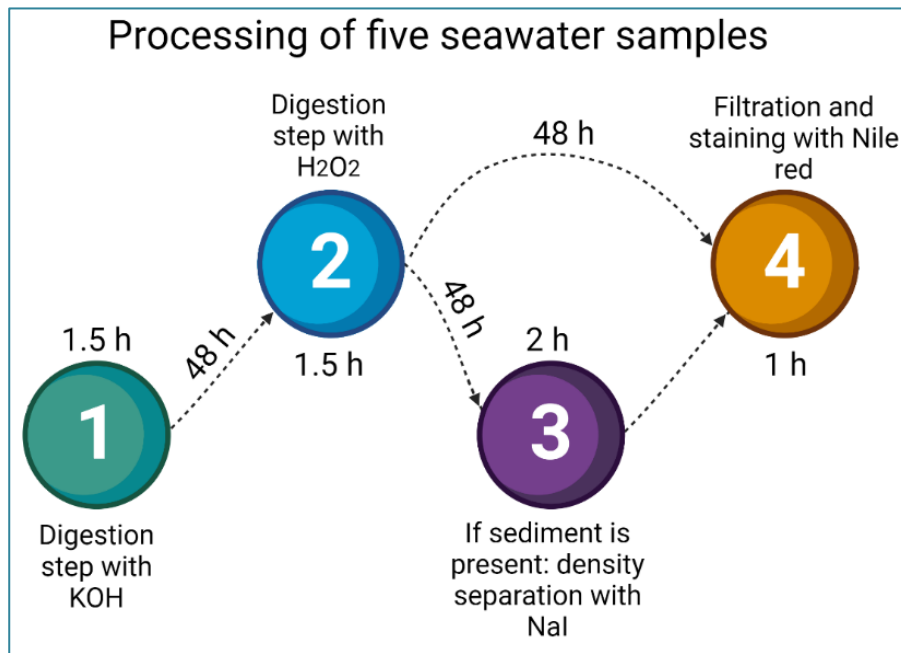


Figure 1: Scheme of all major steps within the protocol, as well as their durations

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 H₂O₂ 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 H₂O₂, (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% H₂O₂ 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 H₂O₂ 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 H₂O₂. 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 , \varnothing 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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Cover photo credit: Mattias Bossaer

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



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 ($< 10 \mu\text{m}$) and NP ($< 0.2 \mu\text{m}$) 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 $> 20 \mu\text{m}$ 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.

Matrix type	MP size	MP density	MP shape	Total duration	Reagents
Sediment	> 20 µm	≤ 1.8g/cm ³	All shapes	6.5 h (5 samples)	<ul style="list-style-type: none"> • H₂O₂ (30 %) • NaI (100 %) • Nile red • Acetone

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, ø 47 mm)
- Glass Pasteur pipette with rubber stop
- Aluminium foil

- Filters to filter NaI, H₂O₂ and Nile red e.g. [Whatman glass microfiber filters](#) (2.7 µm, ø 47 mm)

Other laboratory equipment

- [Metal sieve](#) of ø 5 cm and mesh size 20 µm
- A small metal funnel
- Metal spatula
- Tweezers
- Cotton lab coat and nitrile protection gloves (H₂O₂)
- Milli-Q water

Reagents

- [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 a filtration apparatus, to reduce potential contamination (e.g., Whatman glass microfibre filters - 2.7 µm, ø 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 (Na₂WO₄·2H₂O - 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 Na₂WO₄·2H₂O 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

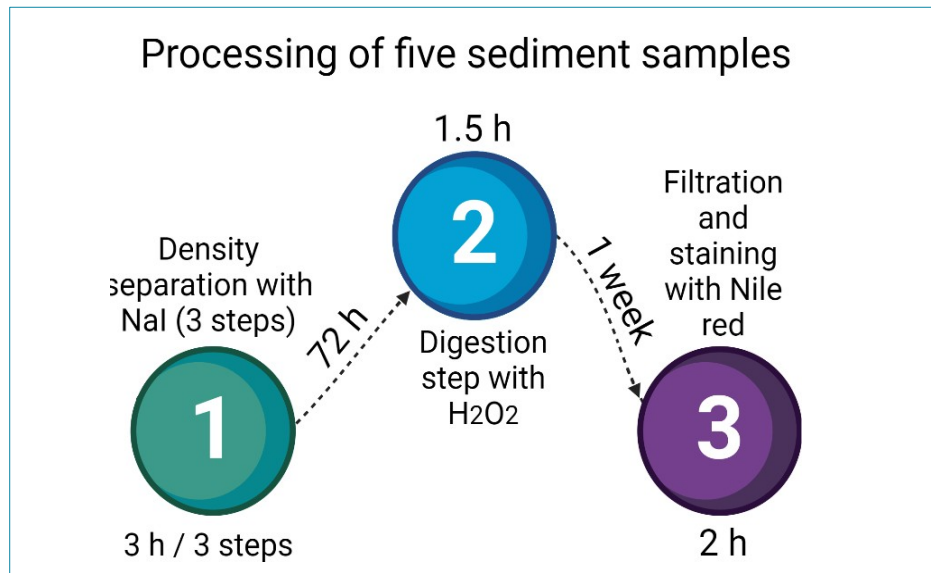


Figure 1: Scheme of all major steps within the protocol, as well as their durations

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 (\varnothing 50 mm, 20 μ m 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 μ m-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 H₂O₂ 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_2O_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_2O_2 (30 %). H_2O_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 $\mu\text{g}/\text{mL}$). 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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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



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).

Matrix type	MP size	MP shape	Working time	Reagents
Biota (mussels and fish GITs)	> 20 µm	All shapes	6-7 h (5 samples)	<ul style="list-style-type: none">• KOH (10 %)• H₂O₂ (30 %)• NaI (100 %)• Nile red• Acetone

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 (ø 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 , \varnothing 47 mm)
- Pasteur pipette with rubber stop
- Conical centrifuge tubes
- Aluminium foil
- [Whatman glass microfibre filters](#) (2.7 μm , \varnothing 47 mm)

Other laboratory equipment

- [Metal sieve](#) of \varnothing 5 cm and mesh size 20 μm .
- Stainless steel filter (20 μm , ring \varnothing 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 - 2.7 μm , \varnothing 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 (Na₂WO₄·2H₂O - 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 Na₂WO₄·2H₂O is used, PVC particles will not be extracted either.

