LABPLAS

Horizon 2020
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Land-Based Solutions for Plastics in the Sea

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D4.1 Guideline for methods for extraction, pre-concentration and purification of SMNPs

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Nº	Participant name	Acronym	Country	Type
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2	UNIVERSIDADE DA CORUÑA	UDC	SPAIN	HES
3	Bundesanstalt fuer Gewaesserkunde	BfG	GERMANY	RTO
4	LABORATORIO IBERICO INTERNACIONAL DE NANOTECNOLOGIA	INL	PORTUGAL	RTO
5	KATHOLIEKE UNIVERSITEIT LEUVEN	KUL	BELGIUM	HES
6	HELMHOLTZ ZENTRUM FUR OZEANFORSCHUNG KIEL	GEOMAR	GERMANY	RTO
7	NATIONAL OCEANOGRAPHY CENTRE	NOC	UNITED KINGDOM	RTO
8	SORBONNE UNIVERSITE	SU	FRANCE	HES
9	OPEN UNIVERSITEIT NEDERLAND	OUNL	NETHERLANDS	HES
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11	ASSOCIACAO PARA O DESENVOLVIMENTO DO ATLANTIC INTERNATIONAL RESEARCH CENTRE	AC	PORTUGAL	RTO
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17	STICHTING RADBOUD UNIVERSITEIT	RU	NETHERLANDS	HES

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Executive Summary:	This document corresponds to Deliverable 4.1. *Guideline for methods for extraction, pre-concentration and purification of SMNPs,* developed in the framework of Task 4.1 of the LABPLAS project. Currently, the detection and identification of small microplastics (<10 µm – 1000 nm) and nanoplastics (1000 nm – 1 nm) (SMNPs) in environmentally relevant samples is highly challenging because of the lack of standardized analytical procedures. Thus, this deliverable covers the optimization and standardization of analytical methods for the extraction, pre-concentration and purification of small microplastics and nanoplastics (SMNPs) from environmentally relevant matrices (water, biota and sediments).

Version	Date	Comments
1.0	17.05.2022	Initial version – Proposed Guidelines for methods for extraction, pre-concentration and purification of SMNPs.
2.0	30.05.2022	Revised and formatted version (open for modification)
3.0	14.02.2023	Modification of the version 2.0 to address comments from the PO and reviewer (open for modification)

Disclaimer

The views and opinions expressed in this document reflect only the authors' views, and not necessarily those of the European Commission.

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ABBREVIATIONS AND ACRONYMS

Abbreviation / Acronym	Description
CCD	Charged-Coupled Device
CLSM	Confocal Laser Scanning Microscopy
CPE	Cloud Point Extraction
EM	Electron Microscopy
ETD	Everhart Thornley secondary Electron Detectors
NIST	National Institute of Standards and Technology
NTA	Nanoparticle Tracking Analysis
PS	Polystyrene
Py-GC-MS	Pyrolysis Gas Chromatography coupled to Mass Spectrometry
SEM	Scanning Electron Microscopy
SERS	Surface-Enhanced Raman Scattering
SMNPs	Small Microplastics and Nanoplastics
SRMs	Standard Reference Materials
TEM	Transmission Electron Microscopy

1 INTRODUCTION

Plastic is pouring from land into our oceans at a rate of nearly 10 million tonnes a year. Once in the sea, plastics fragment into particles moving with the currents and ocean gyres before washing up on the coastline. The smaller the size the higher the risk posed by these particles to organisms and human health. Because small, micro- and nano-plastics (SMNP) cannot be removed from oceans, proactive action regarding research on plastic alternatives and strategies to prevent plastic from entering the environment should be taken promptly. The LABPLAS project is a 48-month project whose vision is to develop new techniques and models for the detection and quantification of SMNPs Specifically, the LABPLAS project will determine reliable identification methods for a more accurate assessment of the abundance, distribution, and toxicity determination of SMNPs and associated chemicals in the environment. It will also develop practical computational tools that should facilitate the mapping of plastic-impacted hotspots and promote scientifically sound plastic governance.

