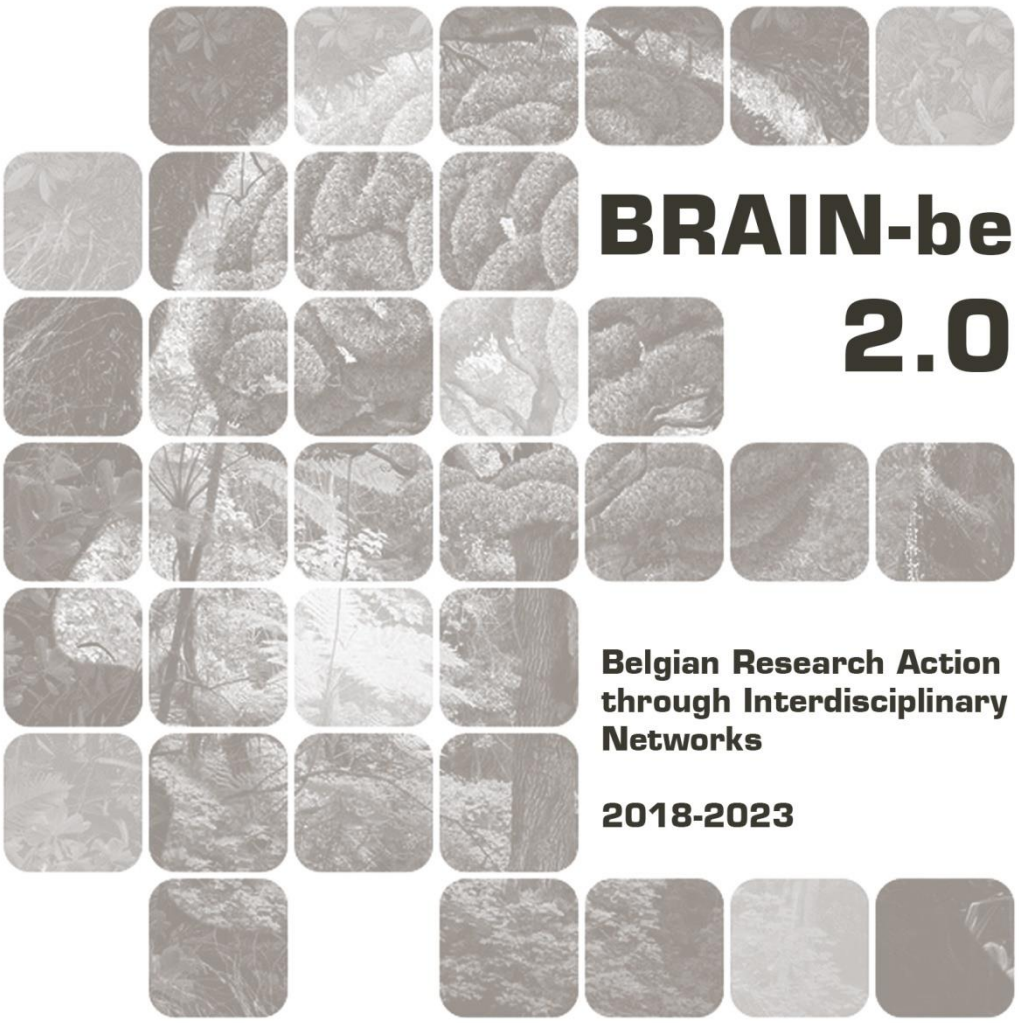
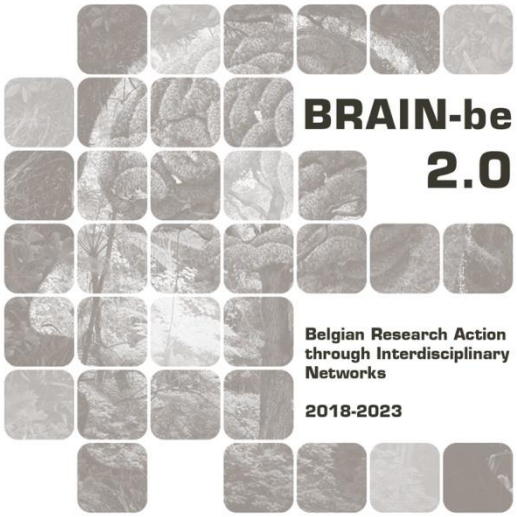


Andromeda

Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment

NELLE MEYERS (ILVO/VLIZ) – BAVO DE WITTE (ILVO) – GERT EVERAERT (VLIZ)

Contribution of ILVO and VLIZ to the JPI OCEANS project ANDROMEDA



NETWORK PROJECT

Andromeda

Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment

Contract – B2/20E/P1/ANDROMEDA

FINAL REPORT

PROMOTORS: BAVO DE WITTE (ILVO)
GERT EVERAERT (VLIZ)

AUTHORS: NELLE MEYERS (ILVO/VLIZ)
BAVO DE WITTE (ILVO)
GERT EVERAERT (VLIZ)

VLIZ pioneer in marine science ILVO ANDROMEDA belspo .be

Published in 2023 by the Belgian Science Policy Office
WTCIII
Simon Bolivarlaan 30 bus 7
Boulevard Simon Bolivar 30 bte 7
B-1000 Brussels
Belgium
Tel: +32 (0)2 238 34 11
http://www.belspo.be
http://www.belspo.be/brain-be

Contact person: Koen Lefever
Tel: +32 (0)2 238 35 51

Meyer, N.; De Witte, B.; Everaert, G. ***Analysis techniques for quantifying nano- and microplastic particles and their degradation in the marine environment.*** Final Report. Brussels : Belgian Science Policy Office 2023 – 61 p. (BRAIN-be 2.0 - (Belgian Research Action through Interdisciplinary Networks))

TABLE OF CONTENTS

PROJECT CONSORTIUM

The Andromeda project is a JPI OCEANS project coordinated by the Université d'Aix-Marseille (MIO, France). The project started officially on the 1st of September 2020 and will end on the 31st of August 2023, with a final meeting in September 2023. The Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) and the Flanders Marine Institute (VLIZ) are the Belgian project partners. Other project partners are: Institut français de recherche pour l'exploitation de la mer (IFREMER, France), Sintef Ocean AS (Norway), Norwegian Institute for Air Research (NIVA, Norway), University of Malta (Malta), University of Gothenburg (Sweden), Helmholtz-Centre for Environmental Research (Germany), University College Cork (Ireland), Instituto Espanol de Oceanografia (Spain), Tallin University of Technology (Estonia), McGill University (Canada), Wageningen University (The Netherlands) and Merinov (Canada).

This project report describes the input of the Belgian project partners to the Andromeda project.

1. INTRODUCTION

Over the past decades, there have been significant developments in the identification and characterization of microplastics (MPs) in marine environmental matrices, including complex matrices such as sediments and biota. These approaches range from simple light microscopy and particle-by-particle spectroscopy to imaging spectroscopy and various statistical algorithms and image analysis tool packages. However, these methods exhibit large variations in their level of comprehensiveness, applied detection modes, pre-treatments, and measurable particle size ranges. Within the Andromeda project, a clear need for validation of laboratory reproducibility and for harmonization (comparability) between methods was identified. Next, the need for a diverse range of MP analysis methods was identified, in order to fulfil a variability of monitoring and research needs. Whereas spectroscopic techniques such as µFTIR or µRaman spectroscopy have become state of the art for identification and quantification of MPs, these techniques are typically expensive and time consuming. When the aim is to identify MPs within large scale research campaigns, monitoring, citizen science or education, there is a need for cost-effective methods, using either in situ measurements or fast laboratory methods for analysis of MPs >50, >100 or >300 µm, depending on the applied methodology. The development and optimization of cost-effective methods is nevertheless challenging, as these techniques should combine savings in time and cost with an acceptable level of accuracy and precision, the detectability of a large variety of polymers, a low risk for contamination and a low risk for mismatching other types of microparticles.

Current microspectroscopic approaches are also not without limitations and uncertainties and thus require further improvement. For example, in imaging FTIR false positives are common for very small particles (<20 µm), where noise in a few pixels can result in incorrect interpretation. Additionally, microspectroscopic approaches are not effective for the analysis of all types of MP pollution. Examples of challenging forms of MP include microfibers from textiles and ropes, tire wear particles (TWPs) and paint flakes from multiple maritime and land sources. Accurate quantification of microfibers is hampered due to background contamination, e.g. from clothing, and due to challenges in accurate identification by µFTIR. A major issue related to their analysis by µFTIR is that microfibers do not lie flat on the analysis area, which makes it difficult to keep the entire fiber in focus. Spectroscopic techniques cannot be applied to TWPs made from pure carbon black as this absorbs all light at all wavelengths. A similar issue occurs for paint flakes, where pigments may absorb light. In these instances, modified or alternative approaches are urgently needed so that such forms of MP pollution can be identified, quantified and risk assessed effectively.

Current widespread existing analytical tools are not suited to the quantification of small MP (<10 µm) and nanoplastic (NP) particles, which are predicted to be the most abundant form of plastic pollution in terms of particle numbers. The main issues are related to the instrumental limits of size detection in the case of µFTIR (~10 µm) and µRaman (~1 µm), as well as the problems associated with extracting NP from complex environmental matrices and removing the background contribution from naturally occurring nano-sized particulates. Even advanced FTIR- and Raman-based approaches are severely limited by the 'searching for a needle in a haystack' dilemma. Current knowledge regarding the potential impact of engineered nanomaterials suggests that plastic particulates in this size range may have a significantly higher risk for eliciting toxic responses in organisms in comparison to micron-sized particulates. This is primarily linked to the increased mobility and potential for transport across biological barriers resulting from their small size. Again, there is a pressing need for the development

of approaches that enable MPs <10 µm and NPs to be identified and quantified in environmental samples, including biota.

Although the majority of MPs in the environment is thought to be transported from terrestrial sources, a certain proportion of MPs result from macroplastic litter fragmentation caused by a combination of UV degradation, mechanical abrasion and microbial degradation. Given that solar radiation, hydrostatic pressure, temperature, wave energy and microbial activity are not homogeneously distributed in the ocean, the effectiveness of these processes varies significantly between specific environments. For example, degradation is considered most intense in coastal environments due to higher UV exposure, seawater dynamics and abrasion induced by sand/coastline. These processes impact the physical characteristics of MP as the surface area and surface characteristics will be altered when larger MPs fragment into small MPs and NPs. Degradation also alters the chemical composition of the plastic through oxidation, resulting in additional challenges from an identification and quantification perspective. For example, the IR-spectrum of weathered plastic will differ from the pristine plastic, making identification more difficult. It is therefore essential to not only consider pristine MPs within analytical method development, but to make use of degraded reference materials (RMs).

Degradation processes also include the gradual leaching of additives such as plasticizers, UV-stabilizers, antioxidants or colorants which may impact marine organisms. This chemical leaching may also affect the analysis of MPs by chemical methods. However, the multitude of degradation processes are slow (order of magnitude of years) under the normal range of conditions encountered in the marine environment. The slow degradation processes complicates the standardized study of the fate of MPs in the environment, quantified data are needed to understand the current and future fate of plastic particles in the marine environment and their associated risk for exposed ecosystems. As a result, there is a need for accelerated degradation methods under laboratory-controlled conditions, in which MPs are subjected to stressors that mimic natural degradation at a higher speed. Such accelerated degradation techniques have the potential to supply the research community with the partially degraded plastic RMs needed for analytical identification and quantification method development.

Microplastic analysis by fluorochromes

The fluorescent staining of MPs using the hydrophobic dye Nile red (NR), frequently used in histology, enables the rapid screening of environmental samples for the presence of MPs using fluorescence microscopy (Maes et al., 2017; Bakir et al., 2020; Erni-Cassola et al., 2017). Based on the solvatochromic nature of NR, whose emission spectrum shifts depending on the polarity of its environment, MPs can be classified into “polar” and “hydrophobic”, based on their polymer characteristics (Maes et al., 2017). The interaction of NR with different polymers varies according to the chemical characteristics of the plastic, which has led to the successful use of NR staining of particles for polymer identification based on fluorescence (Sancataldo et al., 2020; Nel et al., 2021).

NR staining has proven efficient at distinguishing MPs from non-plastic materials such as amphipod carapaces, algae, seaweeds, wood, feathers, mollusk shells, chalk and sand particles using only blue light microscopy filters, or in combination with orange filters (Maes et al., 2017; Shim et al., 2016;

Sturm et al., 2021). This enables the identification of MPs extracted from environmental samples as these, even after digestion and further processing, often contain residual biological material and sediment, which is known to interfere in other MPs identification and characterization methods (Ruggero et al., 2020). Even though previous assessments indicate that NR has low affinity for natural materials frequently observed in the marine environment (Maes et al., 2017), other studies indicated that debris such as chitin-based and natural fibres can still be stained and show fluorescence, leading to a potential misidentification of the particles present (Stanton et al., 2019). To overcome overestimation of the number of MPs identified using fluorescence analysis, authors have suggested co-staining techniques using NR combined with a dye with high affinity for biological samples (Stanton et al., 2019; Maxwell et al., 2020), but this approach would imply further complexity in the sample processing and analysis.

Automation of analysis techniques

Automated approaches for MPs identification and characterization are a rapidly evolving field, which aim to contribute to and enhance environmental observations and monitoring programs. In general, automation methods can strongly reduce the time of sample analysis, decrease human bias, and allow larger datasets to be analysed in a standardized and cost-effective manner. Previously suggested automated methods for MP assessments combined spectroscopic techniques with 1) SVR (support vector machine regression) and 2) PLS-DA (partial least squares discriminant analysis) (Paul et al., 2019; da Silva et al., 2020), 3) random decision forests (Weisser et al., 2021; Hufnagl et al., 2022), and 4) KNN algorithms (k-nearest neighbours) (Kedzierski et al., 2019). However, although promising and quite efficient in identifying MPs, the referred methodologies are still dependent on obtaining outputs based on infrared spectroscopy, which are themselves highly dependent on the access to expensive equipment. Benchtop FTIRs are available from US$25 k onwards (Primpke et al., 2020), but for FTIR microscope systems with options crucial for MP analysis, costs increase quickly. Particle finder systems range from US$100–250 k, not yet considering the liquid nitrogen supply needed for certain detector types. Raman spectrometers are available starting at US$50 k (Primpke et al., 2020).

Classification models have been broadly and successfully used in the field of ecological modelling, e.g., to determine soil moisture of an area, which is of high importance in agriculture (Pekel, 2020), to construct groundwater spring potential maps (Chen et al., 2020), or to allow for plant identification and determination of informative traits to distinguish different plant taxa (Almeida et al., 2020). The use of algorithms eliminates the subjectivity of human sorting and is therefore an unbiased method. A drawback of automated research techniques is often their difficulty to be grasped by non-experts, as they often require expertise in the field of artificial intelligence (AI) and machine learning. Classification models and their branching methodology are easily interpretable while being capable of achieving high accuracy, provided that extrapolation outside the spectral ranges used in the training dataset is avoided. Contrary to black-box models used in machine learning (e.g. neural networks), white-box models have a transparent inner structure and represent information in a visual and clear way. These models can give insight in complex, unbalanced, non-linear data where commonly used exploratory and statistical modelling techniques often fail to find meaningful patterns (De'ath and Fabricius, 2000).

The automation of inexpensive fluorescence staining methodologies as an alternative to often used, spectroscopy-based methodologies has already been demonstrated to be promising for the

development of cost-effective screening methods for MPs analysis (Shruti et al., 2022). Most of these approaches focused on MP detection only (Primpke et al., 2017; Maes et al., 2017; Prata et al., 2019). The interaction between Nile red and different polymers based on their chemical characteristics has not yet been thoroughly explored and shows potential for the development of more advanced high-throughput, cost-effective analysis methods that allow for both MP detection and polymer identification.

2. STATE OF THE ART AND OBJECTIVES

State of the art on analytical methodologies

Existing protocols for the determination of MPs in the marine environment typically comprise sampling and transfer to the laboratory followed by sample pretreatment and analysis. Simple protocols for sampling MPs have been suggested within the frame of the BASEMAN project, however, different sampling methods all have advantages and limitations. To advance our knowledge on MP distribution, cost-effective monitoring approaches are necessary but require further development and new methods to achieve this. A cost-effective method for water samples, for example, would be application of pump-based systems on the ferryboxes (automated on-board measurement systems) which are often included in the marine monitoring programs (Lips & Lips, 2017). Although there are no commonly accepted devices available for MP sampling on ferryboxes, development work has been started. Challenges associated with such unattended sampling devices include filter/sieve clogging and contamination of samples. Within the previous JPI-O projects BASEMAN, EPHEMARE and PLASTOX, there has also been extensive work on sample treatment and preparation, including efficient mild digestion protocols, extraction with heavy density liquids, and treatment of natural matter on filters; all essential prior to detection.

A wide range of analytical techniques have been developed to quantify and identify MPs in environmental samples, each generating different types of information. In a move away from early MP studies that solely relied on visual and physical inspection for classification, increasing emphasis and requirements have been placed on chemical identification (Hidalgo-Ruiz et al., 2012). Vibration spectroscopy techniques (IR and Raman) which probe the chemical bonds in a material are the most commonly used as they can differentiate between polymer types and other synthetic or natural materials. Since the first JPI-O microplastic call (2014), analysis has progressed from handpicking of discrete particles for light microscopy, through basic spectroscopy analysis, to the semi-automatic workflows based on whole filter imaging or the particle-directed point measurements typically used today. It can be anticipated that this research front evolve towards fully automated analytical pipelines, from sampling to database search routines. However, this does require several critical challenges to be resolved. First, there is a need to minimize and remove the sample matrix material without damaging the plastics, where any transfer of MP particles should not lead to their fragmentation or contamination. Furthermore, pristine polymer databases have limited value unless they have been complemented with environmentally weathered materials, and with more challenging materials than pure polymers. It has also been highlighted that the detection and analysis of the more challenging MP types (e.g. TWPs, paint flakes and densely pigmented plastics) benefits from the application of multiple characterization techniques. For example, this can be two different microspectroscopic techniques or complemented light microscopy or hyperspectral imaging combined with e.g. FTIR or Raman analysis (Käppler et al., 2016). Currently, the robust identification of MP in complex environmental samples has not really been demonstrated below 10 µm, although methods such as µRaman have a much finer spatial resolution (≤0.5 µm).

Spectroscopy-based methods offer detailed information on MP down to ~10 µm, but they are often considered too time consuming and costly for routine analysis and monitoring. This points out the need to concurrently develop alternative cost-effective methodologies which must compromise on

robustness and to some degree on the lower size limit for target MP particles. In this context, fluorescent staining methods represent simple, sensitive, and low-cost alternatives to laborious visual sorting or costly spectroscopic techniques (Prate et al., 2019). First introduced by Andrady (2011), to date NR is the most promising lipophilic fluorescent staining dye for MPs where high recovery rates (96.6%) and short incubation times (10-30 min) have been reported (Maes et al., 2017). However, there remain some challenges associated with the approach. Although relatively insensitive to biogenic material (Maes et al., 2017), a thorough digestion step remains indispensable in the procedure. Furthermore, some types of plastic and fibers are difficult to stain with Nile Red (Prate et al., 2019). Hence, future research should focus on an improved matrix removal and clean-up procedure(s), optimizing the use of NR, searching for alternative dyes, and further automating plastic detection.

Detection methods based on hyperspectral imaging offer a second promising cost-effective approach that can be applied either in the lab-scale or in situ. The 1000-2500 nm wavelength range proved to be most applicable for MP analysis in seawater filtrates (Karlsson et al., 2016), compared to other ranges (375-970 nm, 960-1662 nm). The technique has also been successfully applied to the classification of floating plastic debris from disparate regions in relation to polymer type and morphological and morphometric characteristics (Serranti et al., 2018). The success of the chosen spectral region in distinguishing between different polymers has been attributed to the fact that most absorption bands in the same region arise from overtone vibrations of the molecular bonds between hydrogen and carbon (C-H).

In contrast to spectroscopy, mass spectrometry (MS) gives information on the polymer mass fraction rather than a particle count. The most commonly used technique is pyrolysis-gas chromatography-MS (Py-GC-MS), where the sample is thermally degraded and the resulting polymer pyrolysis fragments are chromatographically separated and characterized by MS (Mentenig et al., 2018). Thermal extraction desorption GC-MS (TED-GC-MS) additionally allows the analysis of plastic in environmental samples (≤20 mg) without the need for removing the (in)organic matrix (Dümichen et al., 2017). While both techniques can be applied to small MPs and NPs, they suffer with detection limits that will be too high if not applied in combination with concentration techniques such as cross-flow ultrafiltration (Mentenig et al., 2018). Py-GC-MS and TED-GC-MS are faster than spectroscopic particle analysis but cannot provide information on particle size distribution, number, morphology or aggregation, which must be obtained by other techniques. This highlights the need to combine different methods to fully characterize small MPs and NPs in environmental samples.