7.3 Protocol

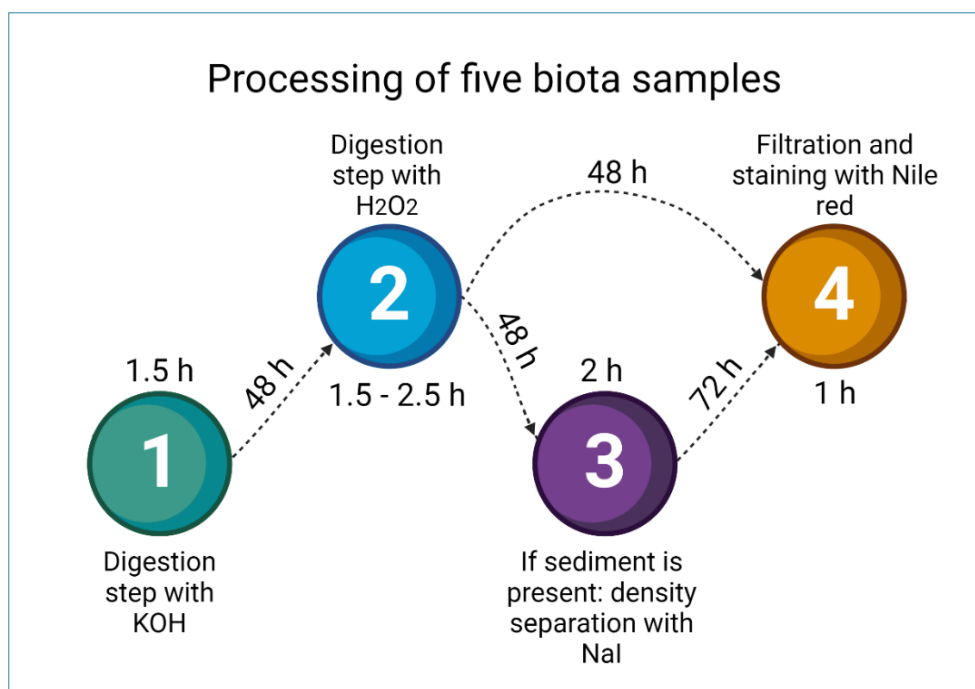


Figure 1: Scheme of all major steps within the protocol, as well as their durations

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_2O_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_2O_2

- A. After rinsing with or without sonication step, add H_2O_2 (30-33%) in a 1:1 ratio to the sample. In this case, add 150 ml of H_2O_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 Nal 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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Cover photo credit: Nelle Meyers

8. Automated microplastic analysis: Nile red staining and random forest modelling

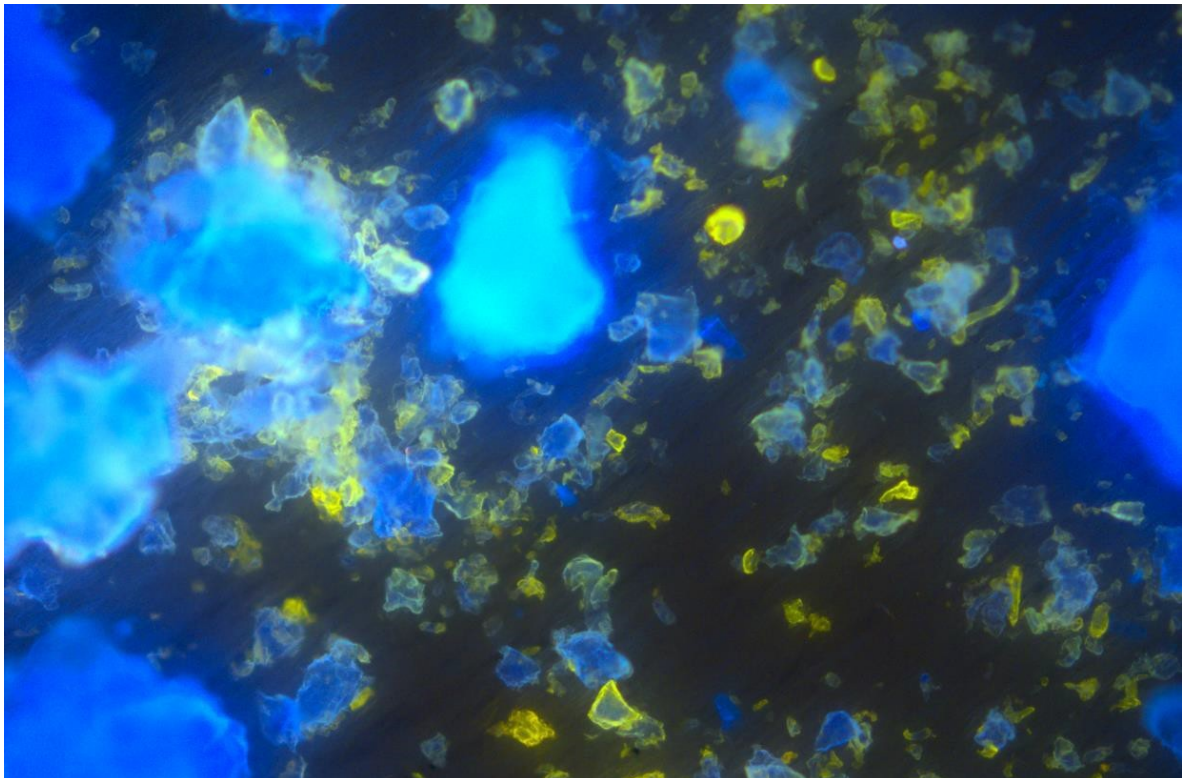
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Deliverable 2.6 Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023



8.1 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, caused by worldwide plastic manufacturing and inadequate waste management. The broad distribution of these global contaminants (2008/56/EC Marine Strategy Framework Directive, Descriptor 10; United Nations Sustainable Development Goal 14 target 14.1.1 [Gigliotti *et al.*, 2019]) has been confirmed in numerous research studies, going from surface waters to the bottom of the ocean and covering all depths, and from Arctic waters to Antarctic waters, including coastal, open and deep sea waters (Zhang, 2017; Pakhomova *et al.*, 2022). Moreover, growing empirical data point to potential adverse effects on organisms after ingestion (Franzellitti *et al.*, 2019). Risk assessment evaluations are needed to comprehend the consequences of MPs on an individual, population, and ecosystem level as well as on human health, and for this, credible MP monitoring data is crucial. Despite recent advancement and diversification of MP analysis techniques, which can provide information on biota exposure to MPs, many approaches are seen as being costly and time-consuming. Furthermore, the multitude of unsolved concerns regarding MP abundance, composition, distribution, fate, and effects in the marine environment highlight the need for more accessible, standardised and reliable analysis techniques.

