Plastics Fate and Effects in the Human Body

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Deliverable report
1.1
Report on selected MP/NP and basic PChem features

Lead beneficiary for this deliverable: CNR

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Vs 1.1	2022-02-21	First draft for the collection of contributes
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Vs 1.3	2022-03-13	Contribute from CSIC
Vs 1.4	2022-03-14	Final draft for internal WP1 revision
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[1]Nature: R = Report, P = Prototype, D = Demonstrator, O = Other

[1]Dissemination level: PU = Public
PP = Restricted to other programme participants (including the Commission Services)
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CO = Confidential, only for members of the consortium (including the Commission Services)
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Executive Summary

Deliverable D1.1 addresses some of the specific objectives of Task 1.1, as summarised below:

- To select a set of relevant micro and nanoplastics (MNP) to maximise the impact of PlasticsFatE by covering as much as possible the compositional/morphological classes of micro-nano plastics (MP/NP) commonly found in food, drinking water and environmental matrices.
- To generate and store a bank of relevant reference and/or certified, well-characterised micro-nano plastics (MP/NP) covering the identified classes of MNP for use in PlasticsFatE and for future projects that will help to characterise or develop strategies to mitigate MP/NP pollution.
- To develop standardized protocols for how to handle, characterize, store and distribute MP/NP.
- To facilitate particle labelling, traceability and detection by means of fluorescent markers.

In response to specific objective 1, a portfolio (1st Tier) of PlasticsFatE MP/NP materials was established that differed in polymer type, source/size, and use scenarios. A classification code for these materials was agreed and they were distributed to WP1 partners for initial characterisation.
In response to specific objective 2, BAM created the PlasticsFatE sample repository, including some primary plastics, some commercial samples and some secondary particles obtained by BAM through cryomilling/aging treatments. Basic particle characteristics were checked by BAM and ISTEC through dynamic imaging and optical or electron microscope characterisation. Possible endotoxin contamination was tested by GAIKER (partner from WP3), confirming that secondary PE and PET particles and HDPE commercial samples (Ceridust from Clariant) were not affected by endotoxin contamination.
In response to specific objective 3, BAM developed and tested protocols for plastics milling and weathering; while ISTEC-CNR and UNITO carried out an intense dispersibility study in order to identify dispersion protocols suitable for characterisation and use in WP1-3. After intense discussion, a two-step process was selected for sample dispersion, (1) a general protocol for the preparation of stock dispersions and (2) the use of specific dispersants for the dilution of stock dispersions in relevant simulant fluids (working dispersions).
In response to specific objective 4, we characterised La-Eu doped PS nanoparticles purchased and distributed by STAMI. Traceability was investigated by Raman-luminescence mapping.
The results from D1.1. were shared/disseminated through the participation in CUSP working group (WG) MS SharePoint/Teams channels and meetings, where Task 1.1 partners are involved: Anna Costa/Magda Blosi as representatives for PlasticsFatE in WG1 (Analytical methods and representative materials) and Korinna Altmann/Dan Hodoraba, as the leaders of WG3 (Inter-laboratory comparisons).

1. Description of task

Task 1.1 Materials selection, labelling, storage, distribution and basic characterisation (M1-36)
Participants: CNR (Leader), BAM, UBT, FHG, CSIC, UBA
T1.1 will organize and implement the selection, labelling, storage, distribution and pre-screening (basic characterization) of the materials to be tested and characterized in WP2-5 and will ensure that the selected materials represent realistic products and situations along the life cycle of MP/NP. Target materials will cover a wide range of polymer material properties, from well-defined processed plastics particles mainly in the size range 1-1000 µm, but also below 1 µm (primary), up to degraded plastic fragments from mechanical and physical-chemical breakdown from larger products (secondary). Due to the complexity of MP/NP in the environment in terms of dimensional variability and physicochemical composition, we will start with synthetic MP/NP samples useful to prepare ad hoc model polluted food, drink and air, before considering more real MP/NP test materials. The target materials, which will also include specific samples with different particle sizes and shapes, will be provided by the consortium partners or, if needed, purchased from specific MP/NP reference repositories (BAM, JRC), and sent to CNR, who will act as central distribution point for receiving, registering, pre-screening and aliquoting and distributing all samples to partners. CNR, in cooperation with other partners will manage the batch-to-batch reproducibility study, to make sure that identical sub-samples enter the characterization process along the WPs. Partners (BAM and UBT), who will produce own test and/or representative materials, will suggest precise and reproducible protocols to perform a basic characterization. UBA will be involved in the selection of relevant materials, the realisation of RM will be realised by BAM. Methods for their physicochemical characterization will be based on state-of-the-art analytical measurement techniques that have been developed in previous projects (LimnoPlast, BASEMAN, RUSEKU, JRC interlaboratory comparison test), in terms of particle size distribution and Zeta potential (DLS/ELS), chemical surface properties (ATR-FTIR, µFT-IR,), additive content (ICPOES) and primary size/shape/morphology (electron microscopy), to provide a sample identity for each MP/NP class. As most characterization procedures are based on optical techniques, CNR will develop fluorescent organometallic markers enabling particles labelling, traceability and detection as useful tool for tracking microplastics during experimental characterization and transfer in tissues, hence maximising the effectivity of exposure and fate assessment. T1.1 will produce the deliverables: **D1.1 Report on selected MP/NP and basic physicochemical features, (M12),** D1.2 Analytical characterization data for the first set of MP/NP samples (M18) and D1.3 Analytical characterization data for the second set of MP/NP samples (M30).