For mass-based analysis, polymers and their decomposition products can be considered as marker compounds providing chemical fingerprints for the plastic material. These chemical fingerprints can be used to quantify polymers in complex samples, including NPs. However, their poor sensitivity and specificity means there is a need to identify and use other chemical markers than the polymer itself. In this respect, both polymer degradation products and polymer chemical additives have been suggested as possible markers. One example case is the detection of TWPs, where analytical strategies involve the determination of either the rubber particles or the additives that serve as markers for tires (Wagner et al., 2018). The major elements considered as markers for TWP are Zn (used as activator in the vulcanization process) and S, originating from various reduced organic sulfur species that are used as vulcanization accelerators. As Zn may have sources besides tire wear, organic Zn is also suggested as marker (Fauser et al., 1999). Potential additives that can be used as TWP markers include

benzothiazoles, the vulcanization agent 1,3-diphenylguanidine, and the antioxidant 6-PPD. Within ANDROMEDA, the aim was to select suitable chemical additives as markers, determine the average content of each marker in a material (e.g. rubber), and quantify the amount in an environmental sample. As the composition of MP particles is not constant, exposures will be based on average additive contents in the plastic materials.

State of the art on nanoplastic detection methods

Based on the particle size distribution data available in the micron range, which indicates a semi-exponential increase in particle number with decreasing size, studies have implied high numbers of NP are likely to be present in environmental samples. Circumstantial evidence certainly seems to show formation of NP in the laboratory through abrasion and degradation (Lambert & Wagner, 2016; Shim et al. 2014) and a review from 2011 stated the near certainty that NP particles are produced during weathering of plastic debris (Andrady, 2011). However, the actual occurrence of NP is still a matter of speculation. Comprehensive reviews concluded no studies have reported established analytical methods to detect NP in either marine or fresh water (Andrady, 2011; Koelmans et al., 2015; Alimi et al., 2018; Mitrano et al., 2021). The ubiquitous presence of natural 'background' nanomaterials (mineral nanoparticles, organic macromolecules and other colloids) in most aquatic environments makes the analysis of NPs very challenging. This is further complicated by the fact that extraction, concentration and identification of NPs in environmental samples remains very difficult.

As a result, there is a need for sensitive, and small size resolution techniques, but most importantly selective detection principles are needed at both the single particle or bulk levels. Under controlled laboratory conditions several techniques that apply to engineered nanomaterials may prove useful for NP fate and effect studies. This includes Nanoparticle Tracking Analysis (NTA), Scanning and Transmission Electron Microscopy (SEM, TEM), Field Flow Fractionation (FFF) and Dynamic Light Scattering (DLS) techniques, each of which have their unique advantages and limitations. For example, SEM-EDX has been used to confirm the presence of NPs in abrasion experiments (Shim et al., 2014), and NTA was applied to characterize NP during the degradation of a polystyrene disposable coffee cup lid (Lambert & Wagner, 2016). Multiple wavelength UV-VIS has been used as a proxy to detect pink-dyed nanoparticles in exposure studies with mussels, where DLS was also used to track the size of the bioavailable aggregates over time (Wegner et al., 2012). A combination of TEM and conventional LM was used to characterize polystyrene NPs and aggregates, respectively (Velzeboer et al., 2014). To achieve detection and quantification of NP in environmental samples, in addition to explore the potential of direct single particle techniques, a multi-step sample treatment approach complemented by rigorous quality control is required followed by application of a multi-instrument platform (imaging, size determination and identification).

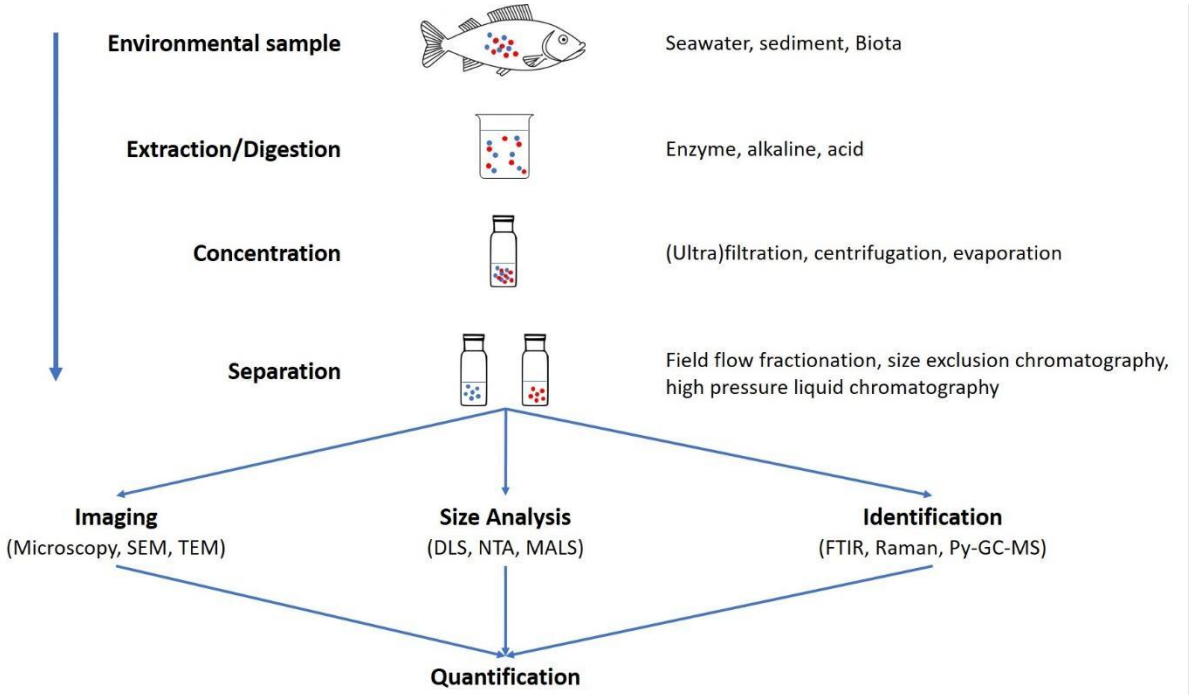


Fig. 1. Overview of analysis step for dtermination of NP and small MP.

State of the art on degradation/fragmentation studies

Most plastics degrade very slowly through a combination of photo-, bio-oxidation and mechanical abrasion, with the major degradation step for poorly degradable polymers (e.g., polyethylene) being UV-initiated oxidation. Photo-oxidation increases the amount of low molecular weight material by breaking bonds and increasing the surface area, where eventually microbes can initiate biodegradation to mineralize the plastic. However, there is little data on the rates at which different polymers degrade and fragment under the broad range of environmental conditions found globally. Plastic degradation processes can generate plastic fragments in the micro- and nano-scale as well as resulting in a significant release of dissolved organic carbon (DOC) in the form of molecules of varying size (Gewert et al., 2018). These can pose a long-lasting contaminant source and potentially transfer additive chemicals to marine organisms when ingested. In addition to threats to living organisms due to fragmentation processes, oxidation may alter fundamental plastic litter characteristics such as density. This can subsequently modify their expected fate in the marine environment by favoring transport to the sediment/deep ocean environment, where very little knowledge is available on MP degradation rate (e.g., significance of pressure, deep sea and sediment microbiology) (Paluselli et al, 2018a; 2018b). Recent research highlighted the importance of the smallest MPs (25-1000 µm) in the plastic debris mass balance, and thus raises the question of the predominance of smaller particles of one or more orders of magnitude (i.e., NPs) (Poulain et al., 2019). However, the extraction, concentration and identification of small MP and NP products in samples from degradation studies remains very difficult (Lambert & Wagner, 2016). In addition to oligomers at different oxidation levels, plastic additives are an important class of chemicals contributing to the molecular scale DOC derived from plastic. These chemicals can leach from the polymer matrix (Paluselli et al., 2019) and have been estimated to contribute ~6% of the global plastic mass. As additives are not chemically bound to the polymer, their release to the surrounding environment is mainly governed by van der Waals interactions after the polymeric matrix has been degraded and opened. A successful understanding of

how MP behave under the combination of physical, chemical and biological processes to which they are subjected to in marine environments is important for assessing their ecological implications. Such information could also provide valuable information towards their possible accelerated degradation under controlled environments.

Andromeda project objectives

The aim of Andromeda is to develop an instrument platform for in situ and cost-effective analysis of MPs, advanced characterization of NP and MP materials and for accelerated MP degradation and degradation characterization.

The main objectives of Andromeda are to:

- Achieve cost-effective analysis of MPs by in situ-methods and low-cost laboratory analysis, including efficient sampling.
- Develop and optimize advanced techniques to measure and quantify small and challenging types of MP particles.
- Investigate the degradation and fragmentation mechanisms of plastic into micro- and NP particles.
- Study the release of additive chemicals during fragmentation and degradation processes.
- Disseminate project results and developed protocols to a range of audiences, including public authorities, the private sector, academia and the general public.

Research focus of ILVO and VLIZ

Whereas the Andromeda project focused on the development, optimization and validation of analysis techniques for a broad range of analysis methods, from large to small MP and even NP, the research focus of ILVO and VLIZ within this project was focused on the development and validation of cost-effective methods. ILVO was the coordinator of work package 2 (WP2) which included optimization of sampling methods, hyperspectral based methods, methods based on chemical markers and fluorescent staining methods. ILVO and VLIZ aimed to optimize cost-effective methods based on fluorescent staining by NR. Methods were optimized to analyse a broad range of MPs by a semi-automated approach in matrices water, sediment and biota. By applying a multi-filter approach, optimized methods allowed identification of plastics versus non-plastics but also allow polymer identification. Protocols were developed and methods were validated. Optimized Nile-Red based methods were then integrated in cross-validation exercises of WP2, including the analysis of sediment samples from the Mediterranean as well as water samples from the Spanish Atlantic coast.

Furthermore, ILVO and VLIZ focused on performing a cost-effectivity analysis of commonly used methods for MP analysis, based on survey data gathered from experts in the field. Concrete and useful recommendations of MP analysis techniques in terms of their cost-effectivity were provided. The resulting predictive tool can support researchers, policy makers and other stakeholders when tasked with choosing between different MP workflows.

3. METHODOLOGY

WP2 management

The project started within the COVID-pandemic hampering physical project and work package meetings during the first project years. ILVO managed WP2 through work package meetings which were held every 2 months. Central themes in the meetings were the follow up of WP milestones and deliverables as well as the production and ordering of reference materials. Within Andromeda, pristine polymers were produced by Carat by cryomilling of large plastics into microplastics, followed by sieving into different size fractions. Different polymer types were made available, in irregular shapes: low-density PE, high-density PE, PVC, PET, PP, PS, PTFE and PUR. A homogenous mixture of these compounds could also be ordered. To obtain natural weathered particles, polymers obtained from Carat were submerged in deep-sea water (by project partner MIO) and coastal environment (by project partner NILU) for a 1-year period.

Data Management

The Flanders Marine Institute (VLIZ) is an ICSU accredited data centre, their core business is to provide data management services for the marine research community. Through the Marine Data Archive (MDA), long-term storage of the project data is assured. The MDA is used by all Andromeda project partners for processed data storage during and after the duration of the project.

All processed results and data from (soon to be) published studies were and will be quality controlled, provided with metadata, and stored according to the principles of FAIR (findable, accessible, interoperable, re-usable). The data format used is either .csv or .txt format for numerical data files. A minimum storage time of 5 years is respected and can be prolonged upon request. VLIZ is an advocate of free data exchange, therefore data is made freely available as much as possible for scientific research both on a national and on an international level. Metadata that illustrate the existence of a data set are always disclosed publicly, unless VLIZ has been explicitly requested not to do so. Datasets are also published by assigning a Digital Object Identifier (DOI) to them. A formal data publication procedure ensures that the dataset is citable and traceable. Lastly, developed codes and macros in R and ImageJ (e.g. of the machine learning models) are stored on GitHub. The data archiving tool Zenodo was and will be used to archive repositories on GitHub, and to issue DOIs for the archives.

Methodology of microplastic analysis by fluorochromes

Machine Learning (ML) models based on pristine MPs

A straightforward, reliable ex-situ methodology to detect and identify MPs, based on a semi-automated, cost- and time-effective approach using pristine MPs was developed (Meyers et al. 2022). This was done by combining machine learning and image analysis of fluorescently dyed MPs. To do so, seven amongst the most abundantly produced plastic polymers worldwide that are commonly observed in marine environmental samples were selected (Geyer et al., 2017; Suaria et al., 2020). Besides their omnipresence and prevalence in the environment that was used as a first criterion, it was ensured a broad range of densities was covered. The selection of reference materials was complemented with ten different non-plastic, nature-based source materials. To create the datasets

(dataset 1 (Meyers et al., 2021a) and dataset 2 (Meyers et al., 2021b)), cryomilled particles of nylon, polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyurethane (PUR) and polyvinyl chloride (PVC), chitin, cotton, flax (raw and bleached), hemp (raw and bleached), silk, wood and wool (alpaca and sheep) were used. All particles were sized between 50 µm to 1200 µm. The plastic polymer types were confirmed using Micro-Fourier Transform Infrared (µ-FTIR) Spectroscopy. These particles were stained with NR dissolved in acetone, filtered upon PTFE filters, and left to dry (Fig. 2).

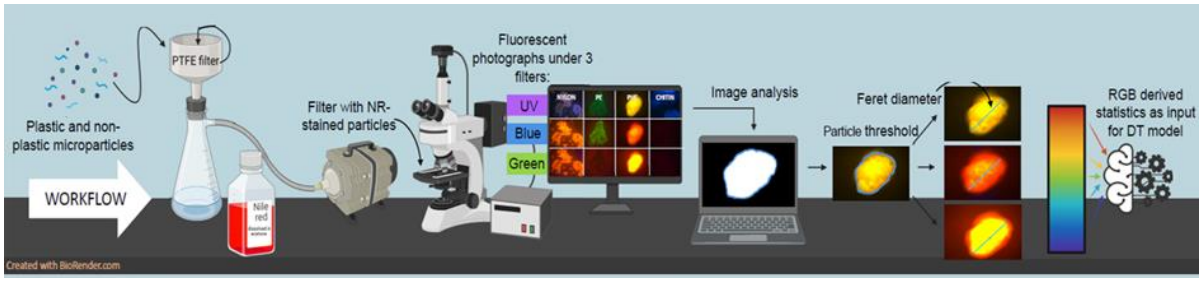


Fig. 2: Microplastics analysis workflow to detect and identify MPs, based on Nile Red staining.

First, images were taken of all fluorescently stained MPs under a blue, green and UV filter using a fluorescence microscope (FM) (Fig. 3). Following this, a two-step semi-automated classification method was created, which combines (RGB)-color quantification based on the automated image analysis of NR-stained particles filters with a supervised machine learning classification tree model, developed in an open-source environment, using the softwares ImageJ and Rstudio (Meyers et al. 2021c).

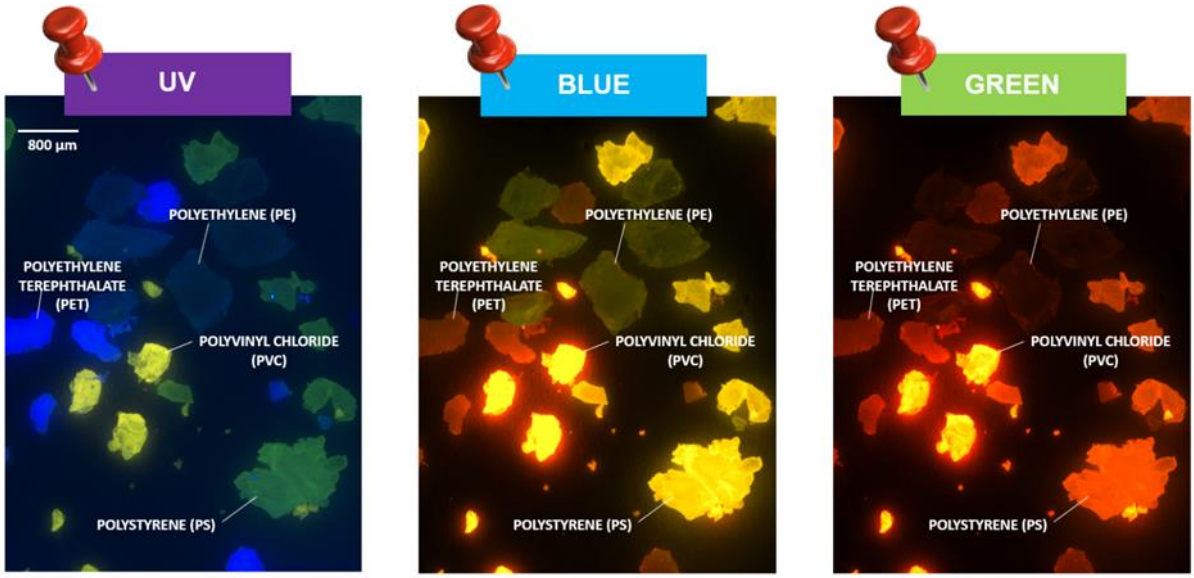


Fig. 3: Fluorescently dyed microplastic polymers, photographed under different fluorescence microscope filters (uv, blue, and green).

Two models were developed: the first one, the Plastic Detection Model (PDM) can predict with high accuracy whether particles are of plastic origin or of non-plastic origin based on their fluorescent colouration, and was trained and tested using RGB colour data from images of both plastic and natural reference particles (n= 2 x 60), and the second one, the Polymer Identification Model (PIM), can

identify plastic polymer types, and this model was trained and tested using RGB colour data of particles of the seven abovementioned plastic polymers (n = 7 x 30).

Model robustness

As proof of principle, the robustness of the developed method for MP identification was verified. To do so, the applicability of the models was tested for plastics and non-plastic particles in environmental matrices (seawater and biota); and for various types of surface and deep sea-weathered MPs. Furthermore, the interlaboratory use of the models was tested, and their limit of detection was determined.

MPs in the marine environment are prone to all sorts of weathering such as UV radiation and mechanical stress, which can alter their chemical composition (REF), and in turn affect their fluorescent colouration after staining with Nile red. As this may result in reduced accuracy of the newly developed ML models, additional tests were done with artificially and eventually naturally weathered plastics. The same pristine MPs as used to build the decision tree models mentioned earlier were subjected to two different types of artificial weathering, performed by Andromeda project partners from the Norwegian Institute for Air Research (NILU) and the Mediterranean Institute of Oceanography (MIO). The first set of pristine MPs underwent surface weathering where they were placed in Norwegian surface waters at sea for one year. The second set of pristine MPs were placed under experimental conditions of 20 MPa and 13°C for one year, mimicking deep sea weathering. As the accuracies of the model predictions decreased for these weathered plastics, the machine learning models based on pristine materials were optimized first. To do so, training and test datasets were expanded from 2 x 60 to 2 x 500 particles, and from 7 x 30 to 7 x 200 particles for the PDM and the PIM, respectively. Next, random forests models were built to verify whether prediction accuracy increased compared to decision trees models. It was decided to use these new models for further MP analyses as accuracies were indeed higher. Results of these robustness tests will be published by the end of the project in Meyers et al. 2023 (in prep.).

To test interlaboratory use of these models, a second set of random forest models was built using images acquired with a fluorescence stereomicroscope (FSM) rather than a microscope, which allows images of whole PTFE filters to be acquired. The PDM dataset consisted of 2 x 420 particles, the PIM of 4 x 135 particles (PE, PET, PS and PVC). By visualizing all MPs on just one series of images (blue, green, and uv), analysis time can be sped up considerably. Next, images acquired with a FM were analysed using FSM-based ML models, and vice versa. This was done to test whether other labs can use the developed MP dataset, or whether compiling new datasets to construct their own PDM and PIM is necessary for accurate MP analysis.

Furthermore, the cut-off size of the random forest algorithm-based PDM and PIM was determined for each MP polymer using a FM. To do so, model performance in terms of the number of pixels analysed was tested, for MP sizes down to 2.8 µm.

Lastly, a detailed analysis protocol was written for the detection of MPs and identification of their respective polymer types using the developed random forest ML models, i.e. the PDM and the PIM (Annex D).