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 MP (< 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 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 semi-automated, cost-effective MP analysis based on machine learning and RGB colour quantification of Nile red stained fluorescent particles. The developed, optimized (Meyers *et al.*, 2022) random forest (RF) models were tested for MPs down to 3 μm and proved accurate for their detection and the identification of their polymer types. The models demonstrated their ability to analyse pristine MPs in various marine matrices, artificially deep sea- and surface-weathered MPs, and ultimately MPs in real marine environmental samples.

8.2 Materials and Equipment

- A. Computer with sufficient storage capacity or access to a cloud storage service, and the open-source programmes ImageJ Fiji, R and R studio installed:
 - Download ImageJ Fiji (<https://imagej.net/software/fiji/downloads>)
 - Download R or R Studio (<https://cran.rstudio.com>)
- B. ImageJ scripts and R codes to create random forest models:
https://github.com/NelleMeyers/RGB_models
- C. Unprocessed PTFE membrane filter (10 µm, ø 47 mm)
- D. Fluorescence microscope (FM) or fluorescence stereomicroscope (FSM)
 - Fluorescence stereomicroscope: e.g. Leica M205 FA Fluorescence stereomicroscope
 - Software: LAS X software

Table 1: Setting specifications LAS X software for a Leica M205 FA Fluorescence stereomicroscope

Filter	Exposure	Gain	Colour Controls			IL Light (%)	Zoom	Iris Control (100%)
			R	G	B			
• Blue filter - 10447407 Filter set ET GFP LP M205FA/M165FC	8.87 s	1.8	2.4	0	7.9	100	1.00	100%
• Green filter - 10450198 Filter set ET CY3 M205FA/M165FC ET545	78.01ms	1.0	2.4	0	7.9	100	1.00	100%
• UV filter - 10450609 Filter set ET UV LP M205FA/M165FC Climate control	8.87 s	1.8	0	21	100	100	1.00	100%

- E. Fluorescence microscope: e.g. LEICA DM 1000 fluorescence microscope
 - Equipped with a camera and a LED for fluorescence
 - Software: LAS Core software (Leica Application Suite version 4.13.0)

Table 2: Setting specifications LAS Core software for a LEICA DM 1000 fluorescence microscope

Filter	Exposure	Gain	Saturation	Gamma	Hue/ Saturation
• Blue filter: filter System I3 S, BP 450–490 nm	2.91 ms	1.0	1.50	0.6	H0 / S100
• Green filter: filter system N2.1 S, BP 515–560 nm	1.01 ms	1.0	1.50	0.6	Auto Colour
• UV filter: filter System A S, BP) 340–380 nm	26 ms	1.0	1.50	0.6	H70 / S100

8.3 Protocol

For the sake of clarity, all **folders** mentioned in this protocol will be in **green**, all **codes** in **red**, all **images** in **blue**, all **generated Excel files** in **purple** and all **working directories** or lines of code in grey.

8.3.1 Particle Selection

To create the predictive models, two separate datasets are needed: one to train the Plastic Detection Model (PDM) to allow for plastic detection, and one to train the Polymer Identification Model (PIM) to allow for polymer identification of plastics. Before image acquisition and analysis, determine the type of particles you want to incorporate in your dataset, and perform a power analysis to know the required size of your training dataset. The size of the dataset will depend on the required statistical power, which depends on the chosen significance level α ; the effect size; and the number of different categories (plastic/non-plastic for the PDM; different polymer types for the PIM).

To create a PDM dataset, photos of fluorescently dyed organic particles representative of the marine environment should be acquired. For example, chitin, cotton, flax, hemp, silk, wood and wool can be used to do so (Meyers *et al.*, 2022). Furthermore, particle size should be representative of MP sizes observed in the marine environment. To obtain small particle sizes, plastics can e.g., be cryo-milled using a cryogenic grinder.

For the plastic particles within both datasets (PDM and PIM), polymer types commonly observed in marine environmental samples should be selected (Suaria *et al.*, 2020). Moreover, ensure a broad range of densities is covered. For example, seven of the most abundant produced plastic polymers worldwide could be included (Geyer *et al.*, 2017): nylon, polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyurethane (PUR) and polyvinyl chloride (PVC). It is strongly advised during the model training phase to use one polymer type per PTFE filter, to avoid additional μ -FTIR analyses of particles, and to allow for rapid image analysis as well as RGB dataset construction. The best practice to ensure model robustness is to use plastics from different manufacturers, as well as multiple PTFE filters per polymer type, to incorporate potential associated variation in fluorescence colouration.

To determine the dataset size needed to train a RF model, the required significance level α , the effect size d (Cohen, 1988) and the number of categories should be considered.

To create the models, particles should be stained as described in Meyers *et al.*, 2024 (a, b, and c). They can be spiked in and recovered from a matrix (seawater, sediment or biota) using the SOPs, with subsequent staining with the fluorescent dye Nile red, or they can be spiked in Milli-Q water and stained with Nile red directly after without performing a sample processing step. For subsequent analysis, particles should be extracted from marine samples as described in Meyers *et al.*, 2024 (a, b and c).

8.3.2 Image Acquisition

- A. Always take photos of the same particle(s) in the same frame in all the settings, making sure the stage does not move and you do not switch to a different magnification. For setting specifications for a Leica M205 FA fluorescence stereomicroscope and a LEICA DM 1000 fluorescence microscope, see Table 1 and 2, respectively.
- B. Each photo series of a particle/number of particles consists of three photos: a photo acquired under a blue filter, under a green filter and under a UV filter. If possible, a bright field photo (no filter) should be acquired as well. For further photo acquisition details, see Section 8.2 'Materials and equipment'.
- C. All photos should be saved in "TIFF" format. Store them under the 'Photos' folder. If taken, bright field photos should be stored in the sample folder (and not under 'Photos'). Generated CSV files will be saved under the 'RGB' folder after photo analysis in ImageJ.