Currently, the detection and identification of small microplastics (<10 µm – 1000 nm) and nanoplastics (1000 nm – 1 nm) (SMNPs) in environmentally relevant samples is highly challenging because of the lack of standardized analytical procedures. Thus, this deliverable covers the optimization and standardization of analytical methods for the extraction, pre-concentration and purification of small microplastics and nanoplastics (SMNPs) from environmentally relevant matrices (water, biota and sediments). These guidelines were developed in the framework of Task 4.1 of the LABPLAS project.

The report describes analytical procedures for sample preparation (extraction, pre-concentration and purification), detection, identification and quantification of SMNPs in the range ≤ 10 µm – 1 nm in environmental samples. A multistep methodology is proposed for sample processing and analysis according to the type of sample (water, biota or sediments). These procedures allow the extraction, pre-concentration and purification of SMNPs with a minimum degradation and effect on their size/shape distribution.

The proposed guidelines will be tested, optimized and validated using samples from LAPBLAS field sampling campaigns. Lab samples and field samples from WP2 (D2.1) field sampling campaigns will be used to test, optimize and validate the proposed guidelines.

The detection of suspected SMNPs is carried out by electron microscopy (EM) and Nile Red labelling using confocal laser scanning microscopy (CLSM). The size (distribution) and chemical composition of the SMNPs extracted from lab and field samples (WP2, D2.1) is analysed by nanoparticle tracking analysis (NTA), scanning electron microscopy (SEM), and Raman spectroscopy. SMNPs are quantified using mass-based Py-GC-MS. Commercially available polystyrene (PS) nanoplastics (SRMs from NIST) with a spherical shape is used to calibrate the described analytical methods and to evaluate the impact of the proposed experimental methods on the size (distribution) and morphology of the SMNPs. Results show low impact for SMNPs dispersed in water and biota samples.

2 SAMPLE PREPARATION FOR SMNPS IN AS-RECEIVED WATER SAMPLES

Water samples included in this guideline comprise seawater and freshwater at different depths.