D1.1: Report on selected MP/NP and basic PChem features [12]
In the report it will be explained how the selection, storage, distribution and basic characterization of material is performed. In addition, some techniques for labeling and traceability of materials will be described.

2. Description of work & main achievements

Specific Objective 1	To select a set of relevant MP/NP to maximise the impact of PLASTICSFATE covering as much as possible the compositional/morphological classes of micro-nano plastics (MP/NP) commonly spread into food, drinking and environmental matrices.

Table 1 provides an overview of the materials that were considered for study in PlasticsFatE, this describes the main source and exposure routes for each. Starting from this list of representative materials we decided to focus on the Tier 1 materials and fewer compositions based on their availability, environmental relevance, different density and aging status, including both primary and secondary particles. Polyolefins, such as polyethylene (PE) and polypropylene (PP), are the most used polymer types in food packing, whilst polyethylene terephthalate (PET) is used for beverage containers. In the Tier 1 portfolio reported in Table 2, we included PE and PET samples whose behaviour in water suspensions is strongly affected by their density, resulting in lower values than that of water in case of PE (around 0.93 g/cm^{-1}) and higher in case of PET (around 1.4 g/cm^{-1}). Pure primary particles are included for their easy commercial supply and utility for method validation. We also included secondary MP produced by cryomilling to better simulate and reproduce real samples and artificially weathered PE, because PE packaging materials that are found in nature are easily

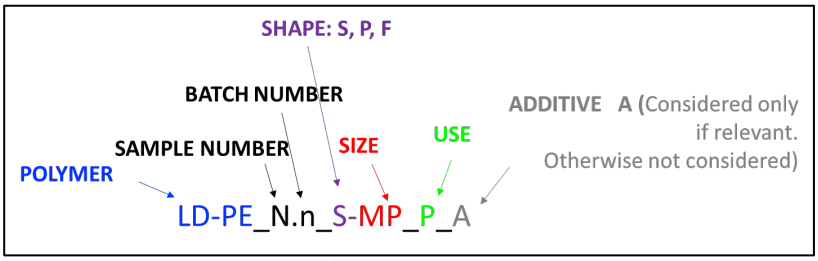
oxidized. A commercial sample of Eu labelled polystyrene was also purchased and included in the list of Tier 1 materials for investigating plastics translocation and fate in *in vitro* and *in vivo* models. All materials selected and included in the order list were labelled with a PlasticsFatE code that includes the main information for sample identification (Figure 1).

Table 1: List of relevant MP/NP in relationship to the main sources and exposure routes.