- D. Using the LAS software to acquire photos, label each photo with a unique code. For each photographed filter area, a series of three images is required, taken under the blue, green and UV filter. Generated CSV files of each photo will be saved under 'RGB' after photo analysis in ImageJ. For the code to run correctly, Photos should be named as followed, in capital letters:

'name of the filter' + '_' + 'number' + '_' + 'colour'
For example: *'PET_1_BLUE'; 'PET_1_GREEN', and 'PET_1_UV'*

In the following example, the construction of an RGB dataset to create a PDM is described, using the script where batch mode is not used. To create a dataset for a PIM, the same procedure should be followed, with the exception that photographs of different plastic polymer particles will be analysed, rather than plastic and organic particles.

8.3.3 Creating Machine Learning Models

8.3.3.1 File Organisation

Create a folder called *'Automated_MP_analysis'*, and four subfolders called *'Coding'*, *'Model_constructions'*, *'Training_datasets'*, and *'Model_visualisations'*. Save the ImageJ code (*'RGB_extraction_batchmode'* or *'RGB_extraction_nobatchmode'*) and the two R scripts (*'RGB_dataset'* and *'PDM_PIM'*) under *'Coding'*. Next, classify your sample images:

1. Batch mode (FM - multiple photos/PTFE filter)
Create three subfolders within *'Model_constructions'*: *'Photos'*, *'Metadata'* and *'RGB'*. Store all images (blue, green, uv) within *'Photos'*.
2. No batch mode (FSM - 1 photo/PTFE filter)
Create subfolders per photographed PTFE filter within *'Model_constructions'*. Within these filter-specific folders, each time create three distinct folders: *'Photos'*, *'Metadata'* and *'RGB'*. Store all filter-specific images (blue, green, UV) within *'Photos'*.

8.3.3.2 ImageJ

- Open ImageJ Fiji.
- Open a new macro through 'Plugins' > 'New' > 'Macro'.
- Paste the ImageJ script *'RGB_extraction_batchmode'* or *'RGB_extraction_nobatchmode'* in the new window

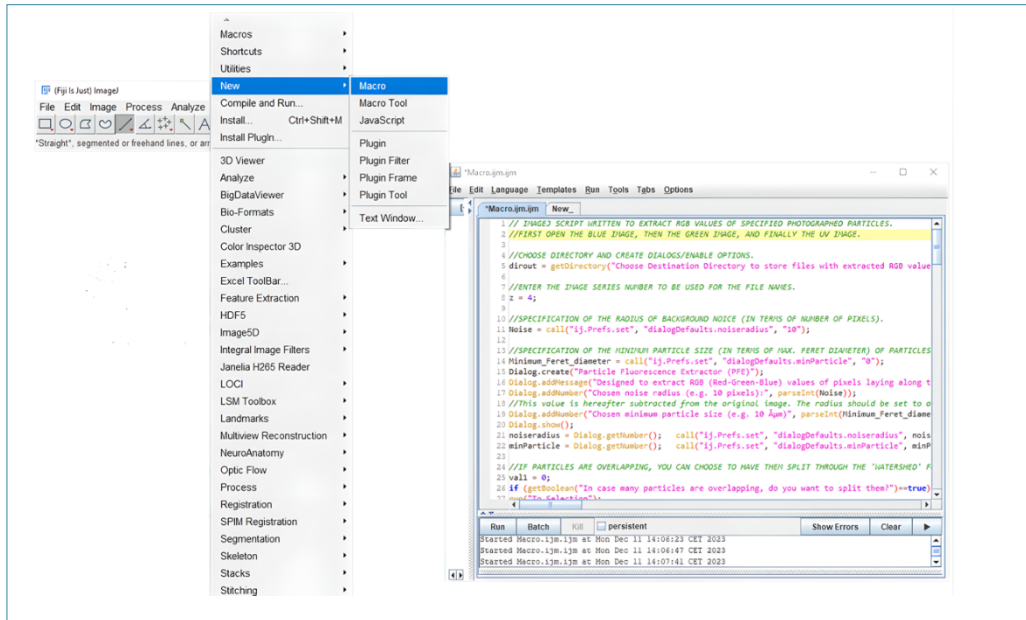


Figure 1: Open script in imageJ

Setting the Correct Scale

- A. Start by setting the correct scale. To do so, use a bright field photo of an unprocessed PTFE filter, acquired with the exact same microscope specifications used for the bright field photo of the actual sample. Open the photo ('File' > 'Open') and use the line-selection tool to draw the diameter of the PTFE filter (see Figure 2). Next, set the scale ('Analyse' > 'Set scale'), enter the known length of the filter diameter under 'Known distance' in μm (47000), enter ' μm ' as unit of length, and make sure 'Global' is checked so that the same scaling factor is used for all photos while the program is opened. Once scaled, it is best to note down the corresponding number of pixels per μm , in case rescaling would be necessary in the future. Once this is done, press 'OK', and close the photo.

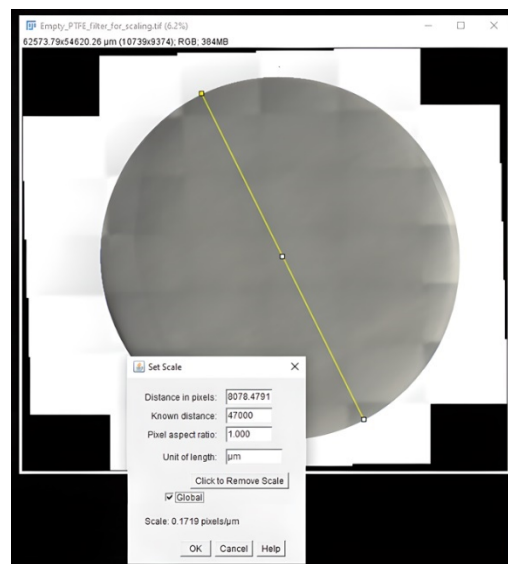


Figure 2: Setting the correct scale

B. Extraction of RGB value

Open the blue filter photo ('File' > 'Open') in ImageJ followed by the green filter and lastly the UV filter (in this specific order)(see Figure 3). Run the macro 'Run' > 'Run'. Uncheck the 'Disable Global calibration' box (and check 'Disable these Messages') to make sure the scaling from the unprocessed PTFE filter is used to scale the photos. Select the 'RGB' folder you just created as a destination directory to store the CSV files with extracted RGB values (dialog box).