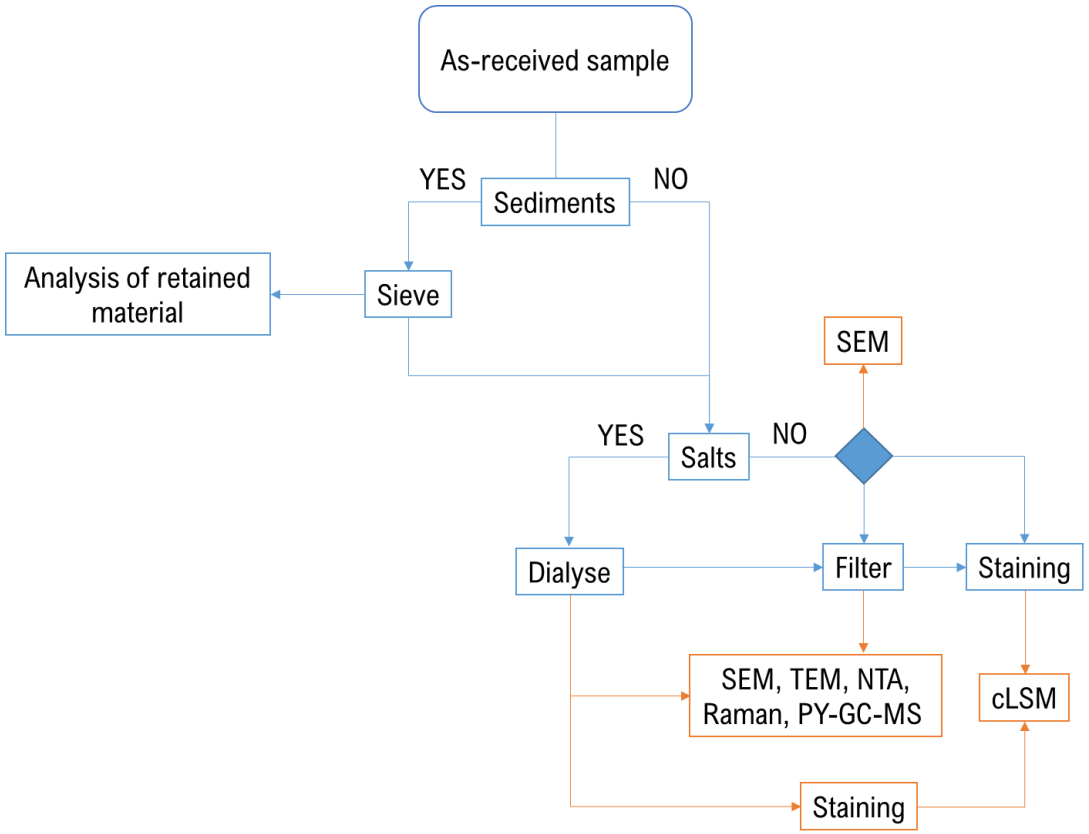

Figure 1. Decision tree for water sample preparation

Specifications	
Sieving	If the samples contain a high level of suspended sediments: Sequentially sieve the samples using steel sieves with a pore size of 500 µm, 100 µm, 50 µm and 20 µm. Set aside an aliquot of sample after each sieving step and the sediments for analysis.
Dialysis	Dialyse water samples to remove the excess salt for 1 hour using MWCO 12.000 Da dialysis cellulose membranes.
Filtration	Filter the dialysate through 1 µm and 0.2 µm nylon membranes (syringe filters, 25 mm and 13 mm respectively). Set aside an aliquot after each step for analysis.
Staining	Incubate each aliquot and the final sample with 10 µg/mL of Nile Red (stock solution of 1 mg/mL Nile Red in ethanol) for 10 min at room temperature under shaking and dark conditions.
Proceed according to described in sections: 5.1 CLSM for SMNPs detection, 5.2 NTA for size distribution determination, 5.3 SEM for size and shape analysis, 5.4 TEM for size and shape analysis, 5.5 Raman spectroscopy for chemical identification and 5.6 PY-GC-MS for chemical identification and quantification	

Observation: Loss of nanoplastics was observed after dialysis using Polystyrene nanoplastics-spiked samples. Therefore, depending on the sample, the user should evaluate whether dialysis is necessary or not. The presence of salts will difficult the analysis of SMNPs using SEM, Raman, and NTA. Salts should be avoided in the TEM.

2.1 CPE for pre-concentration of SMNPs in water samples

Cloud-point extraction (CPE) is a cost-effective sample-preconcentration technique based on the agglomeration and precipitation of micelles when a non-ionic surfactant is heated above its cloud-point temperature.[1]

	Specifications
Step 1: Optional – Sieving	If the sample contains a high level of suspended sediments: Water samples are passed through stainless steel sieves placed in cascade with a pore size of 500 µm, 100 µm, 50 µm and 20 µm.
Step 2: CPE	Disperse the sample in 4 mM Triton-X114 and incubate it in pre-boiled water for 1h.
Step 3: Phase separation	Remove the supernatant (surfactant-poor phase) using glass Pasteur pipettes and redisperse the surfactant-rich phase in 1 mL of ultrapure water.
Step 4: Detection/Identification/Quantification	Proceed according to what is described in sections 5.3 SEM for size and shape analysis, 5.4 TEM for size and shape analysis, and 5.6 PY-GC-MS for chemical identification and quantification.
Step 5: Optional – Staining	Incubate the aliquot of water sample with 10 µg/mL of Nile Red (stock solution of 1 mg/mL Nile Red in ethanol) for 10 min at room temperature under shaking and dark conditions.
Step 6: Optional – Detection/Identification/Quantification	Proceed according to described in section 5.1 CLSM for SMNPs detection and 5.6 PY-GC-MS for chemical identification and quantification.