Source	Polymer type	Main Route	Preparation	Form	Dimension	Provider
I. Particles from mechanical abrasion of packaging material (food and beverage packaging)	Polyethylene terephthalate (PET)		Cryo-milling of virgin material	Irregular shaped particle	50 - 300 µm	BAM
	Polypropylene (PP)		Cryo-milling of virgin material	Irregular shaped particle	1 - 200 µm	BAM
	Polyethylene (HDPE, LDPE)		Cryo-milling of virgin material	Irregular shaped particle	1 - 200 µm	BAM/UBT
	Polystyrene (PS)		Cryo-milling of virgin material	Irregular shaped particle	1 - 200 µm	BAM/UBT
II. Very small particles transported by air (tire abrasion/microfibers from textiles; plastic types from I.)	Styrene-Butadiene/Butadiene (SBR/BR)		Cryo-milling of recycled material	Irregular shaped particle	< 10 µm	BAM
	Polyethylene terephthalate Microfibers (PET)		Milling of virgin/ aged material	Fibres	< 10 µm	BAM, Merck (ITA)
III. Particles intentionally added to products (personal care, paints)	Polyethylene (UHMW-PE)		Commercially available	Spherical particle	Monodisperse distributions (24, 54, 147 µm)	BAM
	Polystyrene (PS)		Commercially available	Spherical particle	Monodisperse distributions (nanoscale ranges)	Spherotech, BS Particles
IV. Particles with unclear but potential toxicological effect (special applications)	Flame retarded, Expandable Polystyrene (FR-EPS)		Cryo-milling	Irregular shaped particle	50 - 300 µm	BAM
	Polyvinylchloride with high additive content (PVC)		Commercially available	Spherical particle	1 - 200 µm	BAM
	Polylactide Blends (PLA-PBAT)		Cryo-milling	Irregular shaped particle	1 – 200 µm	BAM/UBT

As reported in Figure 1, samples were classified by:

- POLYMER TYPE
- SHAPE and SIZE, where primary particles are typically round with micrometric size, whilst secondary and fragmented particles present irregular shapes, with sizes below 10µm difficult to obtain by milling, although progress is being made and we will soon include submicrometric particles supplied by BAM as a reference material. Samples prepared by microemulsion produce regular round, nanosized particles
- USE SCENARIOS, which affects the entry route into the human body
- ADDITIVES - whether these are present or not, including fluorescent dyes or metal doping materials.



Classification	Category
by COMPOSITION 	Low-density-Polyethylene; LDPE
	High-density-Polyethylene; HDPE
	Ultra-high-molecular weight; UHMPE
	Polyethylene terephthalate; PET
	Polystyrene; PS
	Mixture: HDPE/PET
by SHAPE/SIZE 	Nano-emulsion (spherical, 100-300nm); NE
	Primary Microplastics (round shape, 2-6 – 100 µm); P-MP
	Secondary Microplastics (irregular shape, 10/500µm); S-MP
	Microfibers (aorund 10 x 100µm ?); F-MP
by USE SCENARIOS (entry route) 	Foods (Ingestion) F
	Textile, Automotive (Inhalation) TX , AU
	Paints, Waste (Environment); P , W
ADDITIVES	Additives A (only if relevant or with specific information about type and quantity)
	Labelled/dyed L-D
	Labelled/metal L-M (es L-Eu)

Figure 1: PlasticsFatE samples code legend.

Table 2 shows the main information of the PlasticsFatE Tier 1 materials that were distributed to all characterization partners during the first months of the project. In addition to the PlasticsFatE code, the following information was included: suppliers, storage, polymer size (D50) and shape, the physical state (if in powder or dispersed), and aging treatment. Further materials that will be considered in the future, such as smaller size milled samples ≤10µm and reference samples below 100/200nm, will be integrated in the present table, so completing the PlasticsFatE Tier 2 materials portfolio.

 BAM Size: mean diameter 100 – 180 nm Shape: round (primary NP) Available in a few weeks Offer for AURORA, IMPTOX and PLASTCHEAL with reduced price: 100 Euro / glass	Under progress. The production of lower size polyolefins (PE, PP) by BAM with size ≤10 µm and two reference materials that will be distributed by BAM with nanometric size as reference materials (shown on the left).

Table 2: PlasticsFatE portfolio of Tier 1 selected MP/NP

Topic	Polymer type	CODE	Supplier	Storage	Polymer size (D50)	Polymer shape	Powder (P) / Dispersion (D)	Aged
Benchmark	PS	PS_93470720010350_NE_L-Eu	Thermofisher by Distrilab*	now at STAMI	0.3 µm	Spherical	D (1%)	no
Primary	UHMW-PE	UHMWPE_16191_P-MP_P	BAM	BAM	145 µm	round	P	no
	UHMW-PE	UHMWPE_16186_P-MP_P	BAM	BAM	57 µm	round	P	no
	UHMW-PE	UHMWPE_16190_P-MP_P	BAM	BAM	22 µm	round	P	no
	LD-PE	LDPE_16242_P-MP_P	BAM	BAM	< 75 µm	round	P	no
	HDPE	HDPE_296_P-MP_P	Ceridust by Clariant*	1 Kg BAM/ 1 kg ISTEC	5 µm	round	P	no
Secondary	HDPE	HDPE_21181_S-MP_W	BAM	BAM	60 µm	irregular, flat	P	yes
	PET	PET_21180_S-MP_F	BAM	BAM	44 µm	irregular	P	no
	PET	PET_21182_S-MP_F	BAM	BAM	130 µm	irregular	P	no
	PET	PET_21183_S-MF_F	BAM	BAM	70 µm	irregular	P	no

- Technical data sheets uploaded on PlasticsFatE server

Specific Objective 2	To generate and store a bank of relevant reference and/or certified, well-characterised micro-nano plastics (MNP) covering the identified classes for use in PLASTICSFATE and for future projects that will characterise or develop strategies to mitigate MNP pollution.