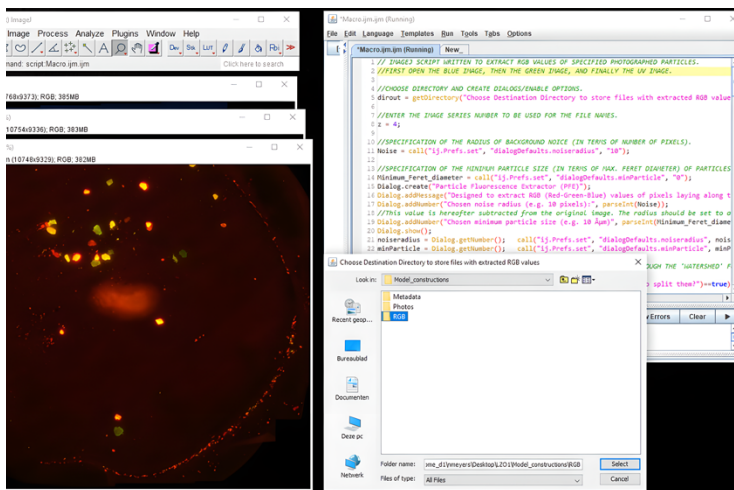


Figure 3: Open the three pictures (blue, green and UV filter) in imageJ, and run the macro

C. A new dialog box pops up. Specify the noise radius the program should use (i.e. from what number of pixels onward a selected area should be considered a particle rather than noise) (see Figure 4) Next, specify the minimum size of the recognised particles for which the RGB values of the pixels on the maximum Feret diameter should be extracted. Enter for example '10' in both fields, then press 'OK'.

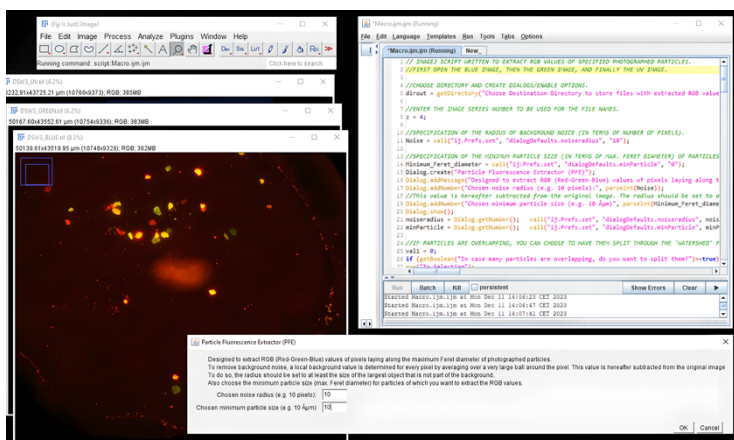


Figure 4: Specify the noise radius and min. particle size for which RGB values should be extracted

D. For the next step, an option is for the program to recognize overlapping particles and split them into separate particles.

Note: If the exact number of particles present is important and the number of overlapping particles is high, it is advised to choose 'no', as unique particles are sometimes mistakenly split into multiple particles.

E. The program asks for the scale to be set. The scale was already specified prior to this step, but to verify the accuracy, the diameter of the filter can be drawn using the line-selection tool (e.g. on the UV photo as borders are clearly visible).

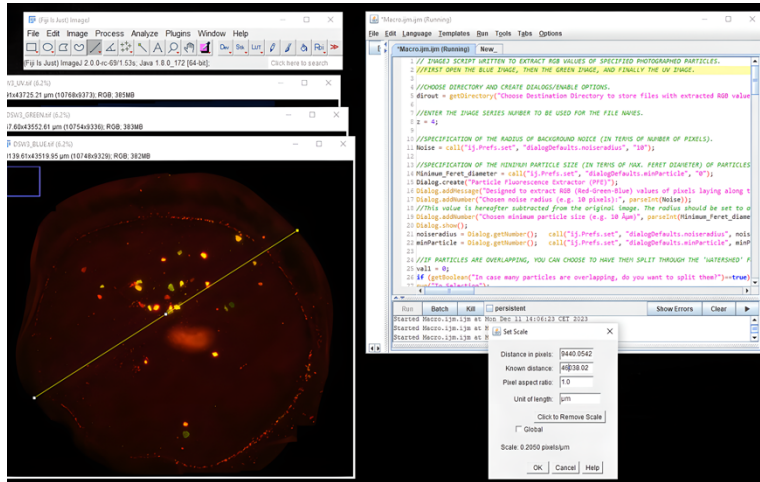


Figure 5: Test whether the scale has been set

Next, press 'OK'. The length shown under 'known distance' should now approximate the diameter of the reference PTFE filter, which is 47000 μm . As circularity of the current filters is not a 100%, the value will deviate slightly from the set value. Don't make any changes, but make sure the 'Global' box is still checked (see Figure 5).

F. The blue filter photo will be processed (the first opened photo). First, a threshold needs to be set to identify all particles present (see Figure 6). To do so, move the upper slider bar until all particles are red. Make sure this specific window is selected, otherwise no changes will be observed when adjusting the slider bar. Next, press 'Apply'. Now, all selected red particles turn white. Following this, press 'Ok'. It is important to respect the order of pressing 'Apply' first followed by 'ok', and not the other way around.

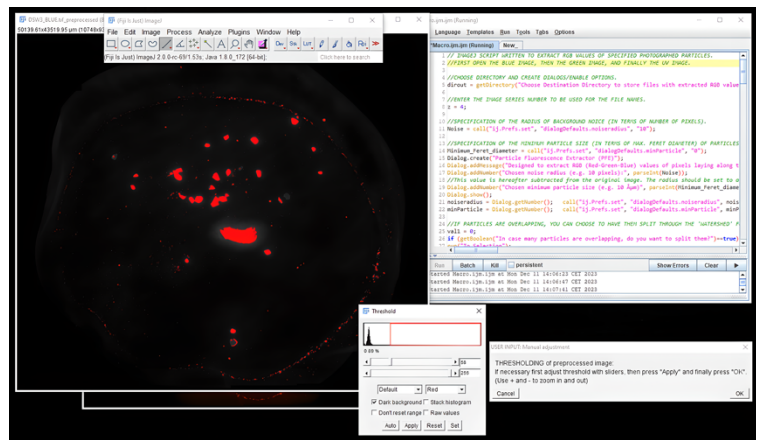


Figure 6: Set the correct threshold

G. Subsequently, all particles will be selected in yellow on the original photos. The progress here can be observed in the menu bar. 'Do you want to move the ROIs (Regions of Interest) for the next picture?' pops up, choose 'yes' for this question (see Figure 7).