Optimization and validation of MP extraction protocols

Protocols were optimised for the extraction of MPs from seawater samples, sediment samples, and biota (mussels and gastrointestinal tracts (GITs) of fish) samples. These protocols ensure the efficient use of the analysis protocol mentioned earlier. The protocols were validated and special attention was given to the analytical quality control and quality assurance associated with the validation of these protocols. Matrices were each time spiked with heterogeneously shaped MPs of different polymer types (PE, PET, PP, PS, and PVC, sized 100-1000 µm) at environmentally relevant concentrations, and samples batches of each matrix were processed over multiple weeks. For the validation, various parameters were considered (recovery, trueness, precision, and (within-lab) repeatability). Furthermore, robustness of the protocols was also verified, e.g. by using GITs of other fish species, non-commercial mussels instead of commercial mussels, by adding organic material to sediment matrices, etc. The mean background contamination was also taken into account, and considered for MP recovery.

Case study dredge disposal site Zeebrugge Oost

To test and validate all developed sample processing and analysis protocols, a case study in the Belgian Part of the North Sea was performed, with a focus on dredge disposal site Zeebrugge Oost. At the same time, a Ferrybox microplastic sampling module, which is an automated sampling device for seawater, was tested to make the sampling process quicker, more affordable and interoperable, in this way creating a fully cost- and time-effective MP analysis workflow.

During a sampling campaign with the RV Belgica in autumn 2022, 14 x 3 sediment samples were acquired with a Van Veen Grab at locations on and near the dredge disposal site, and at reference areas in front of the coast of Oostende (14 locations in total). A seawater sample was also acquired at each of the locations using a ferrybox sampler. During sampling, this sampling system is connected to a water inlet at a depth of ±3m. Intake water is automatically collected with a constant flow rate into a sieve of chosen mesh size (50-300 µm), enabling the collecting of a significant number of MPs. Lastly, biota samples were acquired. Crab pots were filled with bait and left on the dredge disposal site with a buoy attached. They were retrieved with crabs inside after 72h.

All samples were processed according to the developed protocols. Sample analysis using the random forest ML models is ongoing, results will be validated through µ-FTIR analysis of the recovered MPs. Detailed results will be published by the end of the project in Meyers et al. (2023) in prep.

Flow cytometry and Nile red as a robust in-situ MP analysis method

Lastly, the potential of combining flow cytometry with fluorescent staining of MPs with Nile red was explored for the development of an in-situ MP analysis method where MPs down to 1 µm can be detected, and where no sample processing is required.

First, dilution series were prepared to assess the accuracy of the flow cytometer concentration measurement and to quantify the cut-off size. The next step was to develop a suitable staining method. Several experiments were conducted to investigate the potential of the flow cytometer as a detection technique. We tested whether the method was 1) able to detect both autofluorescent microbeads and stained plastic microbeads; 2) able to give accurate estimations of the spiked concentrations of MPs using dilution series of MilliQ; 3) able to detect particles below 20 µm in size

(which is often the cut-off size for µ-FTIR-based techniques); 4) able to distinguish MP polymers based on their emitted fluorescence; and finally 5) able to distinguish MPs from any organic material present, which is crucial if no sample processing step is performed.

Cross-validation

The validity of different MP analytical methods for analysis of water and sediment samples was assessed by applying multiple cost-effective methods on environmental samples, including the developed NR fluorescent method. For the sediment cross-validation exercise, samples were taken by Ifremer in and around the bay of Marseille (France, Fig. 4). Samples were taken with a Reineck corer. The surface layer of sediment (five upper centimeters) has been taken, manually mixed in a big stainless-steel container and packed in stainless steel boxes. In the laboratory, the environmental samples were packed in different glass flasks (500 g wet weight – w.w) with absolute ethanol and sent out to the participating laboratories for analysis. Positive and negative control samples were created by cleaning sediment samples with ZnCl (negative control), followed by spiking of a fixed amount of 300µm-1mm particles of different polymer types (positive control).

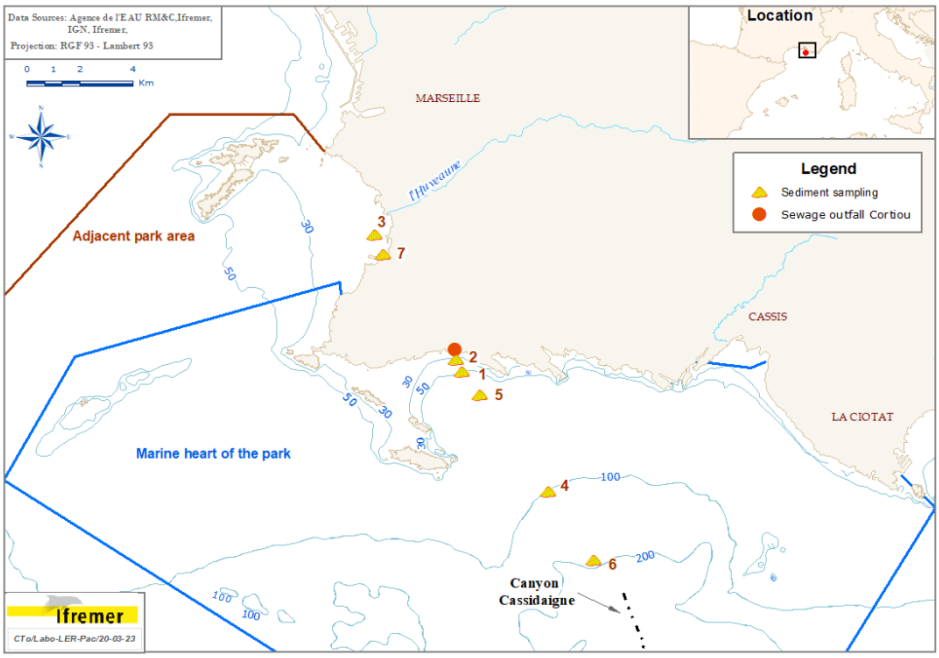


Fig. 4: Sampling sites for sediment samples in the bay of Marseille – French waters in the Western Mediterranean Sea (projection RGF93-Lambert 83).

For the water samples, three transects were conducted at different points along the Galician coast nearby Vigo (Spain). Sampling included a ferrybox sampling, a surface manta net and a depth manta net, the latter simultaneously and at the same depth of the ferrybox sampling (4.8 m). For analysis, each laboratory received one set of ferrybox filter samples, 5 surface manta samples, 5 depth manta samples, a spiked manta sample as positive control, 2 background samples consisting of ferrybox connection scratch and ship paint and 1 negative control filter from the wet lab obtained during sample processing. Identical to the sediment samples, water samples were stored in absolute ethanol after filtration and sent out to the participating laboratories for analysis.

Since the Andromeda project was not yet finished at the day of submitting this report, data processing and reporting on the cross-validation samples was still ongoing on the day of submission of this report. Under the lead of Ifremer (Gerigny et al., in prep.) and EIO (Blanco et al., in prep.), results are intended to be published in the second half of 2023.

Cost-effectiveness of MP analysis methods

Survey

To investigate the cost-effectiveness of commonly used analysis techniques for MPs in seawater, an online survey was performed. The survey data was collected through QuestionPro (www.questionpro.com). The survey was sent around to various European MPs expert groups, professional networks of the authors, and was distributed via social media (Twitter), informed consent was obtained before the start of the survey. The data was collected from September to November 2022. In total, 64 people attempted the questionnaire, and after excluding unusable and unrealistic data, the final dataset consisted of 25-30 responses. A first section of the survey aimed to determine the socio-economic characteristics of the respondents (employment country, sector and position and others). Within the second section of the survey, a detailed hypothetical scenario was developed where we posited a situation in which five seawater samples were acquired with a manta net targeting > 300 µm MPs, on board of a research vessel in the North Sea. For each of the five seawater samples, here called a batch of samples, the inside of the manta net was rinsed with 1L of MilliQ water which was then intercepted in a glass bottle using a metal funnel. Each of the five acquired seawater samples contained 50 MPs of various shapes within the size range of 300 - 1000 µm, and a suspended particulate matter (SPM) content of 25 mg/L (Neukermans et al. 2012). Respondents were asked not to take into account negative and positive control samples. Next, details of the respondents' MPs analysis workflow were investigated, i.e. of the sample acquisition, sample processing (preparing the sample for MP analysis) and sample analysis step (analysis of the MP content). This section of the survey aimed to determine two types of costs, i.e. equipment costs and labour costs. To do so, questions targeted the types of equipment participants used to analyse one batch of seawater samples as defined above, as well as the equipment purchase prices (excluding VAT)/renting costs, the man-hours needed for each step within the workflow as well as the contract types of the people performing the steps, the median gross annual salary of 1) senior researchers and 2) lab technicians with ten years of work experience in the country of employment of the respondent, and the most expensive consumable used for the analysis of the seawater sample batch along with its cost. Responses either had to be chosen from a list of options with the possibility of adding an own option (single-choice and multiple-choice depending on the question), or a slider bar response scale system was used where participants could indicate costs.

Data analysis

The data obtained from the survey performed was standardised, and unrealistic data was removed. To do so, median gross annual incomes higher than double and lower than half the mean wage of the respondents' country of employment (Eurostat, 2022) were excluded from further analyses. Man-hours deemed unrealistic were excluded. Following this, all standardised data was classified into different analysis technique categories based on the most expensive type of analysis equipment used by a respondent.

1. Labour and equipment cost calculations

To calculate the total cost associated with the analysis of one batch of five seawater samples based on equipment usage intensity, sample processing labour and equipment costs as well as sample analysis labour and equipment costs were considered. This was done using the following formulas:

$$Total\ cost = sample\ processing\ labour\ cost + sample\ processing\ equipment\ cost + sample\ analysis\ labour\ cost + sample\ analysis\ equipment\ cost$$

$$Labour\ cost = \frac{median\ man\ hours * gross\ annual\ income}{man\ hours\ per\ year}$$

$$Equipment\ cost = \frac{\frac{median\ equipment\ purchase\ price}{equipment\ depreciation\ time}}{\frac{number\ of\ analysed\ batches}{year}}$$

Labour and equipment costs of the sample processing and of the analysis part of each technique in the country of employment of the respondents were calculated. For the labour costs, median man hours needed to perform each of the two workflow steps were calculated for each of the six major analysis techniques categories as defined earlier. Three different simulations were performed based on the GNI per capita (p.c.) of the countries of employment of the respondents, as defined by the World Bank (World bank, 2021): for lower income European countries (GNI p.c. below 29,620 EUR, calculated at exchange rate of 1 USD = 0.82 EUR on 1/01/2021 through www.xe.com), for middle income European countries (GNI p.c. between 29,620 and 52,681 EUR), and higher income European countries (GNI p.c. above 52,681 EUR).

To calculate labour costs, it was assumed employees had fixed salaries and worked 38 hours per week, during 52.143 weeks, which equalled 1981 working hours per year. Based on the survey results, the most frequently selected contract type to perform each of the two workflow steps was used for further calculations. The gross annual salary of these employees with ten years of work experience was used to calculate the sample processing labour costs and the sample analysis labour costs. To do so, the respondents' GNI p.c. data for the specific contract types was subdivided according to the three groups described above, after which the median gross annual salary per income group was calculated and multiplied with the median man-hours needed for sample processing or for sample analysis within each of the six analysis techniques categories. Total labour cost was calculated by dividing obtained costs for both steps by the man-hours performed per year, followed by determining the sum of the obtained final sample processing and sample analysis costs. Sample processing as well as analysis equipment purchase costs were determined by calculating the median of the sum of the purchase prices (excluding VAT) of all types of equipment used within each of the six analysis techniques categories. As very few survey respondents indicated the rental of equipment rather than purchase, this data was excluded from further analyses.

2. Total sample analysis cost as a function of equipment usage intensity

The depreciated cost of all used equipment (the equipment value after reducing its value when it was new by the total amount of depreciation) was determined. Here, a 20% depreciation rate was used (yearly decrease in monetary value of equipment due to use, wear and tear, or obsolescence), which equals a use of five years, and this for both sample processing and sample analysis equipment. A salvage value (estimated book value of equipment after the depreciation is complete) of 0 EUR was used (Office of Tax Analysis, 1990). The depreciated equipment cost was calculated by dividing it by the number of years of useful life (i.e. five years). To determine the depreciated equipment cost as a function of equipment usage intensity, the total depreciated equipment cost was divided by the

number of analysed sample batches per year, as defined earlier. Calculations were done for a range of 10 to 1000 batch analyses per year. Total MP analysis cost, i.e. the sum of all labour and equipment costs, was then plotted as a function of equipment usage intensity (EUI) for each of the six analysis technique categories, and this for each of the three income groups.

3. Method effectiveness scoring – fit for purpose

The fit for purpose of each of the six analysis technique categories was determined. To do so, a relative ranking system was used where scores were given to each of the methods for eight different effectiveness criteria. The criteria used were based on the abilities/characteristics of each method, and were the following: 1. Confirmative plastic/non-plastic; 2. Physical characterisation of MPs (number/size/shape); 3. MP mass determination; 4. Polymer identification; 5. cut-off size < 300 (1) µm or < 50 µm (2); 6. Characterisation of particles that are challenging to detect: paint flakes, tire wear particles (TWP) Tire wear particle (TWP) characterisation; 7. Whether or not the method is destructive for the analysed sample, and 8. Identification of chemical additives. Scores were given from 0 to 2, where 0 meant the method does not have this ability, 1 meant the ability is not inherent to the method, and 2 meant the ability is inherent to the method. For criterion 5, a method with cut-off size > 300 µm, < 300 µm or < 50 µm was given a score of 0, 1 or 2, respectively. For criterion 7, 2 or 0 was given to non-destructive or destructive methods, respectively. The sum of all scores was defined as the effectiveness score of a method, and theoretically ranged between 0 and 16.

4. Workshops

Two online workshops were designed to present, discuss, and build a consensus around cost-effective MP analysis methodologies for seawater sampling. Both workshops applied the same workshop structure and participatory method, which are detailed in the associated workshop reports (Kopke et al., 2023a and Kopke et al., 2023b). A guided conversation was adapted and applied for both workshops, in which a series of questions was presented to workshop participants to encourage reflection on the presented work and making recommendations that support informed decision making and consider cost effectiveness of sampling, processing, and analyses of MP samples from seawater.

Workshop 1 was implemented with researchers and scientists working in the same field with the aim to validate preliminary analysis outcomes with scientific experts, to discuss the potential applicability of our CEA for research and make recommendations concerning future research. Ten researchers and scientists representing eleven organisations from across eight European countries participated. Workshop 2 was undertaken with eight policy experts and decision makers representing OSPAR, the JPI Oceans secretariat, the Joint Research Centre (JRC), the Royal Belgian Institute of Natural Sciences (RBINS), the Belgian Federal Science Policy Office (BELSPO), the Flanders Marine Institute (VLIZ), the Marine Institute in Ireland (MI), the Marine Environment Division of the Department of Housing, Local Government and Heritage in Ireland and the UK Centre for Environment, Fisheries and Aquaculture Science (CEFAS). Workshop 2 included a summary presentation of Workshop 1 recommendations to allow for further reflection and discussion and aimed to make recommendations on the potential applicability of our CEA in environmental monitoring and in relation to environmental policy and regulations.

4. SCIENTIFIC RESULTS AND RECOMMENDATIONS

Microplastic analysis by fluorochromes: results and recommendations

Machine Learning (ML) models based on pristine MPS

Both models (Fig. 5) showed high accuracies and kappa statistics (measure used to test inter-rater reliability) for the correct classification of pristine test materials (95.8% and 88.1%; κ = 0.92 and 0.86, respectively) and of plastics spiked in marine matrices (overall accuracies between 88.6 % – 97.1 %).

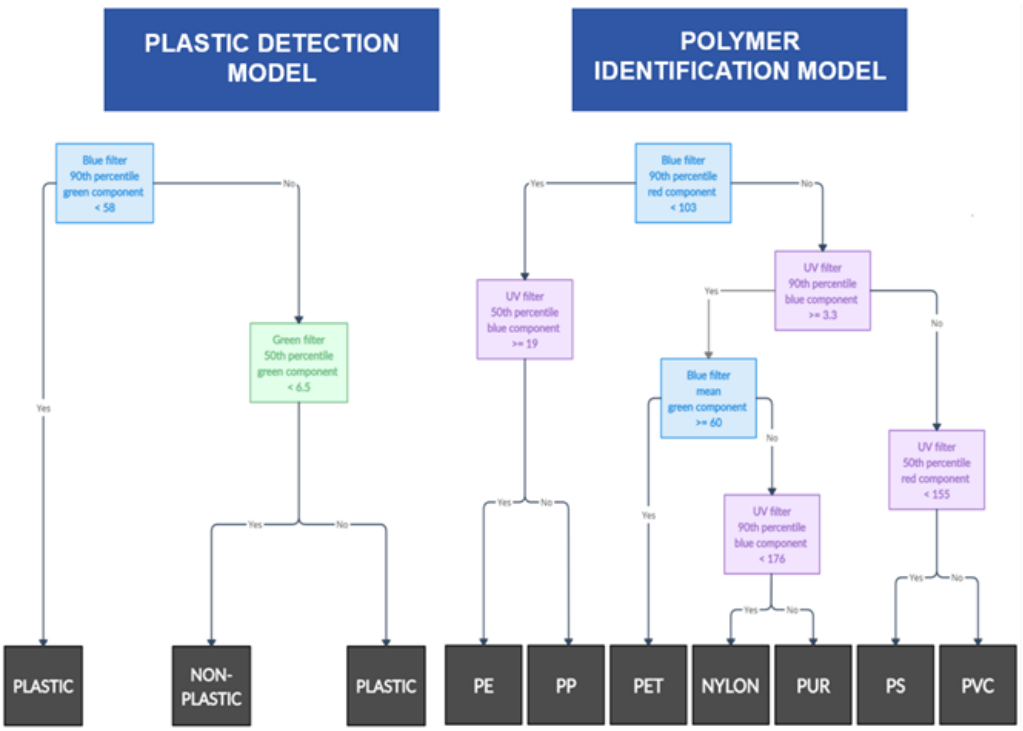


Fig. 5: Developed machine learning models for the detection and polymer identification of microplastics.

Model robustness

After optimising the machine learning models, prediction accuracies of surface and deepsea weathered MPs remained high. Accuracies over > 99% were obtained for the PDM, and over 70% using the PIM for nearly all polymer types except for PET, where fluorescence intensity decreased following both types of weathering. It is therefore advised to either incorporate RGB data of artificially weathered PET particles in training datasets of new models, or to be aware of the potential underestimation of the number of PET particles present in analysed seawater samples.

Inter-laboratory comparison (FM vs. FSM) showed that models in general do not perform well in identifying MPs photographed with microscopes different from the microscope used to train the models. Therefore, in order to use this ML model approach based on automated image analysis of fluorescently stained particles, one needs to generate lab-specific models to be able to acquire reliable analysis results.

Determining the cut-off size of the random forest algorithm-based models showed that pristine MP below 10 µm could still be detected using this analysis technique. For example, a total of 1715 PP and 823 PET particles below 10 µm in size were analysed, where the smallest particle was 2.8 µm (= 12 pixels; 1 pixel = 0.23 µm). Over 90 % and over 80 % of these particles, respectively, were correctly identified by the PIM (Fig. 5). This was also the case for other polymer types. Potentially, even smaller particles can be identified, but this has not been tested so far due to size constraints of the particles available.

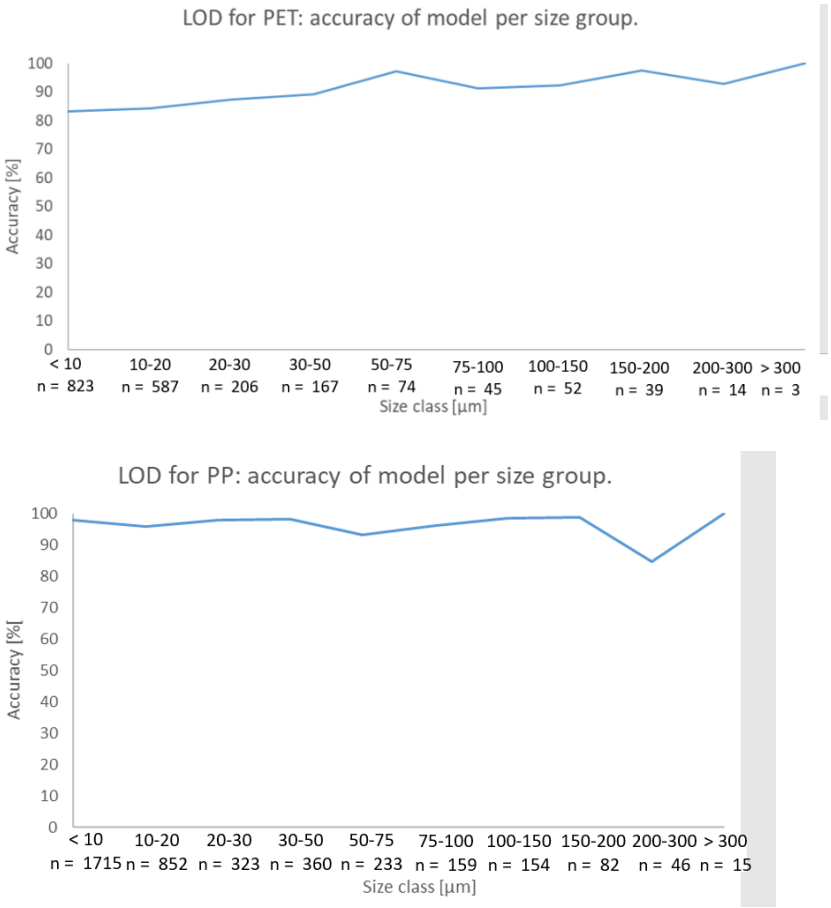


Fig. 6: The cut-off size (LOD) of the random forest algorithm-based models for PET and PP particles.