A step-by-step methodology described in Figure 2 was followed to produce samples that were all characterised for particle size. Secondary particles were prepared by BAM by cryomilling (cryogenic treatment + centrifugal milling) and in one case (sample HDPE_21181_S-MP_W), partly artificially aged before cryomilling. Ageing occurred in a weathering device by higher temperature under UV radiation. All other materials are non-aged. After milling, the materials were sieved by a sieve cascade with mesh sizes of 500, 100 and 50 µm depending on the size distribution wished. The produced powder (large batch) was homogenized in a tumble mixer and well characterized. The obtained MP were given a unique code, as described in Table 2. Primary and secondary samples were distributed to the characterisation partners of the PlasticsFatE consortium.

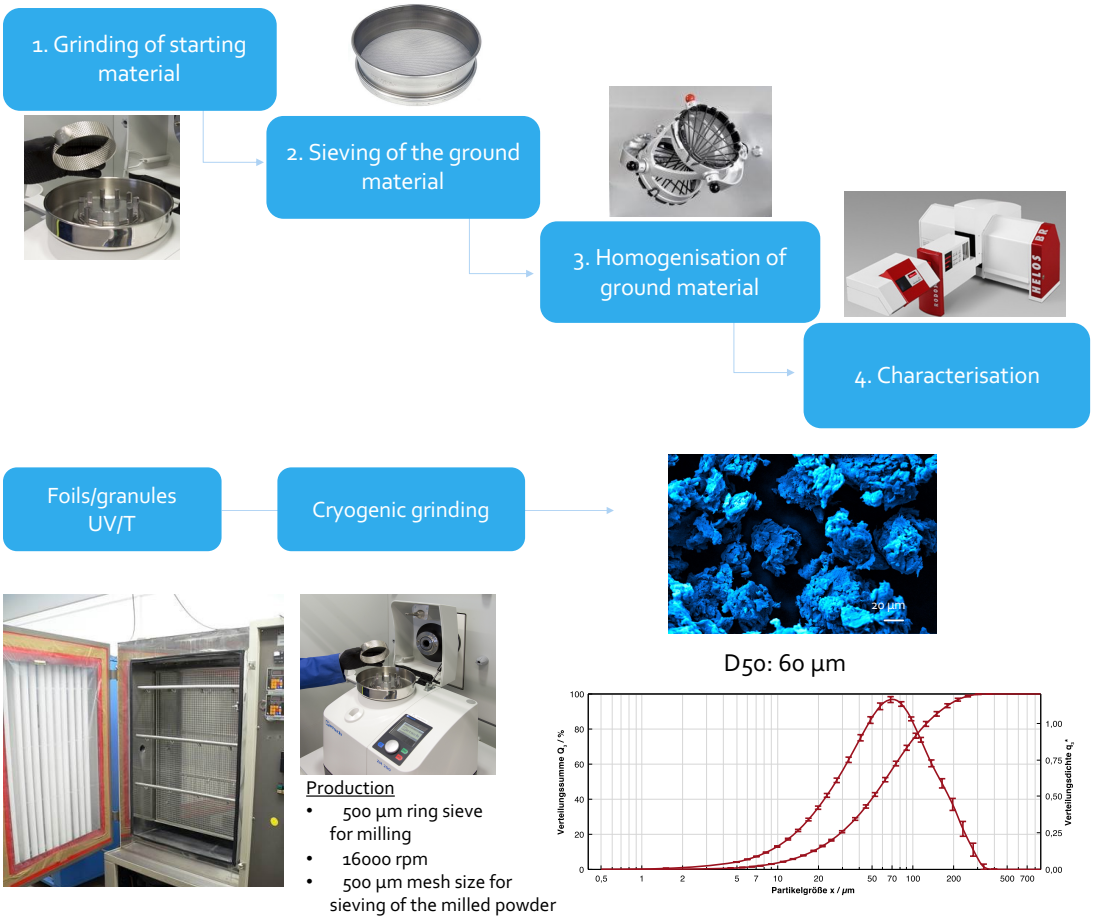


Figure 2: a) step by step methodology followed for preparing secondary test materials; b) weathering example (HDPE foil).