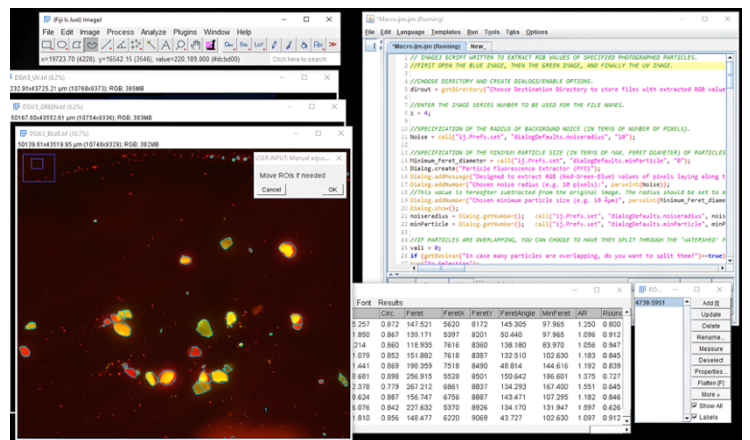


Figure 7: The ROIs are now visible on the original photos

- H. Once the program is ready, all particles will be selected in blue. Select the line selection tool if not selected, put the cursor on a particle, click right and keep it pressed down to move the whole particle selection until the particle outlines match with the particles (see Figure 8).

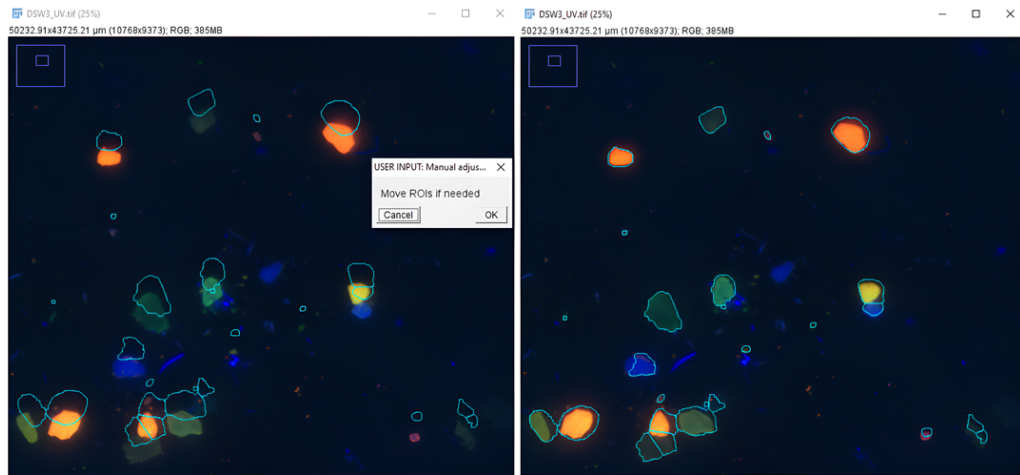


Figure 8: Move to ROIs to the correct position if necessary

It is advised to zoom in while doing so. Next, press 'Ok'. Following this, RGB values files will be generated for all particles on the blue filter.

Note: If the selection would turn yellow, it can no longer be moved. If this happens, go to the 'ROI manager' window and click on the number, which represent the selection. Following this, the selection should turn blue again. Next, choose 'Yes' when 'Do you want to move the ROIs for the next picture?' pops up again, this time for the green filter photo. Move the selection if needed, so that all particles are correctly outlined, then press 'Ok' for 'Move ROIs if needed'. Repeat the same process for the UV filter.

When using the batch mode script, the next series of three photos of a sample (photographed under a blue, green and UV filter) will open, after which the threshold process and extraction of RGB values will be repeated. This process will be repeated until all photos stored in that folder are processed.

8.3.3.4 RStudio

Calculation of RGB-Statistics

- A. Go to the folder 'RGB' of a specific sample a check whether files have been generated.
- o '*Numbered_particles_1.tif*', '*numbered_particles_2.tif*', and '*numbered_particles_3.tif*' are the blue, green and UV filter photos with numbered particles (see Figures 9a, b and c),

respectively. *'PET_4_numbered_particles.tif'* is black-white outline of all analyzed particles, here particles are also numbered (see Figure 9d). When opening the metadata file *'particle_measurements_4_details.xml'*, In column 'A' the particles numbers can be found which correspond with the numbered particles in the *'PET_4_numbered_particles.tif'* photo. Notice however that this numbering does not correspond with the numbering on the *'numbered_particles'* photos. In column 'E', the size (maximum Feret diameter) of the corresponding particles can be found. More particle characteristics can be found in other columns.

- For every particle on the three *'numbered_particles'* photos, a CSV file per colour filter can be found: e.g. for particle **1**, a file *'PET_BLUE_p1.tif'*, *'PET_GREEN_p1.tif'* and *'PET_UV_p1.tif'* can be found.
- These files contain RGB data for each pixel located on the maximum Feret diameter of a selected particle. In the file, each row presents a pixel, where 'X,Y, Red, Green, Blue' stand for the x-coordinate, the y-coordinate as well as the red (R), green (G) and blue (B) value (all ranging between 0-255) of that pixel, respectively.

Note: For the script with batch mode, all numbered colour filters photos as well as all CSV files can be found in the same RGB folder.