A detailed analysis protocol was written for the detection of MPs and identification of their respective polymer types using the developed random forest ML models. This protocol can be found in annex D.

Optimization and validation of MP extraction protocols

Detailed, validated sample processing protocols for the three different matrices (seawater, sediment and biota) can be found in annexes A, B and C.

Case study dredge disposal site Zeebrugge Oost

The developed sample processing protocols were successfully used to extract MPs from the acquired sediment, water and biota samples. MP analysis is currently ongoing, results will be published soon in Meyers et al. 2023 (in prep.).

The sampling tool used to acquire the seawater samples is a largely unexploited opportunity for sample collection where a fairly simple MP sampling module is fitted to the pump system of a vessel. This can be a ferry box which is normally used for observing Essential Ocean Variables (EOV). Their use has been encouraged by experts as they can sample large volumes in a controlled way, cover broad spatial ranges, allow contamination to be estimated and provide accurate flow measurements.

Flow cytometry and Nile red as a robust in-situ MP analysis method

Vacuum filtration combined with image analysis gave the best results when verifying MP concentrations in dilution series. Furthermore, the flow cytometer was able to detect both autofluorescent microbeads and stained plastic microbeads. The best method that provided enough staining to be detected by flow cytometry but that did not affect the structure of any polymers was staining MPs in a 75% Nile red (10 µg) dilution in Milli-Q water. Measurements of the dilution series resulted in an underestimation of the concentrations present, so for now, the method is not reliable for estimating polymer concentrations of samples and should be further optimised. PET particles smaller than 20 µm were successfully measured, showing the ability of the flow cytometer to solve the size limit issue. Flow cytometer measurement of a PE-PS particle mixture resulted in a cytogram in which the two polymers appeared as two separate lines (Fig. 8). Thus, the flow cytometer can distinguish between different types of polymers (Fig. 7).

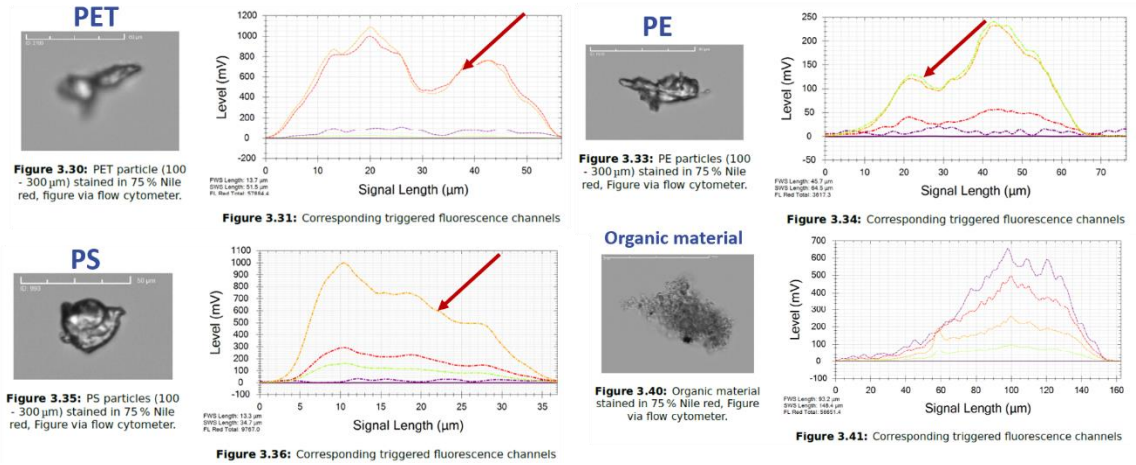


Figure 3.30: PET particle (100 - 300 µm) stained in 75 % Nile red, figure via flow cytometer.

Figure 3.31: Corresponding triggered fluorescence channels

Figure 3.33: PE particles (100 - 300 µm) stained in 75 % Nile red, Figure via flow cytometer.

Figure 3.34: Corresponding triggered fluorescence channels

Figure 3.35: PS particles (100 - 300 µm) stained in 75 % Nile red, Figure via flow cytometer.

Figure 3.36: Corresponding triggered fluorescence channels

Figure 3.40: Organic material stained in 75 % Nile red, Figure via flow cytometer.

Figure 3.41: Corresponding triggered fluorescence channels

Fig. 7. Fluorescent signals of plastic polymers as well as organic material after Nile red staining and flow cytometry-based analysis.

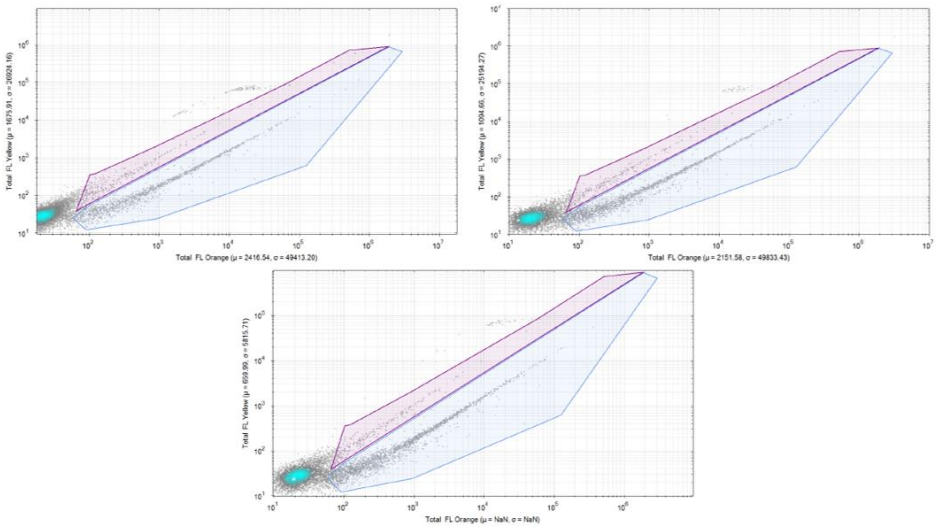


Figure 3.38: Flow cytometry cytogram for the mix of PE and PS particles. PE particles are situated in the purple gate, while PS particles are situated in the blue gate. The dense cloud in the left bottom is considered noise.

Fig. 8. Flow cytometry cytograms for a mix of PE and PS particles.

The final step in development was to distinguish between organic matter and polymers, a requirement to be able to make the measurements in-situ without the need for an extensive processing step. Although the detected stained organic material gave a different fluorescence pattern than the PS particles, the measurement of a mixture of stained organic material and MPs did not yet result in a cytogram with a sharp distinction between the different materials. Further analysis of the data showed that the Phenograph algorithm could distinguish MPs and organic material using the k-nearest neighbors approach.

Cost-effectiveness of MP analysis methods

Based on the standardised data, six different analysis technique categories were created: 1. (fluorescence) (stereo)microscopy ((F)(S)M)), comprising all purely microscopy-based techniques; 2. (stereo)microscopy combined with ATR-FTIR ((S)M+ATR-FTIR); 3. (stereo)microscopy combined with FTIR microspectroscopy ((S)M + µ-FTIR); 4. fluorescence (stereo)microscopy combined with FTIR microspectroscopy F(S)M + µ-FTIR; 5. (stereo)microscopy combined with Raman microspectroscopy ((S)M + µ-Raman); and 6. all GC-MS-based techniques (GMBT).

1. Labour and equipment cost calculations

Half of the respondents indicated that in most cases lab technicians perform the sample processing step, while senior scientists often perform the sample analysis step. Therefore, the indicated gross annual salary of these employees with ten years of work experience was used for labour cost calculations. To calculate labour costs, wage data of respondents employed in Estonia, Poland, Portugal, Spain and Romania was grouped within the *lower wage (LW) European countries* category as defined by the World Bank (World bank, 2021); wage data of respondents employed in Belgium, Finland, France, Germany, Italy, Sweden and UK was grouped within the *middle wage (MW) European countries* category (Fig. 9); and finally wage data of respondents employed in Denmark, Ireland and Norway was grouped within the *higher wage (HW) European countries* category.

2. Total sample analysis cost as a function of equipment usage intensity

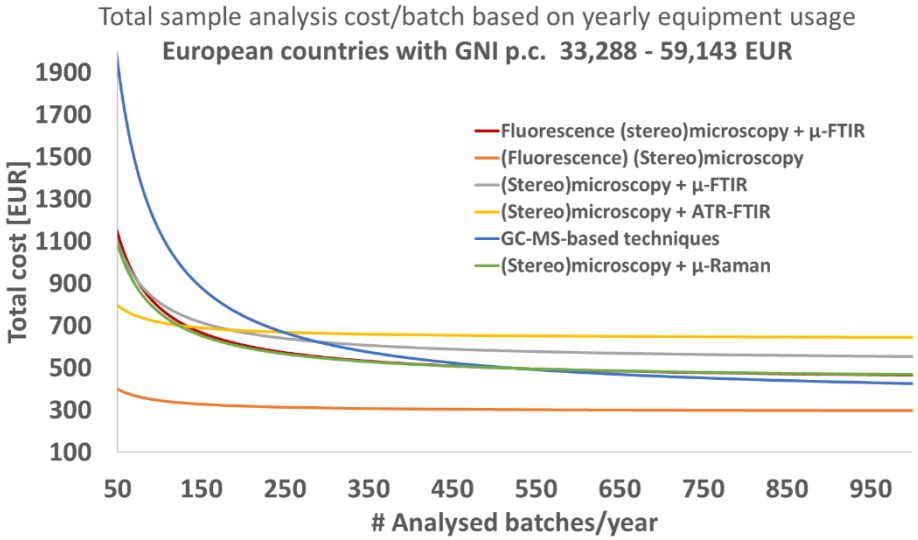


Fig. 9: Total sample analysis cost for each of the six method categories per batch of 5 defined seawater samples, as a function of equipment usage intensity, and this for middle wage European countries with a GNI p.c. of 33,288 - 59,143 EUR.

The cost-effectiveness of MP analysis methods for seawater samples is influenced by multiple factors, including the research questions, the equipment used, the degree of automation, the intensity at which the equipment is being used as well as its depreciation time, the position of the employee, and the country of employment. Generally, the more frequently and intensively the equipment is used, the lower the cost per analysis. Labs that offer services to e.g. customers from industrial sectors or governing bodies such as the European Union often perform routine analyses. For such routine analyses (easily 500 batch analyses per year (b/y)), investment in high-cost equipment is quickly earned back, with costs at a higher EUI mainly determined by the labour costs and hence the man hours needed as well as the position of the analyser. Some analysis equipment requires skilled personnel or additional training, and consequently comes with higher labour costs.

GMBT are very costly when not performed regularly, almost twice as expensive as F(S)M + µ-FTIR- and (S)M + µ-Raman-based techniques for an EUI of 50 b/y (mw countries, Fig. 9). However, with an increase in number of analyses performed per year (50 to 550 b/y), GMBT show the steepest decline in cost (approx. - 75%) and become the most economical option available due to its relatively lower labour costs.

At low EUI, equipment costs contribute relatively seen more on the total cost compared to labour costs. Therefore, for labs that occasionally perform MP analyses, methods with less costly equipment are the obvious choice. For MW countries, at an EUI of 50 b/y, (S)M + µ-FTIR-, F(S)M + µ-FTIR- and (S)M + µ-Raman-based techniques are similar in cost. They are more economical than GMBT, but slightly more expensive than (S)M + ATR-FTIR-based techniques. With a slight increase in the yearly number of processed samples (100 b/y), both F(S)M + µ-FTIR- and (S)M + µ-Raman-based techniques become less expensive than techniques based on (S)M + ATR-FTIR, while (S)M + µ-FTIR becomes more costly than the three aforementioned methods. Overall, (F)(S)M-based methods are by far the most economical methods available, but this comes at the cost of a lower method effectiveness.

The results of the survey and discussions in workshops (see below) have clearly shown the benefits of automation for microscopy-based techniques ((S)M+ µ-FTIR, (S)M+ ATR-FTIR, F(S)M+ µ-FTIR) in reducing the analysis time of MP samples. Automation allows the analysis to be completed more quickly, which can significantly shorten the overall time required for the analysis process. Additionally, automation can help lower the total cost of analysis by reducing the resources needed, such as staff time and personnel expenses.

3. Method effectiveness scoring

To assess the fit of purpose for each technology, effectiveness scores were given to each of the six analysis technique categories based on the abilities of each of the categories, using a relative ranking system, with scores theoretically ranging between 0 and 16 (Table 1). Compared to all other methods, the categories (S)M + µR and GMBT obtained the highest scores of 12-14 and 13, respectively. This is mainly due to their ability to identify tire wear particles. However, physical characterization of MPs is not inherent to GMBT, therefore information on MP number, size and shape can only be obtained when an additional µ-FTIR analysis of a (sub)sample of MPs is performed. Effectiveness scores for (S)M + µR ranged between 12 and 14, depending on whether the cut-off size is < 50 µm or < 300 µm, which in turn depends on whether a microscope or a stereo microscope is used.

Table 1. Effectiveness scoring system of the six method categories for microplastic analysis.

Method	Confirmative plastic/non-plastic	Physical characterization MPs	MP mass determination	MP polymer identifi-cation	LOD < 300 µm (1); < 50 µm (2)	TWP character-isation	Total score
(Fluorescence) (Stereo) microscopy	0	2	1	0-1	1-2	0	4-7
(Stereo) Microscopy + ATR-FTIR	2	2	1	2	0	0	7
(Stereo) microscopy + µ-FTIR	2	2	1	2	1-2	0	8-10
Fluorescence (stereo) microscopy + µ-FTIR	2	2	1	2	1-2	0	8-10
(Stereo) microscopy + µ-Raman	2	2	1	2	1-2	2	10-12
GC-MS-based techniques	2	1	2	2	2	2	11

The analysis technique categories (S)M + µF and F(S)M + µF both have effectiveness scores ranging between 10 and 12, again depending on what type of microscope is being used. Contrary to the previous category, techniques within these categories do give information on physical characterisation of MPs, but mass determination is not inherent to these methods. Furthermore, techniques in these two categories are unable to detect TWP present in seawater samples (Gao et al. 2022). (S)M+AF received a score of 9. The major difference of this category with (S)M + µF is its cut-off size, which received a score of 0 as ATR-FTIR cannot accurately analyse particles below 300 µm in size. Lastly, (F)(S)M received an effectiveness score of 6-9, which is lower than all earlier mentioned scores. One of the factors causing this lower score is the inability of these techniques to be confirmative for the plastic nature of a particle, as well as their inability in most cases to identify MP polymer type. Lastly, as with all of the aforementioned microscopy-based techniques, cut-off size depends on the microscope type.

Evaluation of the method effectiveness can be really nuanced, fit for purpose is crucial here. Depending on the objectives of the research performed, method requirements differ. Research can be focused on e.g. mapping MP pollution, source identification, fate assessment or ecotoxicological evaluations, all with different requirements regarding MP characterisation and quantification, e.g. in terms of their cut-off size and their ability to identify polymer types, to provide information on MP size, shape and colour; and the units in which results are expressed, e.g. mass vs. count (Tan et al. 2022). Many research questions can be addressed using MP count, e.g. identification of MP hotspots, assessing the efficiency of water treatment systems, MP risk assessments, ecotoxicological questions, and so on. On the other hand, microlitter indicator values for MP monitoring nowadays are often reported in g per m^2 for the upper water layer (Masura et al. 2015, Thornton et al. 2022). The effectiveness of a method is therefore inherent to the research goal.

4. Workshops

Participants of both ANDROMEDA workshops expressed that the presented preliminary results should be published to allow for repetition and adaptation of the approach. Adaptation of the approach was discussed in relation to adding additional criteria and context, such as accounting for different MP size classes, incorporating environmental criteria and policy related fields. Both workshop discussions highlighted the relevance of the approach to environmental monitoring programs and the need to build on systems that are in place. However, monitoring featured more prominently in discussion with the policy and decision makers, while the scientists and researchers' discussion focused more on the financial context in which the preliminary results were presented (Kopke et al., 2023ab). The specific recommendations from both workshops can be found in Kopke et al., 2023ab.

5. Recommendations based on the developed predictive tool

At the moment, clear recommendations and guidelines are being written up in terms of the most cost-effective MP analysis methods, and this for various scenarios, based on the performed CEA. The developed predictive tool could help researchers gain insight on which workflows provide the greatest value for money for specific seawater samples and could act as baseline data for researchers. An A1 publication of the results will be submitted by the end of June 2023 (Meyers et al. 2023 in prep.)

Furthermore, the tool can be used to allow for more informed decision-making regarding policy and management. The MSFD requires EU member states to achieve or maintain good environmental status in their marine waters by 2020, including a reduction in marine litter, including MPs. The predictive tool can help member states to assess progress towards this goal by providing a more comprehensive understanding of the costs and techniques used to evaluate distribution of MP pollution. Policy and decision makers (Kopke et al., 2023b) emphasized that the developed tool could help to inform changes in monitoring that may need to be applied to a European-wide scale. Participants suggested the development of a knowledge base using the predictive tool to help evaluate different available technologies and methodologies according to cost and what needs to be detected, to inform the selection of appropriate and feasible technologies and methodologies (Kopke et al., 2023b).

Policy and decision makers made a number of suggestion how our CEA tool could be used and adapted in a policy and monitoring context, which relate to the reproducibility of the methodology, strengths and limitations related to MPs detection, links to source emission measures, size limitation needed

for monitoring, availability of technology in commercial labs, harmonization of existing monitoring programs within and outside of the EU, usability for other matrices (wastewater, industrial emissions, etc) and contribution to filling current knowledge gaps, e.g. in relation to nano-plastics or risks (Kopke et al., 2023b). Overall, the predictive tool developed in this study represents an important step towards a more effective and informed approach to monitoring and managing MP pollution in the marine environment, supporting the implementation of the MSFD and ultimately contributing to the protection of marine ecosystems and human health.

5. DISSEMINATION AND VALORISATION

Scientific dissemination at a national level

- Participation in international conference such as the VLIZ Marine Science day (2021-2023). For a full list of references see 6.Publications

- Press article on our recently published MP analysis method from the public broadcaster of the Flemish Community in Belgium: https://www.vrt.be/vrtnws/nl/2022/02/15/belgische-wetenschappers-forceren-doorbraak-in-onderzoek-naar-mi/ .

Scientific dissemination at an international level

- Protocols for MP extraction from sediment, seawater and biota samples; as well as the protocol for sample analysis using ML models were bundled and shared with project partners.

- Participation in international conferences, such as and MICRO 2022, and SETAC Europe (2021-2023). For a full list of references see 6.Publications

- Two online workshops were designed to present, discuss, and build a consensus around cost-effective MP analysis methodologies for seawater sampling. Both workshops applied the same workshop structure and participatory method, which are detailed in the associated workshop reports (Kopke et al., 2023a and Kopke et al., 2023b). Workshop 1 was implemented with researchers and scientists working in the same field with the aim to validate preliminary analysis outcomes with scientific experts, to discuss the potential applicability of our CEA for research and make recommendations concerning future research. Ten researchers and scientists representing eleven organisations from across eight European countries participated. Workshop 2 was undertaken with eight policy experts and decision makers representing OSPAR, the JPI Oceans secretariat, the Joint Research Centre (JRC), the Royal Belgian Institute of Natural Sciences (RBINS), the Belgian Federal Science Policy Office (BELSPO), the Flanders Marine Institute (VLIZ), the Marine Institute in Ireland (MI), the Marine Environment Division of the Department of Housing, Local Government and Heritage in Ireland and the UK Centre for Environment, Fisheries and Aquaculture Science (CEFAS). Workshop 2 included a summary presentation of Workshop 1 recommendations to allow for further reflection and discussion and aimed to make recommendations on the potential applicability of our CEA in environmental monitoring and in relation to environmental policy and regulations.