[1] X.X. Zhou, L.T. Hao, H.Y.Z. Wang, Y.J. Li, J.F. Liu, Cloud-Point Extraction Combined with Thermal Degradation for Nanoplastic Analysis Using Pyrolysis Gas Chromatography-Mass Spectrometry, Anal. Chem. 91 (2019) 1785–1790. https://doi.org/10.1021/acs.analchem.8b04729.

3 SAMPLE PREPARATION OF SMNPS IN BIOTA SAMPLES

The analysis of biota samples is focused on bivalves.

	Specifications
Step 1: **Specimen collection and preparation**	Samples are stored at – 20 ºC. Frozen samples are lyophilized for 3 days. Crush the sample using a glass rod.
Step 2: **Enzymatic hydrolysis**	Add 0.5 mg/mL of activated papain (papain dissolved in 1.1 mM EDTA, 0.067 mM 2, 3-dimercaptopropanol and 5.5 Mm l-cysteine HCl and 100 mM phosphate buffer pH 6.5) to the mashed mussel. React overnight at 50 ºC under shaking and dark conditions.
Step 3: **Centrifugation**	Transfer the sample to glass centrifugation tubes. Centrifuge at 3500 g for 40 min. Store the supernatant.
Step 4: **Matrix lysis**	Redisperse the pellet in 20 mL of matrix lysis (8 M Urea, 1 % Triton-X114). React overnight at 4 ºC under shaking and dark conditions.
Step 5: **Centrifugation**	Transfer the sample to glass centrifugation tubes. Centrifuge at 3500 g for 40 min. Store the supernatant.
Step 6: **Redispersion of final pellet**	Redisperse the pellet in 5 mL of ultrapure water.
Step 7: **Detection/Identification/Quantification**	Supernatants and final extract proceed according to described in sections: 5.1 CLSM for SMNPs detection, 5.2 NTA for size distribution determination, and 5.6 PY-GC-MS for chemical identification and quantification.
Step 8: Optional - CPE	Proceed according to 2.1 CPE for pre-concentration of SMNPs in water samples.

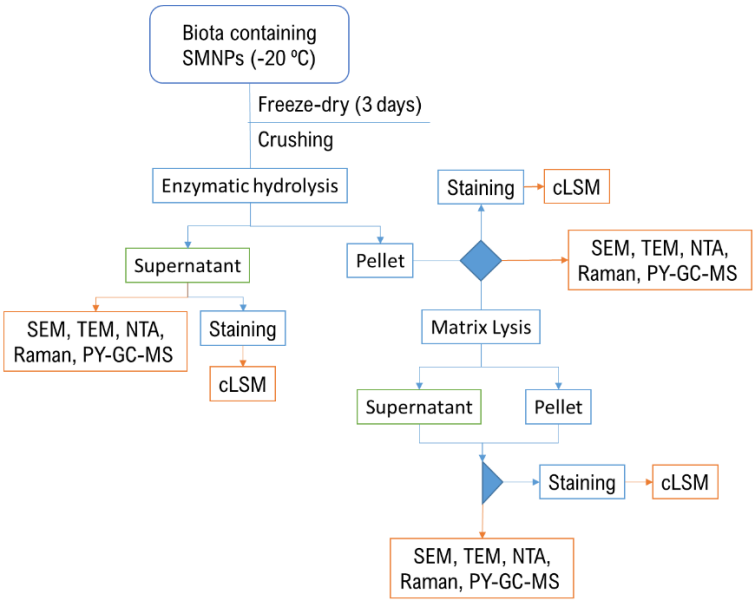

Figure 2. Workflow chart for the preparation of biota samples

4 SAMPLE PREPARATION OF SMNPS IN SEDIMENT SAMPLES

We have focused on establishing analytical methods for water and biota samples due to the possibility of having access to field samples: water samples from WP2 (D2.2) and biota such as mussels, which are regularly available at INL facilities.

Sediment samples for SMNPs analysis will be included in the second sampling campaign (WP2), as after the first sampling campaign, the sediment samples have been established. In addition, guidelines for sample preparation and analysis of SMNPs in suspended sediments will be included due to the high probability of their adsorption to the suspended material. This information is expected to be delivered by the end of the year 2023 (M31) and will be included in an updated version of this deliverable.