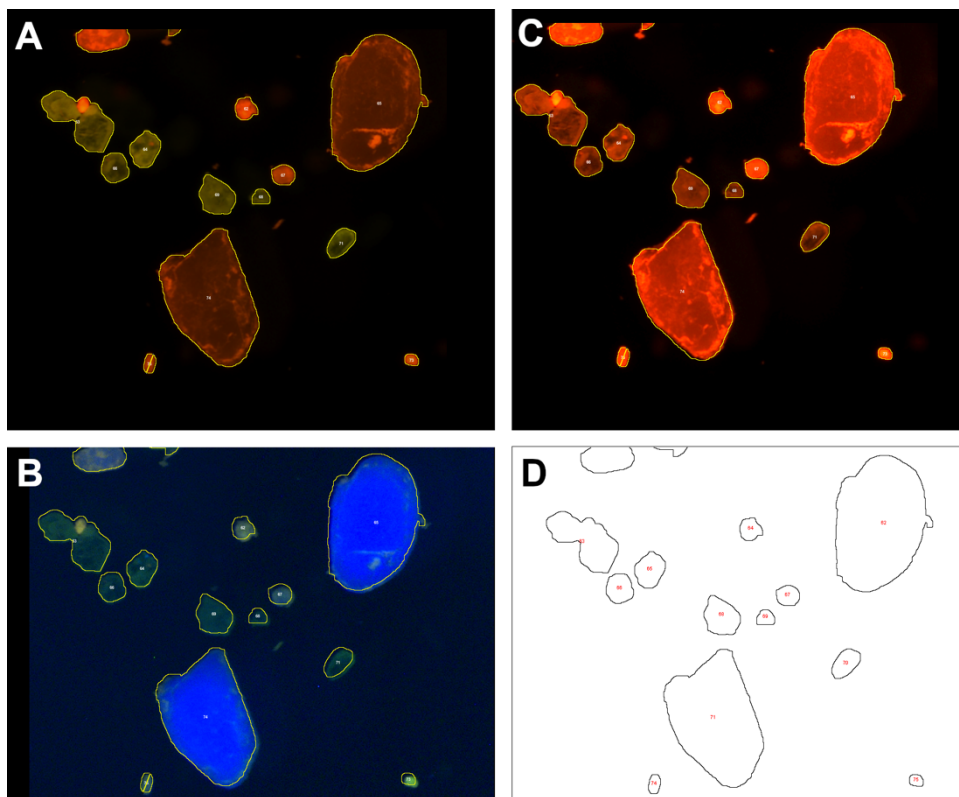


Figure 9: LRTB: 'numbered_particles' for the blue (a), green (b) and (c) UV filter, with all particles numbered, as well as a (d) black-white outline.

B. Open the 'RGB_dataset' script in R. In this step, the mean, median, 10th and 90 percentiles of the RGB value of all analysed pixels belonging to a particle (= per CSV file) will be calculated in R. As a first step, create three new folders within your RGB folder: 'BLUE', 'GREEN' and 'UV'. Next, copy all '_BLUE'-CSV files into the BLUE folder, all '_GREEN'-CSV files into the GREEN folder, and all '_UV'-CSV files into the UV folder.

C. Once the R script is opened, adapt the working directory to the directory where the 'BLUE'-CSV files are stored:

```
setwd("../Automated_MP_analysis/Model_constructions/PET/RGB/BLUE")
```

- Change the working directory again for the Green CSV files:

```
setwd("../Model_constructions /PET/RGB/GREEN")
```

- and do this once more for the UV CSV files further on in the script:

```
setwd("../Model_constructions /PET/RGB/UV")
```

- Next, change the working directory to the location where the UV CSV files are stored after 'Export': `Export<-setwd("../Model_constructions /PET/RGB/UV")`

- Lastly, choose a name for the generated dataset, e.g. 'PDM_RGBdataset':

```
Filename<- paste(Date,"_PDM_RGBdataset.xlsx",sep= "")
```

→ After this, run the whole script. A dataset containing all particle statistics will be generated in the UV folder.

D. Open the generated dataset and delete the first row 'Fulldataset_bluefilter'. Following this, select all statistics (column D until AM, row 3 until the end of the dataset), right click on the selected data and select 'Convert to number'. Lastly, adapt all cells so that the values have 0 decimals. Right now, the dataset with RGB statistics of photographed particles is ready. Each line in the Excel file now contains RGB statistics of an analyzed particle which can be traced back on the analysed photos through its number. As a last step, save the dataset with RGB statistics (e.g. as 'PDM_RGBdataset.xlsx') under the folder 'training datasets'.

E. If desired, a RGB dataset for a PIM can be constructed as a next step, containing RGB statistics of different plastic polymers.

8.3.3.5 ML Model Construction

A. Open the code 'PDM_PIM' in R.

o Adapt the working directory to where the newly generated datasets are stored:

```
setwd("../Model_constructions /Training_datasets")
```

- o Choose a name for the decision tree plot of the generated RF model, e.g.
`'PDM_setseed7': jpeg(file = "Model_visualisations/PDM_setseed7.jpg")`

B. Run the script under `'#Generate_model'`. A random forest model will be generated following randomization of the training dataset. A total of 1000 bootstrap decision trees will be generated (`ntree = 1000`) (see Figure 10), where all 36 variables are randomly sampled as candidates at each split (`mtry = 36`). The confusion matrix, a performance measurement technique, associated with the RF model is also generated, as well as the out-of- bag (OOB) error rate (%), which is a measure for prediction error. In the folder `'Model_visualisations'`, a JPG-file is generated with a plotted decision tree representative of the random forest output. However, be aware that these models are visually complex and often uninterpretable. To generate a more readable friendly model for the purpose of visualisation, a depth parameter can be specified.

```
tree.RGB <- randomForest(  
  Material ~ .,  
  data = RGB_dataset[, -1],  
  ntree=1000,  
  mtry = 36,  
  replace=F,  
  importance = T,  
  keep.forest=T,  
  keep.inbag=T,  
  do.trace=50  
)  
  
print(tree.RGB)  
  
jpeg(file = "PDM_visualisation/PDM_setseed7.jpg")  
plot.getTree(tree.RGB)  
dev.off()
```

Ntree = 1000 → Number of bootstrap replicates (= number of trees to grow). The performance of a RF model will plateau beyond a certain number of trees. For more information, run the script under `'#Model information'`.

Mtry = 36 → Number of variables randomly sampled as candidates for splitting at each node.

Figure 10: RF model specifications in the script

- C.** A 5-fold cross-validation can be performed to validate the performance of the generated model under `'#K-fold cross validation'`.
- D.** To determine the required number of bootstrap trees for accurate results (= as a function of model prediction error), as well as the required minimum training panel size and the required minimum number of parameters, run script under `'#Model information'`.

8.3.4. Microplastic Analysis using Generated Models

To analyse particles of unknown origin within a sample, a RGB dataset of the particles is needed. To create this new dataset, follow the same steps as done to build the model training dataset(s).