- Within the Andromeda project, project partners from the University of Malta launched a new citizen science campaign on MPs on a popular beach in Valletta, Malta. A newly developed app was introduced, which enables the visual analysis of submitted MP photos by a developed algorithm, with all the submitted MP records feeding in a European-wide database. Both ILVO and VLIZ participated in this launch and actively interacted with citizens present. (https://www.um.edu.mt/newspoint/news/2022/04/launching-of-a-new-citizen-science-

campaign-on-microplastics

- Participation in the final JPI Oceans workshop, where our project results will be presented to partners of other plastic related JPI oceans projects.

6. PUBLICATIONS

- **Andromeda publications**

Published

- Meyers, N.; De Witte, B.; Everaert, G.; Hostens, K.; Janssen, C. (2022). Detection and identification of microplastics in biota using Nile red and machine learning: validation of an innovative, cost-effective approach, in: Mees, J. et al. Book of abstracts – VLIZ Marine Science Day, Online event 2 March 2022. VLIZ Special Publication, 88: pp. 20-21

In preparation

- Value for money: a cost-effectiveness analysis of microplastic sampling analytics. Meyers, N.; Everaert, G.; Kopke, K.; Buhhalko, N.; Mattsson, K.; Janssen, C.; De Witte, B. (To be submitted june 2023).
- Publication on model robustness (weathered particles, interlaboratory tests, cut-off size, etc.)
- Publication on the method development and validation for microplastics in biota matrices.
- Publication on microplastic pollution in the Belgian Part of the North Sea, with a focus on badger disposal site Loswal Zeebrugge Oost.

- **Related publications**

- De Witte, B., Catarino, A. I., Vandecasteele, L., Dekimpe, M., Meyers, N., Deloof, D., ... & Torreele, E. (2022). Feasibility study on biomonitoring of microplastics in fish gastrointestinal tracts. Frontiers in Marine Science, 8, 794636.

- **Conference abstracts**

Oral presentations

- Detection and identification of microplastics in biota using Nile red and machine learning: validation of an innovative, cost-effective approach. Meyers, N.; De Witte, B.; Everaert, G.; Hostens, K.; Janssen, C. Vliz Marine Science 2022 (Online). In: Jan Mees and Jan Seys (Eds). 2022. Book of abstracts – VLIZ Marine Science Day, Online event 2 March 2022. VLIZ Special Publication 88. Vlaams Instituut voor de Zee – Flanders Marine Institute (VLIZ): Oostende, Belgium. v + 91 p. https://www.vliz.be/en/imis?module=ref&refid=349866
- Detection and Identification of Microplastics in Biota Using Nile Red and Machine Learning: Validation of an Innovative, Cost-Effective Approach. Meyers, N.; De Witte, B.; Hostens, K.; Janssen, C. SETAC 2022, Copenhagen (DK). https://www.vliz.be/en/imis?module=ref&refid=352362
- Value for money: a cost-effectiveness analysis of microplastic sampling analytics. Meyers, N.; Everaert, G.; Kopke, K.; Janssen, C.; De Witte, B. Vliz Marine Science Day 2023, Bruges (BE). In: Mees, J.; Seys, J. (Ed.) (2023). Book of abstracts – VLIZ Marine Science Day, 1 March 2023, Bruges. VLIZ Special Publication, 90. Vlaams Instituut voor de Zee - Flanders Marine Institute (VLIZ): Oostende. vi + 112 pp. https://www.vliz.be/en/imis?module=ref&refid=361372

Poster presentations

- A novel, automated method to identify microplastic polymers based on fluorescent staining with Nile Red. Meyers, N.; Catarino, A.; Colin, J.; De Witte, B.; Everaert, G. Vliz Marine Science Day (2021) (online). In: Mees, J. et al. Book of abstracts – VLIZ Marine Science Day, Online event 2 March 2021. VLIZ Special Publication, 88: pp. 87. https://www.vliz.be/en/imis?module=ref&refid=333610
- A novel, automated method to identify microplastic polymers based on fluorescent staining with Nile red. Meyers, N.; Catarino, A.; Colin, J.; De Witte, B.; Everaert, G. SETAC 2021 (online).

- Flow cytometry for microplastic observation in the marine environment. Timperman, J.; Meyers, N.; Asselman, J.; Janssen, C.; De Witte, B.; Catarino, A.I.; De Rijcke, M.; Everaert, G. (2022). In: Jan Mees and Jan Seys (Eds). 2022. Book of abstracts – VLIZ Marine Science Day, Online event 2 March 2022. VLIZ Special Publication 88. Vlaams Instituut voor de Zee – Flanders Marine Institute (VLIZ): Oostende, Belgium. v + 91 p. https://www.vliz.be/en/imis?module=ref&refid=349874

- Automated detection and identification of microplastics in biota using Nile red and machine learning: Validation of an innovative, cost-effective approach. Meyers, N.; De Witte, B.; Everaert, G.; Hostens, K.; Janssen, C. MICRO 2022 (Online).

- Value for money: a cost-effectiveness analysis of microplastic sampling analytics. Meyers, N.; Everaert, G.; Kopke, K.; Buhhalko, N.; Mattsson, K.; Janssen, C.; De Witte, B. SETAC 2023, Dublin (IE). https://www.vliz.be/en/imis?module=ref&refid=363941

- **Workshops**

- Kopke K., Meyers N., Dozier A., Fitzgerald E., Power O-P., Agnew S., Everaert G., De Witte B., (2023a). Scientist Perspectives on the Cost-Effectiveness of Microplastic Analysis Methods for Seawater Samples: ANDROMEDA Workshop 1 Event Summary & Participant Recommendations on Cost-effectiveness. JPI Oceans project. https://www.andromedaproject.net/publications

- Kopke K., Meyers N., Dozier A., Fitzgerald E., Power O-P., Agnew S., Sempéré R., (2023b). Policy & Decision Makers Perspectives on the Cost-Effectiveness of Microplastic Analysis Methods for Seawater Samples: ANDROMEDA Workshop 2 Event Summary & Participant Recommendations on Cost-effectiveness. JPI Oceans project. https://www.andromedaproject.net/publications

- **Other**

Master thesis

Developing and optimizing a time-efficient method for in-situ observation of microplastics in the marine environment using flow cytometry. Timperman, J.; Meyers, N.; Janssen, C.; De Witte, B.; Catarino, A. I.; De Rijcke, M.; & Everaert, G. (2022).

Bachelor theses

Microplastics in het mariene milieu: voorkomen op plastic hotspots in Belgische en Spaanse kustwateren. Alshammari, R.; Meyers, N.; De Witte, B.; Salliau, S. (2023).

Validation of a Nile red-based method for determining microplastics in the gastrointestinal tract of fish. Terryn, E.; Meyers, N.; De Witte, B.; Salliau, S. (2022).

Validatie van een vernieuwende methode gebaseerd op fluorescentie voor de analyse van microplastics in mosselen. Lescrauwaet, C.; Meyers, N.; De Witte, B.; Salliau, S. (2022).

Optimalisatie van een innovatieve fluorescentiemethode voor de analyse van microplastics in biota. Demaegdt, S.; Meyers, N.; De Witte, B.; Salliau, S. (2021).

Vergelijking van de bestaande methode bij ILVO met een alternatieve methode op basis van hydrofobe ijzerpartikels voor de bepaling van microplastics in mosselen. Salenbien, N.; Meyers, N.; De Witte, B.; Hooland, E. (2020).

7. ACKNOWLEDGEMENTS

Andromeda is a JPI Oceans funded project, with the support of following international partners: Belgium: the Belgian Federal Science Policy Office (BELSPO), France: The National Research Agency (ANR), Estonia: Ministry of the Environment of the Estonia Republic (MoE) and the Estonian Research Council (ETAg); Germany: Federal Ministry of Education and Research (BMBF), Ireland: Marine Institute, and the Dept of Housing, Planning, and Local Government (DHPLG); Malta: Malta Council for Science and Technology (MCST); Norway: The Research Council of Norway (RCN); Spain: Spanish State Research Agency (AEI); Sweden: the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS). Project Coordinator: richard.sempere@mio.osupytheas.fr.

Ship time on RV Belgica was financed by Belspo and RBINS OD Nature, ship time on the Simon Stevin was financed by VLIZ. We also want to thank the project partners of the JPI Oceans Project Andromeda, the laboratory technicians of the ILVO marine analytical laboratory and the VLIZ Ocean and Human Health team for support.

8. REFERENCES

Alimi, O.S.; Budatz, J.F., Hernandez, L.M.; Tufenkji, N. Microplastics and Nanoplastics in Aquatic Environments: Aggregation, Deposition, and Enhanced Contaminant Transport. Environ. Sci. Technol., 2018, 52(4), 1704-1724.

Almeida, B.K., Garg, M., Kubat, M., Afkhami, M.E., 2020. Not that kind of tree: assessing the potential for decision tree–based plant identification using trait databases. Appl. Plant Sci. 8. https://doi.org/10.1002/aps3.11379.

Andrady, A.L. Microplastics in the marine environment. Mar. Pollut. Bull., 2011, 62(8),1596-1605.

Bakir, A., Desender, M., Wilkinson, T., van Hoytema, N., Amos, R., Airahui, S., Graham, J., Maes, T., 2020. Occurrence and abundance of meso and microplastics in sediment, surface waters, and marine biota from the South Pacific region. Mar. Pollut. Bull. 160. https://doi.org/10.1016/j.marpolbul.2020.111572.

Chen, W., Li, Y., Tsangaratos, P., Shahabi, H., Ilia, I., Xue, W., Bian, H., 2020. Groundwater spring potential mapping using artificial intelligence approach based on kernel logistic regression, random forest, and alternating decision tree models. Appl. Sci. 10. https://doi. org/10.3390/app10020425.

da Silva, V.H., Murphy, F., Amigo, J.M., Stedmon, C., Strand, J., 2020. Classification and quantification of microplastics (<100 μm) using a focal plane array-Fourier transform infrared imaging system and machine learning. Anal. Chem. 92. https://doi.org/10.1021/ Acs.analchem.0c01324.

De'ath, G., Fabricius, K.E., 2000. Classification and regression trees: a powerful yet simple technique for ecological data analysis. Ecology 81, 3178. https://doi.org/10.1890/ 0012-9658(2000)081[3178:CARTAP]2.0.CO;2.

Dümichen, E.; Eisentraut, P.; Bannick, C.G.; Barthel, A.K.; Senz, R.; Braun, U. Fast identification of microplastics in complex environmental samples by a thermal degradation method. Chemosphere, 2017, 174, 572-584. 2017.

Erni-Cassola, G., Gibson, M.I., Thompson, R.C., Christie-Oleza, J.A., 2017. Lost, but found with Nile red: a novel method for detecting and quantifying small microplastics (1 mm to 20 μm) in environmental samples. Environ. Sci. Technol. 51, 13641–13648. https://doi.org/10.1021/acs.est.7b04512.

Eurostat (2022) Auxiliary indicators (population, GDP per capita and productivity). Average full time adjusted salary per employee (NAMA_10_FTE). Downloaded on 2023 January 4 (last update: 19-12-2022). Statistics | Eurostat (europa.eu).

Fauser, P.; Tjell, J.C.; Mosbaek, H.; Pilegaard, K. Quantification of tire-tread particles using extractable organic zinc as tracer. Rubber Chem. Technol., 1999, 72(5), 969-977.

Gewert, B.; Plassmann, M.; Sandblom, O.; Macleod, M. Identification of Chain Scission Products Released to Water by Plastic Exposed to Ultraviolet Light. Environ. Sci. Technol. Lett., 2018, 5(5), 272–276.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. available at http://advances.sciencemag.org/ doi:10.1016/j.ecolmodel.2010.08.013.

Hidalgo-Ruiz, V.; Gutow, L.; Thompson, R.C; Thiel, M. Microplastics in the Marine Environment: A review of the methods used for identification and quantification. Environ. Sci. Technol., 2012, 46(6), 3060-3075.

Hufnagl, B., Stibi, M., Martirosyan, H., Wilczek, U., Möller, J.N., Löder, M.G.J., Laforsch, C., Lohninger, H., 2022. Computer-assisted analysis of microplastics in environmental samples based on μFTIR imaging in combination with machine learning. Environ. Sci. Technol. Lett. 9, 90–95. https://doi.org/10.1021/acs.estlett.1c00851.

Käppler, A.; Fischer, D.; Oberbeckmann, S.; Schernewski, G.; Labrenz, M.; Eichhorn, K.-J., Voit, B. Analysis of environmental microplastics by vibrational microspectroscopy: FTIR, Raman or both? Anal. Bioanal. Chem., 2016, 408(29), 8377-8391.

Karlsson, T.M.; Grahn, H.; Van Bavel, B.; Geladi, P. Hyperspectral imaging and data analysis for detecting and determining plastic contamination in seawater filtrates. J. Near Infrared Spectrosc., 2016, 24, 141-149.

Kedzierski, M., Falcou-Préfol, M., Kerros, M.Emmanuelle, Henry, M., Pedrotti, L., Bruzaud, S., 2019. Amachine learning algorithmfor high throughput identification of FTIR spectra: 1 application on microplastics collected in the Mediterranean Sea 2. available at https://www. elsevier.com/open-access/userlicense/1.0/ doi: 10.1016/j.chemosphere.2019.05.113.

Koelmans, A.A.; Besseling, E.; Shim, W.J. Nanoplastics in the Aquatic Environment. Critical Review, in Marine Anthropogenic Litter, Bergmann, M.; Gutow, L.; Klages, M., 2015, Springer International Publishing: Cham. p. 325-340.

Kopke K., Meyers N., Dozier A., Fitzgerald E., Power O-P., Agnew S., Everaert G., De Witte B., (2023a). Scientist Perspectives on the Cost-Effectiveness of Microplastic Analysis Methods for Seawater Samples: ANDROMEDA Workshop 1 Event Summary & Participant Recommendations on Cost-effectiveness. JPI Oceans project.

Kopke K., Meyers N., Dozier A., Fitzgerald E., Power O-P., Agnew S., Sempéré R., (2023b). Policy & Decision Makers Perspectives on the Cost-Effectiveness of Microplastic Analysis Methods for Seawater Samples: ANDROMEDA Workshop 2 Event Summary & Participant Recommendations on Cost-effectiveness. JPI Oceans project.

Lambert, S.; Wagner, M. Characterisation of nanoplastics during the degradation of polystyrene. Chemosphere, 2016, 145, 265-268.

Lips, I.; Lips, U. The importance of Mesodinium rubrum at post-spring bloom nutrient and phytoplankton dynamics in the vertically stratified Baltic Sea. Front. Mar. Sci., 2017, 4, 1-16.

Maes, T.; Jessop, R.; Wellner, N.; Haupt, K.; Mayes, A.G. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. Sci. Rep., 2017, 7, 44501.

Masura, J., Baker, J., Foster, G., & Arthur, C. (2015). Laboratory Methods for the Analysis of Microplastics in the Marine Environment: Recommendations for quantifying synthetic particles in waters and sediments.

Maxwell, H.S., Melinda, K.F., Matthew, G., 2020. Counterstaining to separate nile red-stained microplastic particles from terrestrial invertebrate biomass. Environ. Sci. Technol. 54, 5580–5588. https://doi.org/10.1021/acs.est.0c00711.

Meyers, N.; De Witte, B.; Everaert, G.; Hostens, K.; Janssen, C. (2022). Detection and identification of microplastics in biota using Nile red and machine learning: validation of an innovative, cost-effective approach, in: Mees, J. et al. Book of abstracts – VLIZ Marine Science Day, Online event 2 March 2022. VLIZ Special Publication, 88: pp. 20-21

Meyers, N., Catarino, A.I., Declercq, A., Brenan, A., Devriese, L., Michiel, Vandegehuchte, de Witte, B., Janssen, C., Everaert, G., 2021a. RGB-statistics derived from Nile red-stained reference plastics for the construction of the PDM (Plastics Detection Model). Mar. Data Arch. https://doi.org/10.14284/512.

Meyers, N., Catarino, A.I., Declercq, A., Brenan, A., Devriese, L., Vandegehuchte, M., deWitte, B., Janssen, C., Everaert, G., 2021b. RGB-statistics derived from Nile red-stained reference plastics for the construction of the PIM (Polymer identification Model). Mar. Data Arch. https://doi.org/10.14284/511.
Meyers, N., 2021c. NelleMeyers/RGB_models: updated release RGB models for microplastic analysis. available atZenodo. https://zenodo.org/record/5596531.

Mintenig, S.M.; Bäuerlein, P.S.; Koelmans, A.A.; Dekker, S.C.; van Wezel, A.P. Closing the gap between small and smaller: towards a framework to analyse nano- and microplastics in aqueous environmental samples. Environ. Sci-Nano, 2018, 5(7), 1640-1649.

Mitrano, D. M., Wick, P., & Nowack, B. (2021). Placing nanoplastics in the context of global plastic pollution. *Nature Nanotechnology*, *16*(5), 491-500.

Nel, H.A., Chetwynd, A.J., Kelleher, L., Lynch, I., Mansfield, I., Margenat, H., Onoja, S., Goldberg Oppenheimer, P., Sambrook Smith, G.H., Krause, S., 2021. Detection limits are central to improve reporting standards when using Nile red for microplastic quantification. Chemosphere 263. https://doi.org/10.1016/j.chemosphere.2020.127953.

Neukermans, G., Ruddick, K., Loisel, H., & Roose, P. (2012). Optimization and quality control of suspended particulate matter concentration measurement using turbidity measurements. Limnology and Oceanography: Methods, 10(12), 1011-1023.

Office of Tax Analysis (1990), "Depreciation of Scientific Instruments," Washington, Office of Tax Analysis, U.S. Department of the Treasury, March.

Paluselli, A.; Aminot, Y.; Galgani, F.; Net, S.; Sempéré, R. Occurrence of Phthalate Acid Esters (PAEs) in the Northwestern Mediterranean Sea and the Rhone River. Prog. Oceanogr.,2018a, 163, 221–231.

Paluselli, A.; Fauvelle, V.; Schmidt, N.; Galgani, F.; Net, S.; Sempéré, R. Distribution of Phthalates in Marseille Bay (NW Mediterranean Sea). Sci. Total Environ., 2018n, 621, 578–587.

Paluselli, A.; Fauvelle, V.; Galgani, F.; Sempéré, R. Phthalate Release from Plastic Fragments and Degradation in Seawater. Environ. Sci. Technol., 2019, 53,166–175.

Paul, A., Wander, L., Becker, R., Goedecke, C., Braun, U., 2019. High-throughput NIR spectroscopic (NIRS) detection of microplastics in soil. Environ. Sci. Pollut. Res. 26. https://doi. org/10.1007/s11356-018-2180-2.

Pekel, E., 2020. Estimation of soil moisture using decision tree regression. Theor. Appl. Climatol. 139, 1111–1119. https://doi.org/10.1007/s00704-019-03048-8.

Poulain, M.; Mercier, M. J.; Brach, L.; Martignac, M.; Routaboul, C.; Perez, E.; Desjean, M. C.; ter Halle, A. Small Microplastics as a Main Contributor to Plastic Mass Balance in the North Atlantic Subtropical Gyre. Environ. Sci. Technol., 2019, 53(3), 1157-1164.

Prata, J.C.; da Costa, J.P.; Duarte, A.C.; Rocha-Santos, T. Methods for sampling and detection of microplastics in water and sediment : a critical review. Trac-Trend Anal. Chem., 2019, 110, 150-159.

Prata, J.C., Reis, V., Matos, J.T.V., da Costa, J.P., Duarte, A.C., Rocha-Santos, T., 2019. A new approach for routine quantification of microplastics using Nile red and automated software (MP-VAT). Sci. Total Environ. 690, 1277–1283. https://doi.org/10.1016/j.scitotenv.2019.07.060.

Primpke, S., Christiansen, S.H., Cowger, W., de Frond, H., Deshpande, A., Fischer, M., Holland, E.B., Meyns, M., O'Donnell, B.A., Ossmann, B.E., Pittroff, M., Sarau, G., Scholz-Böttcher, B.M., Wiggin, K.J., 2020. Critical assessment of analytical methods for the harmonized and cost-efficient analysis of microplastics. Appl. Spectrosc. 74. https://doi.org/10.1177/0003702820921465.