5 SMNPS IDENTIFICATION/QUANTIFICATION

5.1 CLSM for SMNPs detection

5.1.1 Small microplastics (10 µm to 1 µm) and nanoplastics (1 µm to 0.2 µm)

Analyse the SMPs by Confocal Laser Scanning Microscopy (CLSM) to get their size, size distribution (estimative) and relative abundance (number of SMPs per mm^2).

Incubate the aliquot of the sample with 10 µg/mL of Nile Red (stock solution of 1 mg/mL Nile Red in ethanol) for 10 min at room temperature under shaking and dark conditions. Take 25 µL of the sample with a glass pipette and place it over a glass slide previously rinsed with ultrapure water and ethanol 70%. Cover the sample with a round glass coverslip (of 0.17 mm thickness, if possible) previously rinsed with ultrapure water and ethanol 70% and seal the edges with nail polish.

Image the sample in a CLSM with an oil immersion objective of at least 40X magnification (63X when possible) registering; transmission, fluorescence from Nile Red (559 nm excitation, 635 nm emission) and, if possible, Z-scan of fluorescent area. A full scan through the coverslip area should be made, and at least 5 areas should be analysed when fluorescent SMPs are detected in a homogeneous distribution. If not homogeneous, the full area should be covered.

Observation: Plastics will concentrate during water evaporation. To avoid agglomeration, increase the sample stability and increase fluorescence imaging resolution, the sample can be mixed 50:50 with glycerol.

Samples investigated: 100 nm and 200 nm Polystyrene and ≤ 20 µm PHB were detected using the described approach.

5.2 NTA for size distribution determination

5.2.1 Nanoplastics (1000 nm to 50 nm)

Analyse the particles by Nanoparticle tracking analysis (NTA) to get their size, size distribution and the concentration of particles in the dispersion (particles/mL).

Load a 1 mL plastic syringe with the sample to be analysed (i.e., filtered or extracted from biota) and adjust the infusion rate to 100. Record a minimum of 5 videos with a duration of at least 60 seconds (total frames

analysed = 1498). Rinse the NTA with 1 mL of ultrapure water between replicates and unmount the cell holder after each sample analysis.

Tween-20 can be used to stabilize the particles if sedimentation or poor colloidal stability is observed. The final concentration should be adjusted to values below the toxicity threshold, and control experiments should be included.

Observation: Sample concentration, flow rate and duration of the recorded videos might require optimization depending on the nature and size of the nanoplastics.

Samples investigated: Field samples from WP2, field samples from WP2 spiked with polystyrene nanoparticles, artificial seawater and freshwater spiked with polystyrene nanoparticles and ≤ 20 μm or ≤ 250 μm PHB containing samples were successfully analysed using this protocol.

5.3 SEM for size and shape analysis

5.3.1 SMNPs (< 10 μm to 200 nm)

10 μL of sample is drop cast on a silicon wafer and dried at room temperature under dark conditions inside a closed box to minimize the contamination. SEM analysis is performed by using high vacuum conditions and Everhart Thornley secondary electron detectors (ETD). All samples are analysed using an accelerating voltage of 3 or 5 kV.

Observation 1: the lower size limit will depend on the nature of the SMNPs.

Samples investigated: Field samples from WP2, field samples from WP2 spiked with polystyrene nanoparticles, artificial seawater and freshwater spiked with polystyrene nanoparticles, ≤ 20 μm and ≤ 250 μm PHB, ≤ 250 μm PLA, ≤ 250 μm PBAT containing samples were successfully analysed using this protocol.

Observation 2: Field samples from WP2 except for water from the North Sea, field samples from WP2 except for water from the North Sea, spiked with polystyrene nanoparticles, and artificial seawater and freshwater spiked with polystyrene nanoparticles, were analysed without being dialysed to avoid any possible loss of nanoplastics. Samples from the North Sea were subject to dialysis as the amount of salt made impossible their analysis by SEM. ≤ 20 μm and ≤ 250 μm PHB, ≤ 250 μm PLA, and ≤ 250 μm PBAT containing samples were visualised before and after dialysis.