- A. First, create a new subfolder within *'Automated_MP_analysis'* called *'MP analyses'*. Create subfolders in the same way as done for the model construction (*'Photos'*, *'Metadata'*, and *'RGB'*). Use PTFE filter-specific folders if batch mode is not used.
- B. Create a RGB dataset in the same manner as done before, this time of unknown particles photographed after sample extraction and staining with Nile red. In the dataset, replace the content in the column *'Material'* by *'Unknown'*. Give the dataset an appropriate name, e.g. *'sample1_RGBdataset.xlsx'*.
- C. Open the code *'PDM_PIM'* in R.
 - Under *#Generate model'*, make sure the first working directory is again set to where the model training datasets are stored: `setwd("../Model_constructions/Training_datasets")`, Create a read_excel()-function for this dataset: `RGB_dataset <- read_excel("PDM_RGBdataset.xlsx")`.
 - Under *#Model prediction*, adapt the working directory to the directory where the RGB dataset created from the analysed sample is stored, e.g. `setwd("../Analyses/sample1/RGB/UV")`. Create another read_excel()- function for the generated dataset of unknown particles:
`Unknown_dataset <- read_excel("sample1_RGBdataset.xlsx")`.
 - Chose a name for the Excel file with particle predictions which will be generated when running this code. It is advised to use the name of the dataset, and to add *'_MP_predictions'*, followed by the model used to perform the predictions, e.g. `"sample1_RGBdataset_MP_predictions_PDM'.xlsx"`
`Filename<- paste(Date," sample1_RGBdataset_MP_predictions_PDM'.xlsx.",sep=""`
`"")`
- D. Run the complete code. Following this, an Excel file will be generated with model predictions on the plastic or non- plastic identity of the unknown particles, in case the PDM dataset is being used. If the PIM dataset is used, predictions of polymer types will be generated for these particles (see Figure 11).

Particle_number	Material	Predicted Material	Particle_number	Material	Predicted Material
1	UNKNOWN	PLASTIC	1	UNKNOWN	PET
2	UNKNOWN	PLASTIC	2	UNKNOWN	PET
3	UNKNOWN	PLASTIC	3	UNKNOWN	PVC
4	UNKNOWN	ORGANIC	9	UNKNOWN	PS
5	UNKNOWN	ORGANIC			
6	UNKNOWN	ORGANIC			
7	UNKNOWN	ORGANIC			
8	UNKNOWN	ORGANIC			
9	UNKNOWN	PLASTIC			
10	UNKNOWN	ORGANIC			
11	UNKNOWN	ORGANIC			
12	UNKNOWN	ORGANIC			

Figure 11: Particle identity predictions from PDM (left) and PIM (right) of an unknown sample dataset.

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Cover photo credit: Nelle Meyers

9. Photo-oxidation of Microplastic Materials in Water

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Deliverable 2.5 Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment, as part of the ANDROMEDA project, 2023



9.1 Scope of Protocol

This protocol details an experimental set-up for studying environmental photodegradation of MP materials in water using a solar simulator. The protocol is applicable to all aqueous matrices and to particles in size ranges that can be captured on available filters. Due to the exposure vessel depth limitations, only surface degradation can be investigated.

9.2 Materials and Equipment

Equipment	Name	Manufacturer
<ul style="list-style-type: none">Exposure Chamber	<ul style="list-style-type: none">Atlas Suntest CPS+	<ul style="list-style-type: none">Atlas Material Testing Solutions
<ul style="list-style-type: none">Light Source	<ul style="list-style-type: none">Xenon lamp (1500 kW)	<ul style="list-style-type: none">Atlas Material Testing Solutions
<ul style="list-style-type: none">Exposure VesselsClimate control	<ul style="list-style-type: none">Quartz glass test tubesCooled and ventilated Room or Chamber, or the SunCooler add-on	
<ul style="list-style-type: none">Sterile Filters		
<ul style="list-style-type: none">Exposure Media	<ul style="list-style-type: none">Seawater/synthetic freshwater/other aqueous media	
<ul style="list-style-type: none">Temperature Probe		
<ul style="list-style-type: none">MP Filter (glass or stainless steel)		
<ul style="list-style-type: none">Ultrapure water		

9.3 Protocol

At SINTEF Ocean, UV exposure is performed using an Atlas Suntest CPS+ exposure chamber fitted with a Xenon lamp and a natural sunlight filter. Exposure vessels are

cylindrical test tubes made of Quartz glass (30-50 mL), capped with fitted glass corks. Other systems may be used, but care must be taken to account for comparability. A SunCooler or a climate room/chamber with appropriate ventilation may be necessary to achieve environmentally realistic exposure temperatures.

1. Prepare and sterile filter (0.22 μm) the exposure media (e.g., seawater, synthetic freshwater, or other aqueous media)
2. Accurately weigh the material that is to be exposed and place in Quartz tubes. Top the tubes up with sterile media leaving 10% headspace of air and cap them tightly to avoid leaks.
3. Place the tubes horizontally in the exposure chamber and start exposure using desired settings.
4. Add one tube with just water and a temperature probe to record temperature during the exposure period.
5. On several occasions during exposure (daily or weekly, depending on total experiment duration), gently turn the tubes and switch places in the chamber to ensure homogenous exposure. To avoid oxygen depletion, it is recommended to open the tubes to allow aeration of the samples.
6. Depending on the purpose, samples may either be sub-sampled during exposure or sacrificed at the end of exposure.
7. Upon sampling, filter the entire sample using an appropriate size filter (e.g., glass filter or stainless steel, 1-5 μm or other pore size depending on material size and desire to collect fragmented MPs) to separate degraded MPs and media. Rinse collected fibers using ultrapure water and preserve according to the analysis that will be performed.
8. Depending on the experimental intensity and exposure period, use the below equations to calculate simulated environmental exposure times.

9.3.1 Calculation of the Total UV Irradiance and Simulated Number of Exposure Days

These equations are sourced from Gewert *et al.* (2018) and applied like in Sait *et al.* (2020). The total irradiance for the exposure periods is calculated using the experimental intensity (W/m^2) and hours of exposure:

$$\text{Total irradiance exposed} = \text{Intensity } [\text{W}/\text{m}^2] \times \text{hours of exposure}$$

The simulated number of exposure days under mean European UV irradiance is calculated using a European mean irradiance $\approx 1200 \text{ kWh}/(\text{m}^2\cdot\text{year})$, 5% of which is considered UV light giving a mean UV irradiance of $60 \text{ kWh}/(\text{m}^2\cdot\text{year})$.

$$\text{Simulated days} = \frac{\text{Total irradiance exposed}}{\text{Mean European UV irradiance}} \times 365$$

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