Sancataldo, G., Avellone, G., Vetri, V., 2020. Nile red lifetime reveals microplastic identity. Environ Sci Process Impacts 22, 2266–2275. https://doi.org/10.1039/D0EM00348D.

Serranti, S.; Palmieri, R.; Bonifazi, G. ; Cozar, A. Characterization of microplastic litter from oceans by an innovative approach based on hyperspectral imaging. Waste Manag., 2018, 76, 117-125.

Shim, W.J.; Song, Y.K.; Hong, S.H.; Jang, M. Producing fragmented micro- and nano-sized expanded polystyrene particles with an accelerated mechanical abrasion experiment. , in 23rd Annual SETAC Meeting. 2014: Basel, Switzerland. p. 238.

Shruti, V.C., Pérez-Guevara, F., Roy, P.D., Kutralam-Muniasamy, G., 2022. Analyzing microplastics with Nile red: emerging trends, challenges, and prospects. J. Hazard. Mater. 423, 127171. https://doi.org/10.1016/j.jhazmat.2021.127171.

Stanton, T., Johnson, M., Nathanail, P., Gomes, R.L., Needham, T., Burson, A., 2019. Exploring the efficacy of Nile red in microplastic quantification: a costaining approach. Environ. Sci. Technol. Lett. 6. https://doi.org/10.1021/acs.estlett.9b00499.

Sturm, M.T., Horn, H., Schuhen, K., 2021. The potential of fluorescent dyes—comparative study of Nile red and three derivatives for the detection of microplastics. Anal. Bioanal. Chem. 413. https://doi.org/10.1007/s00216-020-03066-w.

Suaria, G., Achtypi, A., Perold, V., Lee, J.R., Pierucci, A., Bornman, T.G., Aliani, S., Ryan, P.G., 2020. O C E A N O G R A P H Y Microfibers in oceanic surface waters: a global characterization. http://advances.sciencemag.org/.

Ruggero, F., Gori, R., Lubello, C., 2020. Methodologies for microplastics recovery and identification in heterogeneous solid matrices: a review. J. Polym. Environ. 28, 739–748. https://doi.org/10.1007/s10924-019-01644-3.

Tan, S., Taylor, J. A., & Passeport, E. (2022). Efficient Prediction of Microplastic Counts from Mass Measurements. ACS ES&T Water, 2(2), 299-308.

Ter Halle, A.; Jeanneau, L.; Martignac, M.; Jardé, E.; Pedrono, B.; Brach, L.; Gigault, J. Nanoplastic in the North Atlantic Subtropical Gyre. Environ. Sci. Technol., 2017, 51(23), 13689-13697.

The World Bank, World Development Indicators (2012). GNI per capita, Atlas method (current US$). Retrieved from https://data.worldbank.org/indicator/NY.GNP.PCAP.CD?view=map&year=2021.

Thornton Hampton, L. M., Brander, S. M., Coffin, S., Cole, M., Hermabessiere, L., Koelmans, A. A., & Rochman, C. M. (2022). Characterizing microplastic hazards: which concentration metrics and particle characteristics are most informative for understanding toxicity in aquatic organisms?. Microplastics and Nanoplastics, 2(1), 1-16.

Velzeboer, I.; Kwadijk, C.J.A.F.; Koelmans, A.A. Strong Sorption of PCBs to Nanoplastics, Microplastics, Carbon Nanotubes, and Fullerenes. Environ. Sci. Technol., 2014, 48(9), 4869-4876.

Wegner, A., Besseling, E.; Foekema, E.M.; Kamermans, P.; Koelmans, A.A. Effects of nanopolystyrene on the feeding behavior of the blue mussel (Mytilus edulis L.). Environ. Toxicol. Chem., 2012, 31(11), 2490-2497.

Weisser, J., Beer, I., Hufnagl, B., Hofmann, T., Lohninger, H., Ivleva, N.P., Glas, K., 2021. From the well to the bottle: identifying sources of microplastics in mineral water. Water (Switzerland) 13. https://doi.org/10.3390/w13060841.

Wagner, S.; Hüffer, T.; Klöckner, P.; Wehrhahn, M.; Hofmann, T; Reemtsma, T. Tire wear particles in the aquatic environment - a review on generation, analysis, occurrence, fate and effects. Water Res., 2018, 139, 83-100.

ANNEXES

A. MP extraction from seawater samples – Standard Operating Procedure

1) Objective

To isolate microplastic particles down to 20 µm from seawater samples in a rapid, cost-efficient and validated way using digestion steps (KOH + H2O2) and density separation steps (NaI).

2) Material and equipment

Material:

Glassware
- Conical flask
- Closed petri dish
- Large glass slide

Filtration
- Filtering apparatus or filtration manifold set (for Whatmann filters):
  - (Filtration manifold set)
  - Glass funnel(s) with dust cover
  - Fritted glass base(s) with stopper(s)
  - Aluminium clamp(s)
  - Vacuum pump
  - Rubber tubing
  - Büchner flask (1 L)

Laboratory machinery
- Centrifuge
- Multi-position digital magnetic hotplate stirrer
- µ-FTIR and software – for questions on how to use this equipment, please consult the user manual.

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

Other laboratory equipment

- Metal sieve(s)
→ If little sediment is present, use a sieve of ⌀ 10 cm and small mesh size, e.g. 20 µm. If a lot of sediment is present, start with a larger sieve of ⌀ 20 cm and similar mesh size. If subdivision into size classes is desired, stacked large sieves can be used, e.g. of 5 mm, 1 mm, 250 µm and 20 µm.

- A large and a small metal funnel
- Lab support stand with 2 utility clamps
- Magnetic stirring bar (8 mm)
- Tweezers
- Cotton lab coat, nitrile protection gloves and lab goggles (µ-FTIR).
- Milli-Q water
- Bucket (5 L) to recover filtered salt solution/to catch filtered seawater.

Reagents:

- Acetone
- Hydrogen peroxide, (33 %)
- Potassium hydroxide (10 %)
- Nile Red stain
- Liquid nitrogen (µ-FTIR)

3) Procedures

3.1) High concentration of organic matter: digestion

If the concentration of organic matter is too high to allow direct filtration, a digestion step prior to filtration (and density separation) needs to be performed. If this this is not the case, skip this step.

1. Set up the sieving system using a lab support stand, a large stainless steel funnel, a small stainless funnel, two clamps to mount the 2 funnels and the sieve of ⌀ 10 cm (20 µm)* (Fig. 1).
2. Place the small sieve in the mounted small funnel, and position the largest funnel above the smallest one until the tip of the largest funnel almost touches the sieve inside the smallest funnel.
3. Sieve the sample carefully and rinse the bottle multiple times until all sample material is gone.
4. Carefully transfer the sieved material into a conical flask by placing the sieve upside down in a funnel mounted above the flask, and by washing the sieve thoroughly with approx. 100 mL MilliQ water (e.g. with a wash bottle).
5. Add potassium hydroxide (KOH, 10 % solution) made with Milli-Q water or filtered water to the sample at a 1:3 volume sample:solution ratio, add a magnetic stirring rod and leave the sample to digest for 48h on a magnetic hotplate stirrer at 60°C and 150 rpm.
6. Sieve the digested solution again and transfer to a new conical flask with approx. 100 mL Milli-Q water following the same protocol.
7. Add hydrogen peroxide (H_2O_2, 33 % solution) to the sample at a 1:1 volume sample:solution ratio, add a magnetic stirring bar and leave the sample to digest for another 48h on a magnetic hotplate stirrer at 60°C and 150 rpm. If not all organic matter is digested after this step, leave the solution to digest for another 24 h. If needed an additional digestion step using hydrogen peroxide (H_2O_2, 33 % solution) can be added following the same procedure.

Notes:

1. If the sediment content is high, samples can be sieved using a larger sieve first, after which the sampled material is transferred to a smaller sieve in different sub steps using the sieve setup. The large sieve is placed upside down in the upper funnel as is done for the normal procedure.

2. If subdivision into size classes is desired, stacked large sieves can be used, after which the sample material of each sieve transferred to smaller sieves of similar mesh size using the sieve setup.

*3. If the starting sample volume is small, the first sieving step is redundant and the KOH solution can be added at a 1:3 volume sample:solution ratio.

3.2) Presence of sediment: density separation

If the seawater sample contains sediment, density separation steps using a saturated sodium iodide solution (NaI – 1.8 g/cm³, Frias et al. 2018) are necessary to extract all plastics. Because of the high density of the salt solution, plastics with a density below 1.8 g/cm³ should float. Sodium tungstate dehydrate ($Na_2WO_42H_2O$ – 1,4 g/cm³) or even sodium chloride (NaCl - 1,2 g/cm³) solutions can be used as more economical alternatives, but plastics with relatively high densities such as polycarbonate (PC - 1.20-1.22 g/cm³), polyurethane (PU - 1.20-1.26 g/cm³), polyethylene terephthalate (PET - 1.38-1.41 g/cm³) and polyvinyl chloride (PVC - 1.38-1.41 g/cm³) will not be separated when using NaCl.

$Na_2WO_42H_2O$ cannot be used to extract PVC particles either. If the sample does not contain sediment, skip this step.

1. Set up the sieving system (Fig. 1) using a laboratory stand, a large stainless steel funnel, a small stainless funnel, two clamps to mount the funnels above each other and a sieve of ⌀ 10 cm (20 µm). See notes under 3.1.
2. Place the small sieve in the mounted small funnel, and position the largest funnel above the smallest one until the tip of the largest funnel almost touches the sieve in the smallest funnel.
3. Sieve the sample carefully and rinse the bottle multiple times until all sample material is gone.
4. Carefully transfer the sieved material into a falcon tube mounted below the small funnel: place the sieve upside down in the upper funnel and wash the sieve thoroughly with approx. 50 mL sodium iodide solution in a wash bottle.
5. Centrifuge the sieved material at 1000 rpm (216 x g-force) for 5 minutes
6. Carefully filter the supernatant using the filtering apparatus or filtration manifold set and intercept the salt solution so that it can be recycled.
7. Suspend the pellet again using the recycled salt solution. Centrifuge again using the same settings, and filter the supernatant on the same filter. Make sure the centrifugation step is repeated at least 3 times.

3.3) Filtration

Sample filtration is performed using the filtration setup or filtration manifold set (Fig.), a vacuum pump and filters compatible for µ-FTIR analysis, such as PTFE membrane filters (10 µm).

1. Set up the manifold system and assemble the tubing correctly so that a vacuum is created (see Fig. 1).
2. Carefully place a filter on the membrane holder(s), clamp the funnel(s), open the valves if using the manifold set and turn on the filtration pump.
3. Wash the walls of the filter apparatus thoroughly to ensure all plastic particles are recovered on the filter.
4. Switch off the pump again once the sample is filtered. Leave the filter in place.

3.4) Nile Red staining

1. While still on the filter apparatus, cover the filter with 1 mL Nile Red (10 µg/mL acetone) using a glass Pasteur pipette. Ensure that the walls of the filter apparatus are covered to recover lost particles onto the filter, and verify that the whole filter is covered with Nile Red. Leave the particles to stain for 10-15 minutes.
2. Turn on the pump and rinse the filter thoroughly with Milli-Q water to remove the acetone.
3. Switch off the pump and carefully remove the clamp and the funnel.
4. Carefully transfer the filter to a labelled, clean glass slide using clean tweezers. Place the glass slide in a labelled petri dish and leave it to dry. Make sure the petri dish is covered with a lid to avoid airborne plastic contamination until fluorescence microscopy analysis takes place.
5. For µ-FTIR analysis, leave the filter to dry for at least one week at room temperature (21°C).

B. MP extraction from sediment samples – Standard Operating Procedure

1) Objective

To isolate microplastic particles down to 20 µm from sediment samples in a rapid, cost-efficient and validated way using density separation steps (NaI) and a digestion step (H_2O_2).

2) Material and equipment

Material per sample:

Glassware
- 3 beakers
- Closed petri dish
- Large glass slide

Filtration
- Filtering apparatus or filtration manifold set (for Whatmann filters):
  - (Filtration manifold set)
  - Glass funnel with dust cover
  - Fritted glass base with stopper
  - Aluminium clamp
  - Vacuum pump
  - Rubber tubing
  - Büchner flask (1 L)

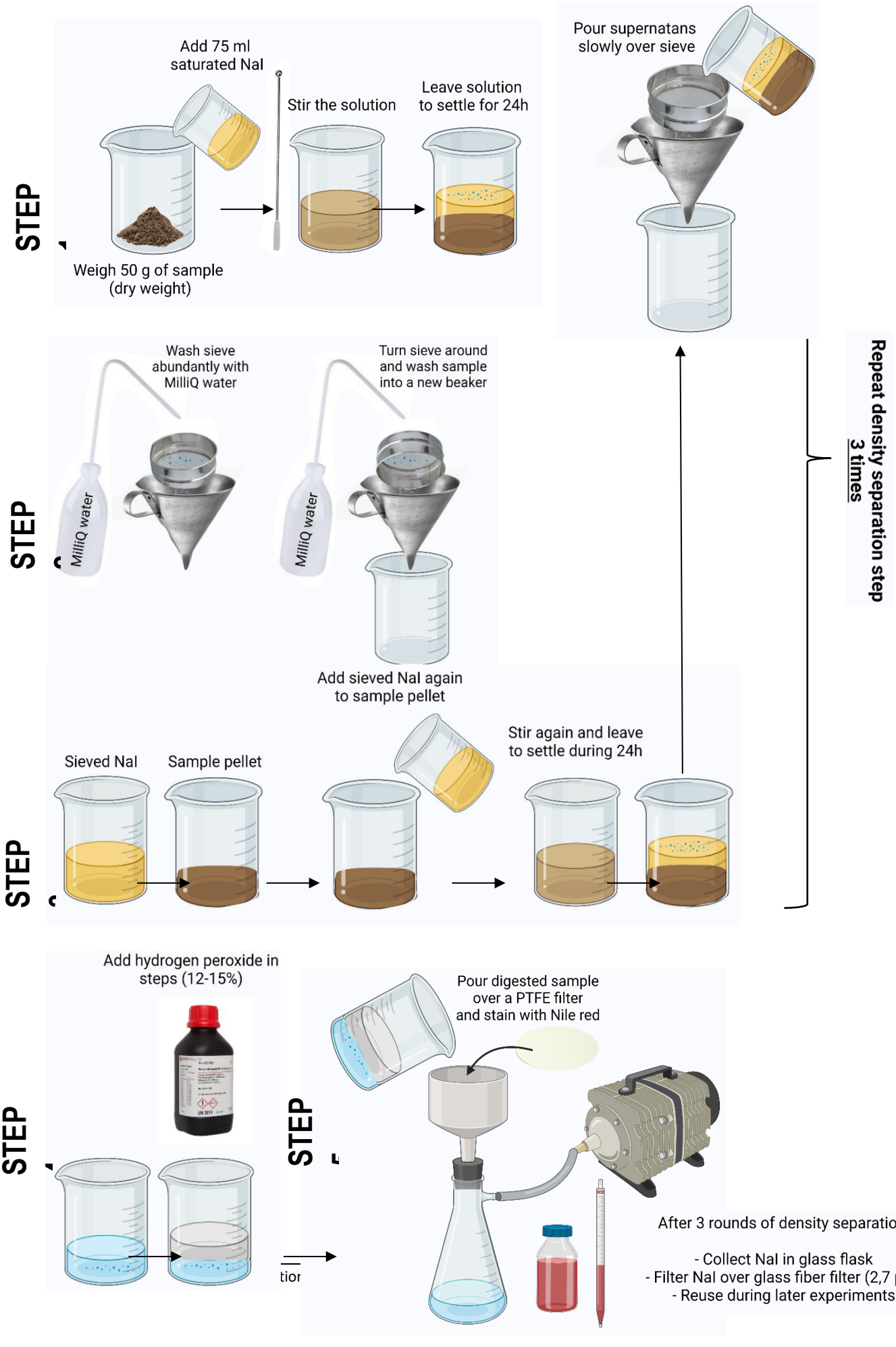
Laboratory machinery
- µ-FTIR and software: if quality check is required. For questions on how to use this equipment, please consult the user manual.

Laboratory consumables
- Filters compatible for µ-FTIR analysis, e.g. PTFE membrane filters (10 µm, ⌀ 47 mm)

Other laboratory equipment
- Metal sieve of ⌀ 5 cm and mesh size 20 µm.
- A small metal funnel
- Tweezers
- Cotton lab coat; nitrile protection gloves and lab goggles (µ-FTIR).
- Milli-Q water
- Metal spatula

Reagents:
- Acetone
- Hydrogen peroxide, (33 %)
- Sodium Iodide (100%)
- Nile Red stain
(- Liquid nitrogen (µ-FTIR))

3) Procedures



- Add a large amount of sediment to a glass beaker (at least 150 g). note down the weight of the sample, of the beaker and of both together. Cover the glass beaker with a petri dish, so that the pouring end is the only non-covered part where evaporation will take place. If the moisture level is really high, you can choose to spread out the sediment in a large petri dish (e.g. 200 mm) and cover this with aluminium foil.

- Put the glass beaker/petri dish in a drying oven for at least 24h, at 50°C. Depending on the level of moisture in the sample, you will need to leave it for another 24h.

- Weight 50g of the dried sample in a new glass beaker (preferably a not too broad beaker, otherwise the NaI-level will be low, complicating the density separation process.

- Prepare a saturated NaI solution with MilliQ water (1793 g/L). You will need around 75 ml/sample. Be aware that a large quantity of NaI needs to be dissolved, best way to do this is by adding NaI to a glass beaker filled with the appropriate volume of MilliQ water in small steps. While doing so the glass beaker should contain a rotating stirring rod and should be put on a magnetic plate (rpm can be high) and should be heated to 50°C to speed up to dissolution process. Caution! Once the solution is saturated, it will turn yellow and can leave very distinct yellow stains behind.

- Add 75 ml of NaI (cooled down) to your sediment sample. Mix with e.g. a spoon, so that most MPs are in suspension, and leave the sediment to settle overnight (24h). Make sure the beaker is FULLY covered, e.g. with aluminium foil. If not covered completely covered you risk drying up of the sample, causing crystallisation and a lower NaI level in your beaker, complicating the density separation process.

- After 24h but before sieving the supernatans, gently push any remaining material that is stuck to the sides of your glass beaker into the settled solution. Because NaI us such a dense salt, most plastic will be floating and are thus separated from the sediment.

- Place a small sieve (20 µm mesh or smaller, Ø 50 mm) in a small metal funnel. Hold this construction above a new glass beaker, and poor the supernatans with floating MPs and organic material into the sieve after 24h of sample settlement. Make sure to stop the pouring on time so that no sediment goes onto the sieve. If you pour slowly, the floating particles will flow into your beaker faster than the sediment.

- Put the beaker containing the saturated NaI which you've just sieved aside and cover it. Take a new glass beaker, put the funnel and sieve on the beaker and pour MilliQ water ABUNDANTLY over the sieve containing the sample to remove ANY traces of the NaI. It is very important to clean the sample as good as possible as the smallest trace of NaI causes a exothermic reaction with a lot of effervescence when added to H_20_2. This needs to be avoided at all costs as it may ruin your sample.

- Once clean, place the funnel with sieve in a new glass beaker (preferably a large one, e.g. 500 ml). Turn the sieve around in the funnel and wash the sieve with MIlliQ, so that the sample is captured in the glass beaker. Wash the edges of the sieve above the funnel to make sure all traces of the sample are washed into the glass beaker. Also rinse the funnel as MPs may be stuck in it. Try to accomplish the transfer of the sample into the glass beaker using max. 50 ml. Cover this glass beaker.

- Pour the saturated NaI back on the sample pellet, stir the sample again and leave it to settle for another 24h.

- Repeat the described procedure two more times (= 3 density separations in a total of 72h), where you add the washed sample to the beaker with the sample you've washed before. You will end up with a beaker containing your sample (MPs + organic material) which you've washed from your sieve at three different points in time.

- Collect your saturated NaI in a glass bottle instead of pouring it on your sample pellet after the 3 round of density separation. Once you've recovered the NaI from all your samples, before recycling it for later use, make sure to filter the whole solution over a 2,7 µm filter using the filter apparatus.