5.4 TEM for size and shape analysis

5.4.1 Nanoplastics (200 nm to 5 nm)

The samples are prepared by drop casting 5 μL of sample on lacey carbon-coated copper grids and drying at room temperature under dark conditions inside a closed box to minimize contamination. TEM images are obtained with a TEM instrument operating at an acceleration voltage of 80 - 100 kV.

Observation: the lower size limit will depend on the nature of the SMNPs. Damages in the samples caused by the beam were observed during the analysis.

Samples investigated: ≤ 20 μm PHB containing samples were analysed but damages in the samples caused by the beam were observed during the analysis.

5.5 Raman spectroscopy for chemical identification

Plastic-contaminated water samples are placed in a substrate fabricated using anisotropic and spherical gold nanoparticles for chemical identification. SERS and Raman's spectra are acquired using a mini-confocal Raman microscope (600 and 1200 lines mm-1 grating and a CCD camera) and excited with a 785 nm or 633 nm excitation laser line. The laser power at the sample should be $\leq$ 5 mW.

Samples investigated: Field samples from WP2, field samples from WP2 spiked with polystyrene, Polyethylene, Polypropylene and PET nanoparticles, and artificial seawater and freshwater spiked with polystyrene, Polyethylene, Polypropylene and PET nanoparticles, were analysed using Raman and SERS. ≤ 20 μm PHB containing samples were also analysed.

Observation 1: However, although promising, the results obtained using the anisotropic gold nanoparticles were not satisfactory according to the standards described in LABPLAS.

Observation 2: Polystyrene and PET nanoparticles were detected using SERS on the spherical gold nanoparticles and Raman. Polyethylene, Polypropylene were not detected either by Raman or SERS.

5.6 PY-GC-MS for chemical identification and quantification

Proceed according to D3.1, section 5 Microplastics Identification/Quantification, sub-section 5.2 Thermoanalytical methods: Pyrolysis-GC-MS (PY-GC-MS).

Py-GC-MS measurements are performed with a micro-furnace pyrolyzer EGA/Py-3030D equipped with an autoshot sampler (both Frontier Labs, Japan) attached to an Agilent gas chromatograph with a DB-5MS column coupled to an Agilent MSD mass spectrometer.

To quantify SMNPs correctly by Py-GC-MS, a determination of specific indicator compounds is required. The most abundant and polymer-specific pyrolysis products were chosen. The calibration was performed by weighing (≈40 µg – 1000 µg) directly into pyrolysis cups. Anthracene d10 was added as an internal standard.

Samples investigated: Field samples from WP2, artificial seawater and freshwater spiked with polystyrene, and ≤ 20 μm PHB, and biota samples containing ≤ 20 μm PHB or polystyrene nanoparticles were successfully analysed.

6 SAMPLE PRESERVATION AND HANDLING IN THE ANALYTICAL PROCEDURES

Samples must be collected, shipped, and stored within the shortest time possible to prevent or minimize degradation and/or contamination. Only glass (water samples and/or biota) or stainless steel (biota) containers and glassware should be used. As-received and treated samples should be stored under dark conditions at 4 ºC and – 20 ºC, respectively.

7 CROSS-CONTAMINATION CONTROLS AND QA/QC

- Negative (blanks) and positive (spiked samples) controls and air contamination must be included.
- Ultrapure water must be used in all solutions and dispersions as well as washing processes.
- All glassware, sieves, filters, etc.. should be cleaned by holding them (in the case of sieves) in a bunsen-burner flame or ultrasonic bath (in the case of nylon membranes) or by placing them (in the case of glassware, except volumetric flasks) in an oven at > 200 ºC for 2 h and be rinsed with ultrapure water. Avoid using plastic items whenever possible.
- Appropriate QA/QC for analytical methods should include sufficient replicates, determination of recovery rates of the method, procedural controls should be analysed alongside the sample series, and calculation and consideration of uncertainties/confidence levels.