- Next, organic material will be removed by adding H_20_2 (12-15%) to your sample. Take a volume H_20_2 which corresponds to 40-50% of your sample volume. For example, if your sample is now 150 ml, you will add a volume of H202 of 60-75 ml to the sample. Before adding the H_20_2, make sure to have a large beaker filled with some cold water in it ready. Add the h_20_2 STEP BY STEP by adding SMALL VOLUMES of H_20_2 each time. Like this, you avoid the exothermic reaction from taking place. You will observe some effervescence and the sample will likely turn yellow, but as long as the foaming doesn't rise a lot and the beaker doesn't become really hot, it is fine. In case you do observe a lot of foaming and the beaker getting hot, place the sample in the beaker filled with cold water. The cold water will slow down the exothermic reaction. Make sure to stay in the vicinity of your samples for at least half an hour and check them regularly until the exothermic reaction has slowed down. Leave the samples to digest for a week.

- Filter the samples over a PTFE filter (20 µm mesh) using a filtration apparatus. Rinse abundantly with MilliQ water. Add 1 ml of Nile red dissolved in acetone (10 µg/ml) homogeneously to the filter. Leave the soak for 15 min, then rinse abundantly with MilliQ. Transfer the filter to a labeled petri dish and leave to dry for 24h before photographing them under a fluorescence (stereo)microscope using the appropriate protocol.

Contamination measures to be taken:

- Wash all equipment/glassware you use 3x with tap water and 3x with MilliQ water.
- Cover your sample at all times.
- Always wear a cotton lab coat.
- Perform the experiment in a laminar flow chamber that's switched on.

- When switching between samples always clean the equipment you've used before using it for another sample.
- If possible, perform your experiments when not a lot of people are in the lab, to avoid airborne MP contamination. If people are present, make sure everyone is wearing a lab coat (or at least note down the colours/materials of the clothes other people in the lab are wearing).
- Make sure to take blanks while you are 1) sampling and 2) processing your samples.

C. MP extraction from biota samples – Standard Operating Procedure

1) Objective

To isolate MP particles down to 20 µm from biota samples such as mussels or fish gastrointestinal tracts (GITs) in a rapid, cost-efficient and validated way using a double digestion step (KOH and H_20_2) with the option of adding a density separation step.

2) Material and equipment

Material per sample:

Glassware
- 4 beakers
- Closed petri dish
-Glass slide

Filtration
Large filtering apparatus or filtration manifold set (with glass base stopper, fitting the stainless steel sieve)
(for Whatmann filters):
- (Filtration manifold set)
- Glass funnel with dust cover
- Fritted glass base with stopper (large)
- Aluminium clamp (large)
- Vacuum pump
- Rubber tubing
- Büchner flask (1 L)

Laboratory machinery
- µ-FTIR and software: if quality check is required. For questions on how to use this equipment, please consult the user manual.

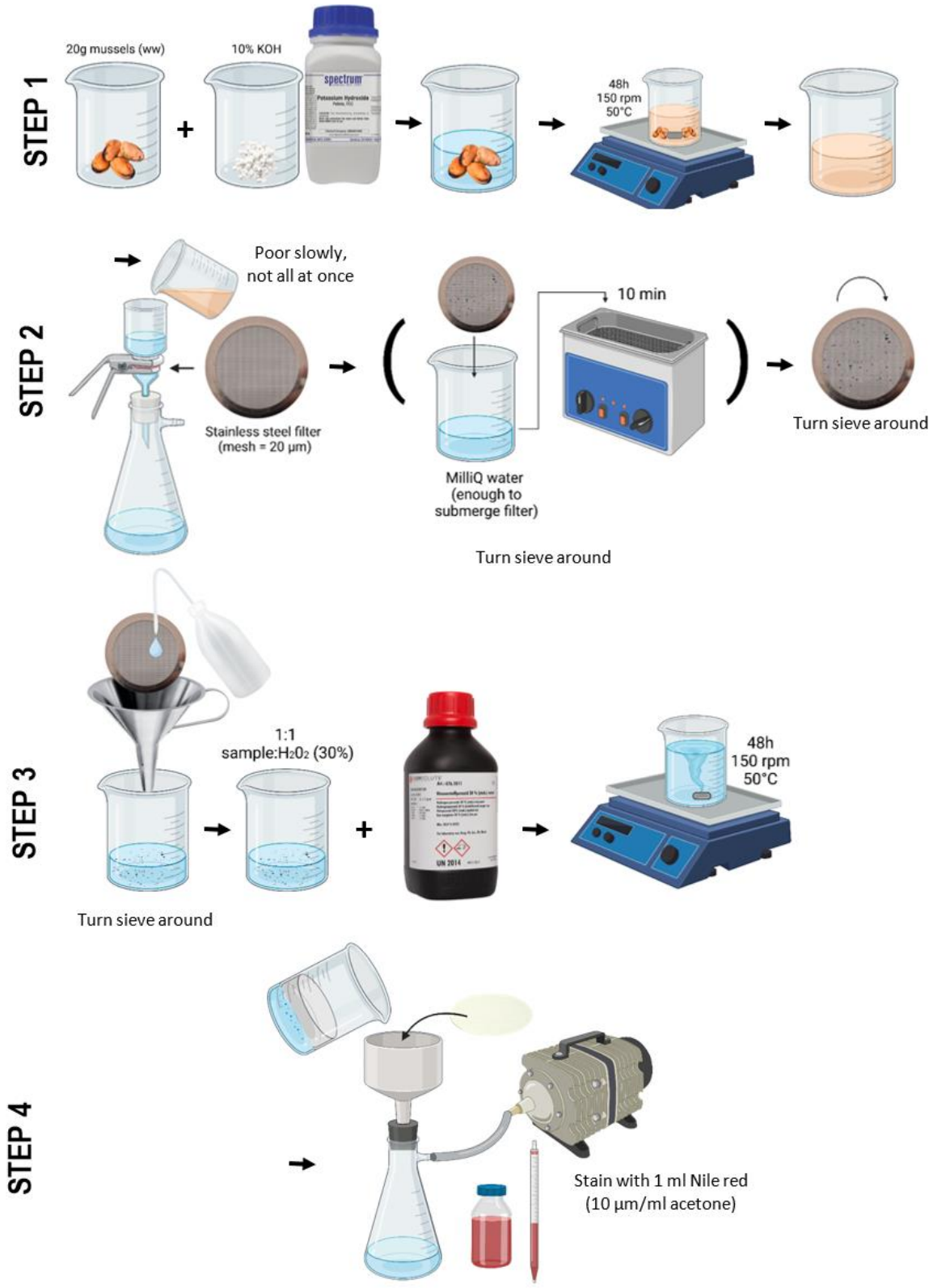
Laboratory consumables
- Filters compatible for µ-FTIR analysis,
e.g. PTFE membrane filters
(10 µm, ⌀ 47 mm)
- Pasteur pipette with rubber stop
- Aluminium foil

Other laboratory equipment

- Stainless steel sieve of ⌀ 5 cm and mesh size 20 µm.
- A small metal funnel
- Tweezers
- Cotton lab coat; nitrile protection gloves and lab goggles (µ-FTIR).
- Milli-Q water
- Metal spatula/spoon

Reagents:
- Acetone
- Hydrogen peroxide, (33 %)
- (Sodium Iodide (NaI, 100%))
- Nile Red stain
(- Liquid nitrogen (µ-FTIR))

3) Procedures



- Add 20g mussels (wet weight) to a glass beaker. You can easily open (partly frozen) mussels by wedging an oyster knife in the hinge of the mussel and then cutting through the muscles.

- Cover the glass beaker with a petri dish at or with aluminium foil at all times to avoid MP contamination.

- Add 200 ml potassium hydroxide (KOH, 10% solution) made with Milli-Q water or filtered water to the sample, add a rinsed magnetic stirring rod, cover the beaker again and leave the sample to digest for 48h on a magnetic hotplate stirrer at 60°C and 150 rpm.

- Filter the semi-digested sample over a stainless steel filter of 20 µm using a filtration apparatus attached to a pump and Büchner flask. Do this by very slowly pouring the sample over the middle of the stainless steel filter. Make sure the filtration apparatus with filtered sample is covered when not working.

- In case the filter still contains a lot of organic material that sticks to the filter (often the case for fish GITs of larger fish), it is advised to do an additional sonication step to loosen the particles present. If not, go straight to the 'rinsing step without sonication'. To do so, put the stainless steel filter carefully into a beaker that is slightly larger (2L) so that the filter can lay flat on the bottom of the beaker (lower it slowly with a metal spatula/spoon underneath), with the side containing the filtered particles facing upwards (). Add 70 ml of MilliQ water to the beaker (so that the filter is completely covered) and place the beaker in an ultrasonic bath for 10 min at a low frequency (around …) for 10 min. Make sure the beaker is covered and cannot fall over in the bath.

- Carefully take the filter out of the beaker using the metal spatula/spoon and vigorously rinse the filter with 30 ml MilliQ water into a new beaker using a volumetric pipette (10 ml, 3x) (or a wash bottle). Ideally, you try to stick to the minimum rinsing volume needed to effectively clean the filter, so that the volume of H_20_2 needed in the next step is kept low (as well as the associated costs).
- Pour the water (70 ml) from the beaker that contained the filter into this new beaker as well. Rinse the first beaker 5 times with MilliQ water with the volumetric pipette (or a wasg bottle, around 50 ml in total). In this way, the sample volume should now be 150 ml.

- Rinsing step without sonication: If no sonication took place, rinse the stainless steel filter into a new beaker using 150 ml of MilliQ in total using a wash bottle.

- After rinsing with or without sonication step, add H_20_2 (ca. 30%) in a 1:1 ratio to the sample. In this case, add 150 ml of H_20_2. Add a magnetic stirring rod to the sample, cover the beaker again and leave the sample to digest again for 48h on a magnetic hotplate stirrer at 60°C and 150 rpm.

If a lot of sediment is present in your sample, perform a density separation step as described underneath. If not, skip this step and go straight to the 'filtration step without density separation'.

- Perform the step in the ultrasonic bath again in the same way. This time you however replace MilliQ water with a saturated NaI-solution: fill the beaker containing the stainless steel filter with 70 ml NaI, and rinse the filter as well as the beaker with NaI.

- Leave the beaker containing the sample + NaI and let the sediment settle overnight (24h). Make sure the beaker is FULLY covered, e.g. with aluminium foil. If not covered completely covered you risk drying up of the sample, causing crystallisation and a lower NaI level in your beaker, complicating the density separation process.

- After 24h but before sieving the supernatans, gently wash any remaining material that is stuck to the sides of your glass beaker into the settled solution with a little NaI. Because NaI is such a dense salt, most plastic will be floating and are thus separated from the sediment.
- Filtration step with density separation: Carefully filter the supernatans of the settled NaI-solution over a PTFE filter (20 µm mesh) using a filtration apparatus. Pour slowly and make sure no/very little sediment gets on the filter. Before rinsing, switch to another Büchner flask, so that the satured NaI can be recycled. After switching flasks, rinse the PTFE-filter abundantly with MilliQ water. Add 1 ml of Nile red dissolved in acetone (10 µg/ml) homogeneously to the filter. Leave the soak for 15 min, then rinse abundantly with MilliQ. Transfer the filter to a labeled petri dish and leave to dry for 24h before photographing them under a fluorescence (stereo)microscope using the appropriate protocol.

- Filtration step without density separation: filter the samples over a PTFE filter (20 µm mesh) using a filtration apparatus. Rinse abundantly with MilliQ water. Add 1 ml of Nile red dissolved in acetone (10 µg/ml) homogeneously to the filter. Leave the soak for 15 min, then rinse abundantly with MilliQ. Transfer the filter to a labeled petri dish and leave to dry for 24h before photographing them under a fluorescence (stereo)microscope using the appropriate protocol.

Contamination measures to be taken:

- Wash all equipment/glassware you use 3x with tap water and 3x with MilliQ water.
- Cover your sample at all times.
- Always wear a cotton lab coat.
- Perform the experiment in a laminar flow chamber that's switched on.
- When switching between samples always clean the equipment you've used before using it for another sample.
- If possible, perform your experiments when not a lot of people are in the lab, to avoid airborne MP contamination. If people are present, make sure everyone is wearing a lab coat (or at least note down the colours/materials of the clothes other people in the lab are wearing).
- Make sure to take blanks while you are 1) sampling and 2) processing your samples.

D. Semi-automated MP analysis based on fluorescent imaging and ML models – Standard Operating Procedure

1) Objectives

To detect and analyse MPs extracted from various matrices, using random forest machine learning models.

2) Material and equipment

Computer with sufficient storage space and the programs ImageJ Fiji and R studio installed.
Download ImageJ Fiji (https://imagej.net/software/fiji/downloads)
Download R Studio (https://cran.rstudio.com/)
R codes and ImageJ macros: newest versions will be published on Github soon.

3) Procedures

1. Folder arrangement

1.1. Batch mode

Use the '*Batch mode*' script when you have a lot of photo series (blue, green and uv) which contain a low number of particles (<100) . If your photos are very large (250 Mb and over), it is advised not to use batch mode as this may cause the program to block (go to '*1.1.2 No batch mode'*).

1.2. No batch mode

Use the '*No batch mode*' script when you have few photos of large size (250 MB and over) containing a high number of particles (>100).

Create a folder called '*Automated_MP_analysis*', and three subfolders called '*Analyses*', '*Codes*' and '*Training_datasets*'. Within the latter, store the training dataset to run the models ('*PDM_FSM_ILVO_final.xlsx'* and '*PIM_FSM_ILVO_final.xlsx'*). Save the ImageJ code ('…') and the two R scripts ('…' and '…') under '*Codes*'. Next, create two new subfolders within the '*Analyses*' folder called '*PDM_visualisation*' and '*PIM_visualisation*'. Next, classify your sample images. If the batch mode script is used, create three subfolders within '*Automated_MP_analysis*': '*Photos*', '*Metadata*' and '*RGB*'. Store all images within 'Photos'.

If the non-batch mode script is used: Within '*Analyses*', create your sample-specific folders (e.g. '*LZO1*') and create a '*Photos*' as well as a '*RGB*' subfolder within each of these folders.

2. Image analysis

2.1 Naming of photos

For each photographed filter you have three photos, taken under the blue, green and UV filter. Store these under the 'Photos' folder per sample. If taken, brightfield photos should be stored in the sampel folder (here 'LZO1'). Generated csv-files will be saved under '*RGB*' after photo analysis in ImageJ.
For the code to run correctly, Photos should be named as followed: '*name of the filter*' +_+ '*number*' +_+ '*colour*' (in capital letters). '*number*' here can e.g. be your filter number if you have multiple filters per sample: 1 = filter 1; 2 = filter 2, etc. Example of names for each of the filters:

'*SeawaterLZO1_1_BLUE*'; '*SeawaterLZO1_1_GREEN*' and '*SeawaterLZO1_1_UV*'. It is important that the name exists of three parts separated by two '_'.

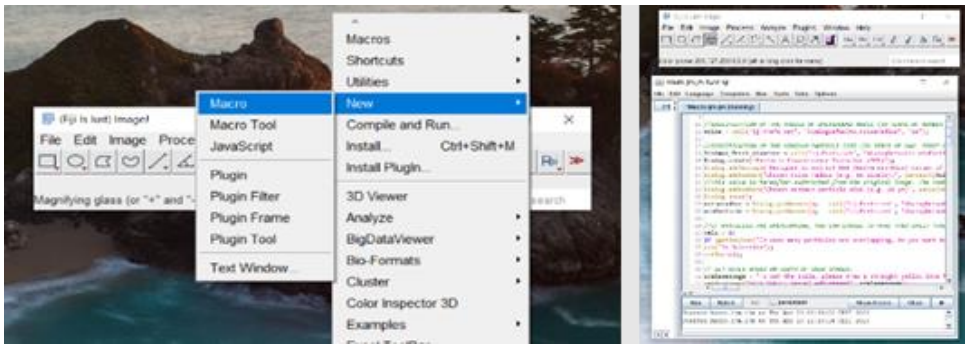
2.2 Batch mode

Use corresponding script.

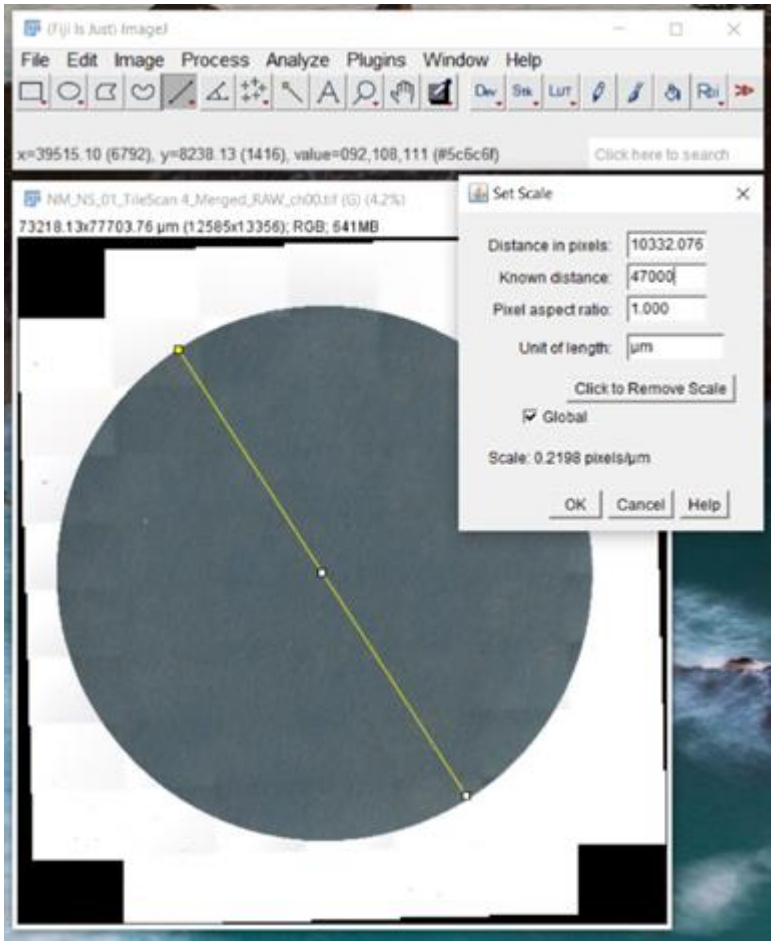
2.3 Analysis

2.3.1. ImageJ Fiji

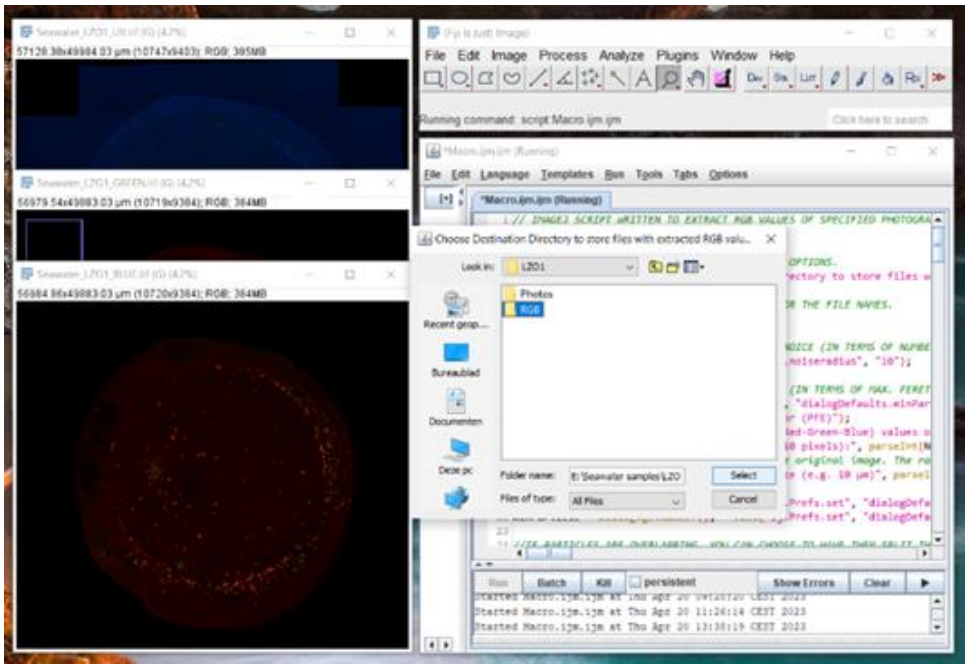
Open ImageJ Fiji. Open a new macro through 'Plugins' > 'New' > 'Macro'. Paste the script in the new window.



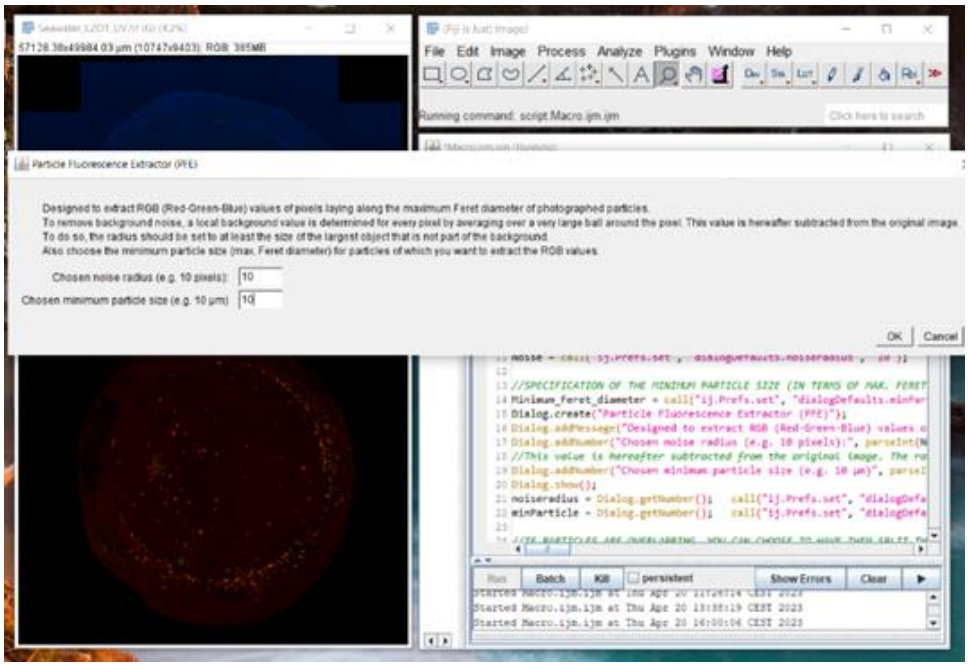
First, the correct scale needs to be set. To do so, use a brightfield photo of an unprocessed PTFE filter, acquired with the exact same microscope specifications as for the brightfield photo of the actual sample. To do so, open the photo ('File' > 'Open') and use the line-selection tool to draw the diameter of the PTFE filter. Next, set the scale ('Analyse' > 'Set scale'), enter the known length of the filter diameter under 'Known distance' in µm (in this case 47000 µm = 47 mm), enter 'µ' as unit of length, make sure 'Global' is checked so that this scaling is used for all photos while the program is still opened. Once scaled, it is best to note down the corresponding number of pixels per µm (here: 0.2198 pixels/µm), in case rescaling would be necessary in the future. Once this is done, press 'OK', and close the photo.



Open the blue filter photo ('File' > 'Open') in ImageJ followed by the green filter and lastly the UV filter (in this specific order). 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).

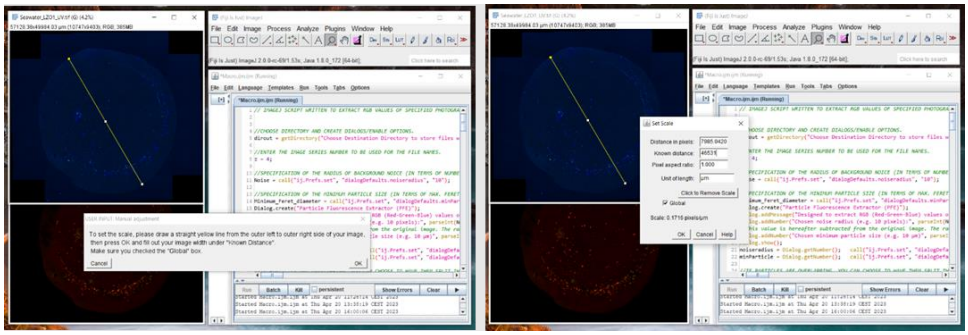


Next, a new dialog box pops up. Here, you first specify the noise radius the program should use (i.e. from what number of pixels onward a selected area should be considered a particle rather than noise). Next, you specify the minimum size of the recognized particles for which the RGB-values of the pixels on the Feret diameter should be extracted (e.g. '10' in both fields), then press 'OK'.



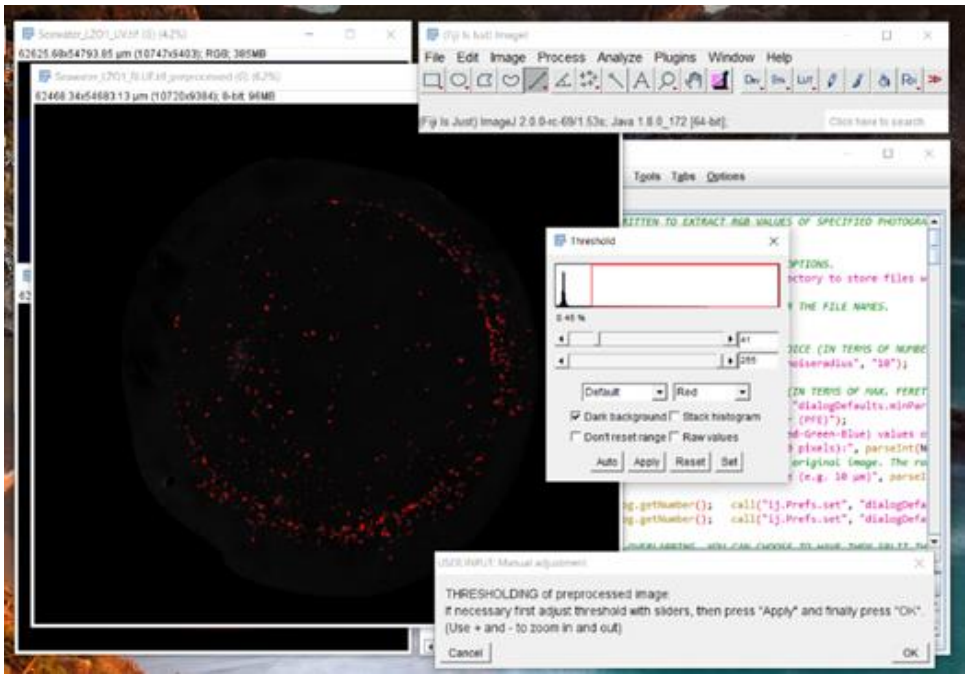
For the next step, you can choose for the program to recognize overlapping particles and split them into separate particles. (If the exact number of particles present is important and the number of overlapping particles is high, it is advised to choose 'no', as particles are sometimes mistakenly split into multiple particles).

Next, the program asks for the scale to be set. As The scale was already specified prior to this, but to verify the accuracy you draw the diameter of the filter (e.g. UV filter as borders are better visible) using the line-selection tool. Next, you press 'OK'. The length shown under 'known distance' should now approximate the diameter of the reference PTFE filter (47000 µm). As circularity of the current filters is not a 100%, the value will deviate a bit. Don't make any changes, but make sure the 'Global' box is still checked.

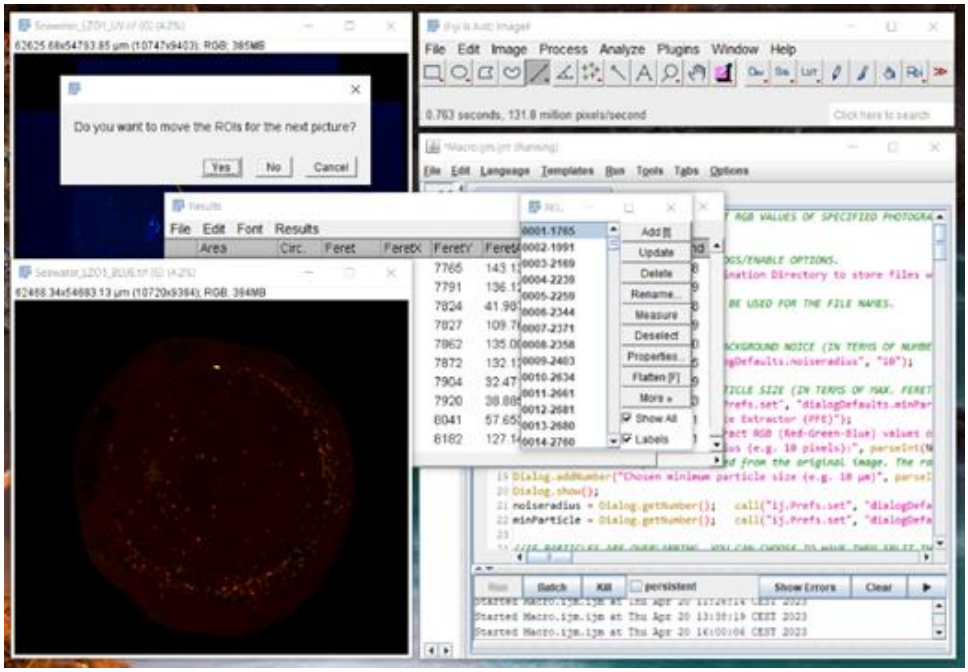


Hereafter, the blue filter photo will be processed (always the first opened photo): a threshold needs to be set to identify all particles present. To do so, move the upper slider bar until all particles are red. Make sure this specific window is selected, otherwise you won't observe any changes when adjusting the slider bar. Next, press 'Apply'. Now, all selected red particles turn white. Following this,

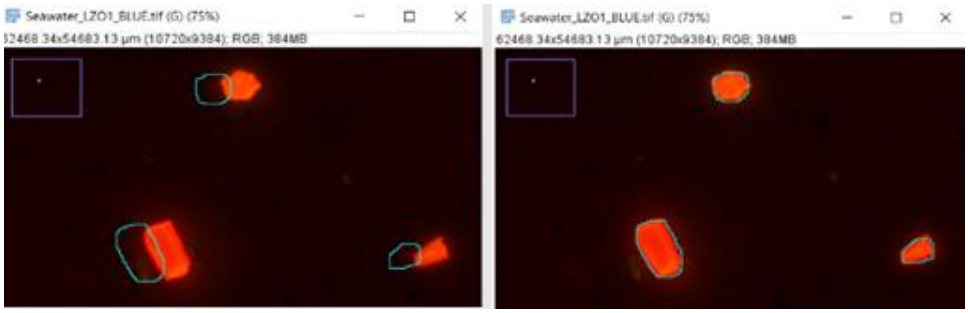
press ‘ok’ (it is important to respect the order of pressing ‘Apply’ first followed by ‘ok’, and not the other way around).



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.



Once the program is ready, all particles will be selected in blue. Select the line selection tool if not selected, then put your 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. You can zoom in to do so if necessary (advised).



Next, press 'ok'. Following this, RGB-values files will be generated for all particles on the blue filter. If the selection would turn yellow, you won't be able to move it anymore. 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.

Note: when using the batch mode script, the next series of three photos of a sample (blue, green and uv) 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 the '*Photos*' are processed.

2.3.2. R Studio

2.3.2.1 Generating RGB dataset

'*No batch mode*' script

Go to the folder 'RGB' of a specific sample.

_particle_measurements_4_details.csv	20/04/2023 16:24	CSV-bestand van ...	70 kB
_particle_measurements_4_details.xls	20/04/2023 16:24	Microsoft Excel 97...	70 kB
numbered_particles_1.tif	20/04/2023 16:24	TIF-bestand	294.717 kB
numbered_particles_2.tif	20/04/2023 16:24	TIF-bestand	294.689 kB
numbered_particles_3.tif	20/04/2023 16:24	TIF-bestand	296.057 kB
Seawater_4_numbered_particles.tif	20/04/2023 16:22	TIF-bestand	98.241 kB
Seawater_LZO1_BLUE.tif_4_p1.csv	20/04/2023 16:24	CSV-bestand van ...	3 kB
Seawater_LZO1_BLUE.tif_4_p2.csv	20/04/2023 16:24	CSV-bestand van ...	1 kB
Seawater_LZO1_BLUE.tif_4_p3.csv	20/04/2023 16:24	CSV-bestand van ...	1 kB
Seawater_LZO1_BLUE.tif_4_p4.csv	20/04/2023 16:24	CSV-bestand van ...	2 kB

'*numbered_particles_1.tif*' is the blue filter photo with numbered particles, '*numbered_particles_2.tif*' is the green filter photo with numbered particles, and '*numbered_particles_3.tif*' is the uv filter photo with numbered particles. Seawater_4_numbered_particles.tif is black-white outline of all analysed particles, here particles are also numbered. When opening the metadata file '*particle_measurements_4_details.xls*', In column 'A' the particles numbers can be found which correspond with the numbered particles in the *'Seawater_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 (Feret diameter) of the corresponding particles can be found.

For every particle on the three 'numbered_particles' photos', a csv-file per filter can be found, e.g. for particle 1, a file '*Seawater_LZO1_BLUE.tif_4_p1.tif*', '*Seawater_LZO1_GREEN.tif_4_p1.tif*' and '*Seawater_LZO1_UV.tif_4_p1.tif*' can be found.

These files contain RGB data for each pixel located on the 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.

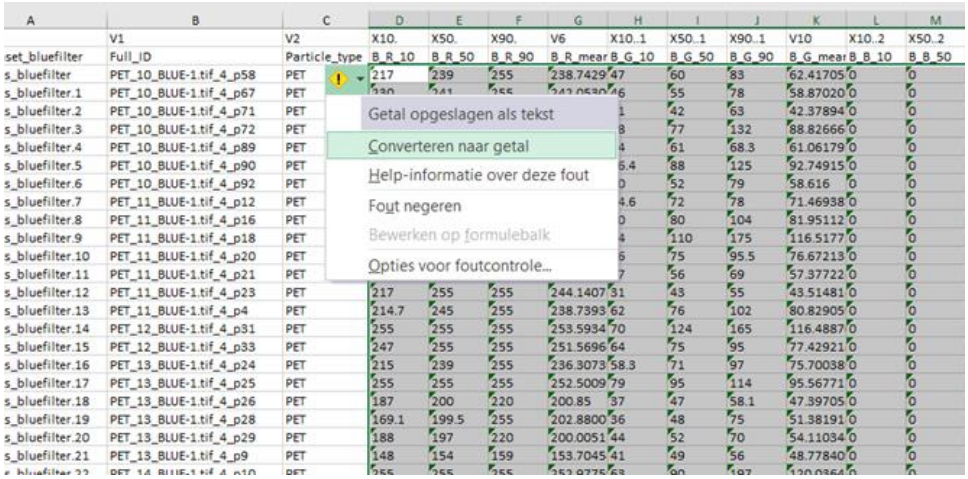
In the next step, the mean, median, 10th and 90 percentile (statistics) of each time the red, green and blue value of all analysed particle pixels (= per csv file) will be calculated in R.

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.

Next, open the '*Rcode_creation_RGBdataset*' script in R. First, adapt the working directory to the directory where the BLUE csv-csv-files are stored (setwd("…/Automated_MP_analysis/LZO1/RGB/BLUE").

Also adapt the working directories for the Green csv-files (setwd("…/Automated_MP_analysis/LZO1/RGB/GREEN") and the UV csv-files (setwd("…/Automated_MP_analysis/LZO1/RGB/UV") further on in the script. Also change the working directory to the location where the UV csv-files are stored after 'Export'. Lastly, choose a name for the generated dataset (e.g. *Seawater_LZO1_RGBdataset*' → Filename<- paste(Date," _Seawater_LZO1_RGBdataset'.xlsx",sep= "")). Now run the whole script. After this, a dataset containing all particle statistics will be generated in the UV folder.

Next, open a new Excel-file and copy the heading (first row) of the file '*Heading_RGB_dataset.xlsx*' in the first row of this file. Save this file using the same name followed by '_2' ("Date_Seawater_LZO1_RGBdataset_2"). Once this is done, open the dataset generated in R studio ("Date_Seawater_LZO1_RGBdataset"), and copy all particle-specific names in column B underneath 'Full_ID' in the new dataset ("Date_Seawater_LZO1_RGBdataset_2") under 'Full_ID'. Following this, replace all empty cells underneath 'Material' with 'unknown'. Next, 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'. Next, adapt all cells so that the values have 0 decimals. To complete the dataset, copy the adapted selection and paste it in the new Excel-file underneath the heading. Right now the dataset with RGB statistics of photographed particles is ready. Developing a dataset of known particles to train and test your own ML model is done the same way, except the content of all cells under 'Particle type' in the generated dataset is copied and pasted under 'Material' in the new dataset.



2.3.2.2 Model predictions

2.3.2.2.1 Plastic Detection Model

For the last step, open the '*Rcode_model_prediction_ILVO_FSM'* code in R.

Adapt the code under 'Set working directory' to the location where the training dataset is located.
→setwd("…/Automated_MP_analysis/Trainingdatasets")

Adapt the code under 'Training dataset' to the PDM dataset:

"RGB_dataset <- read_excel("PDM_FSM_ILVO_final.xlsx")". Make sure to put a '#' in front of "RGB_dataset <- read_excel("PIM_FSM_ILVO_final.xlsx")".

Adapt the code under 'Unknown dataset' ("Date_Seawater_LZO1_RGBdataset_2.xls") to the location where the newly created dataset with RGB values is located.

→setwd("…/Automated_MP_analysis/LZO1/RGB/UV")

Adapt the code under 'To be analysed dataset' to the name of the RGB dataset.

→unknown_dataset <- read_excel("Date_Seawater_LZO1_RGBdataset_2'.xlsx")

Adapt the name of the dataset under 'Filename dataset predicted particle identities' using the name of the RGB dataset.

→ Filename<- paste(Date,"2023-04-13_ Seawater_LZO1_RGBdataset_2_predictions.xlsx",sep= "")

Following this, run the complete code. A file will be generated with predictions from the Plastic Detection Model (PDM) on the plastic or non-plastic identity of the particles.

2.3.2.2.2 Polymer Identification Model

Repeat the same steps as for the PDM, but change the name of the training dataset to the PIM dataset:

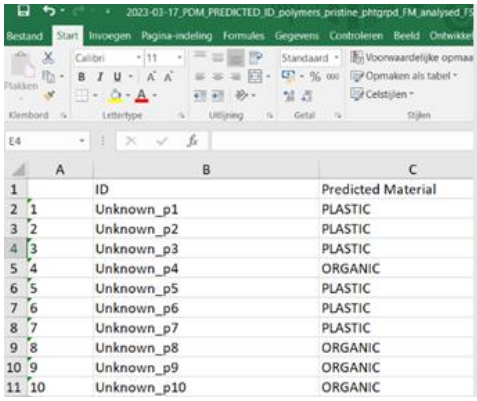
"RGB_dataset <- read_excel("PIM_FSM_ILVO_final.xlsx")". Put an '#' in front of "RGB_dataset <- read_excel("PDM_FSM_ILVO_final.xlsx")".

In the generated Excel-file with PIM-predicted polymer types of the plastic particles, ignore the polymer predictions of particles identified as 'organic' by the PDM.

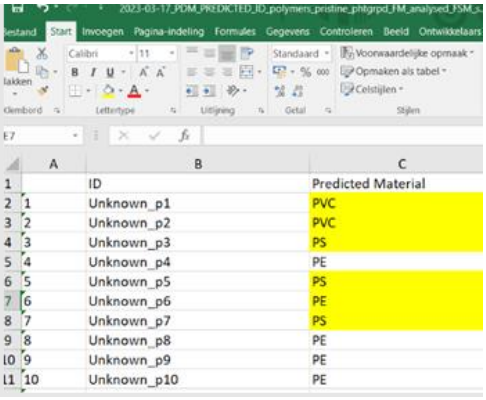
```
tree.RGB <- randomForest(
  Material ~ .,
  data = RGB_dataset[, -1],
  ntree=1000,
  mtry = 36,
  replace=F,
  importance = T,
  keep.forest=T,
  keep.inbag=T,
  do.trace=50

)

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). This should not be set to too small a number, to ensure that every input row gets predicted at least a few times.

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



	A	B	C
1		ID	Predicted Material
2	1	Unknown_p1	PLASTIC
3	2	Unknown_p2	PLASTIC
4	3	Unknown_p3	PLASTIC
5	4	Unknown_p4	ORGANIC
6	5	Unknown_p5	PLASTIC
7	6	Unknown_p6	PLASTIC
8	7	Unknown_p7	PLASTIC
9	8	Unknown_p8	ORGANIC
10	9	Unknown_p9	ORGANIC
11	10	Unknown_p10	ORGANIC



	A	B	C
1		ID	Predicted Material
2	1	Unknown_p1	PVC
3	2	Unknown_p2	PVC
4	3	Unknown_p3	PS
5	4	Unknown_p4	PE
6	5	Unknown_p5	PS
7	6	Unknown_p6	PE
8	7	Unknown_p7	PS
9	8	Unknown_p8	PE
10	9	Unknown_p9	PE
11	10	Unknown_p10	PE