The process of milling was optimised by changing parameters. The starting material (mainly granules) needed to be pre-cooled by liquid nitrogen before milling, to be below the glass transition

temperature of the individual polymer type, which makes material brittle and stiff. Once in the mill, there are several effects which have influence on the final milled powder (Figure 3). The granules are cut at the rotor teeth, before the particles bounce against the sieve walls and are punched through the holes. These effects during grinding leads to the round like shape of the final produced particles. Potentially, the particles can unfold in liquid solvent, and this will be carefully analysed in future.

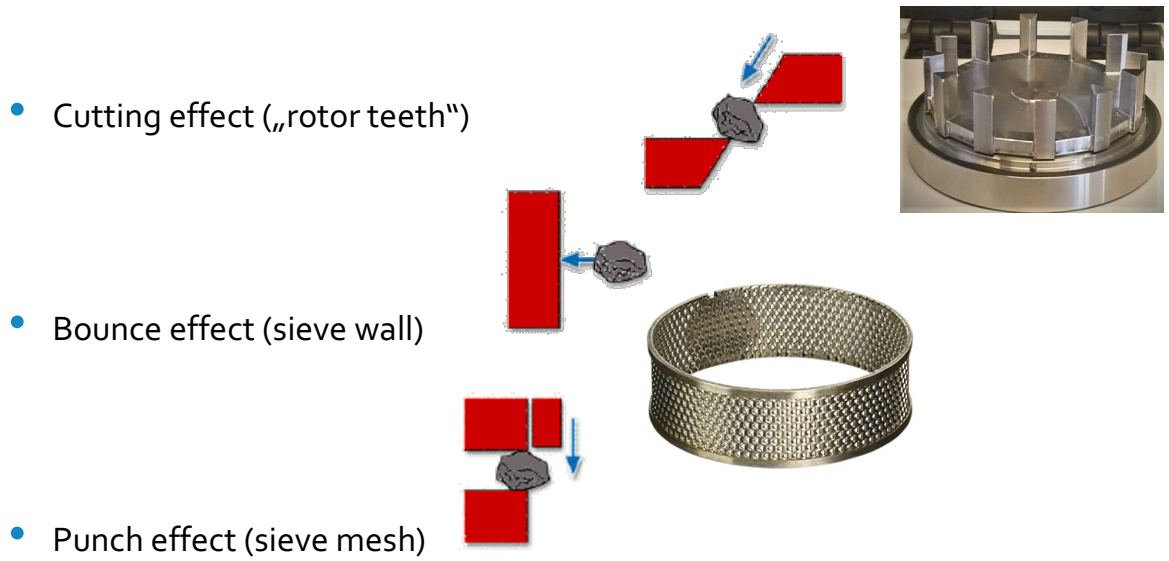
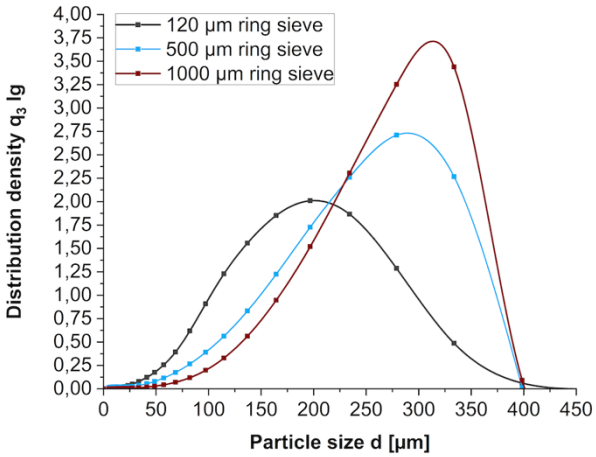


Figure 3: Schematic description of the forces that act on the starting material during milling with a centrifugal mill.

The centrifugal milling process was optimised, varying different grinding parameters such as: mesh size of the sieves at three hours milling time as reported in Figure 4. It was decided to use the 500 µm sieve, because a high yield was obtained. The 120 µm sieve led to smaller particles, but the sieve became clogged very often, which reduced the yield dramaically. A large sieve (1000 µm mesh size) led to bigger particles, as shown in Figure 4.

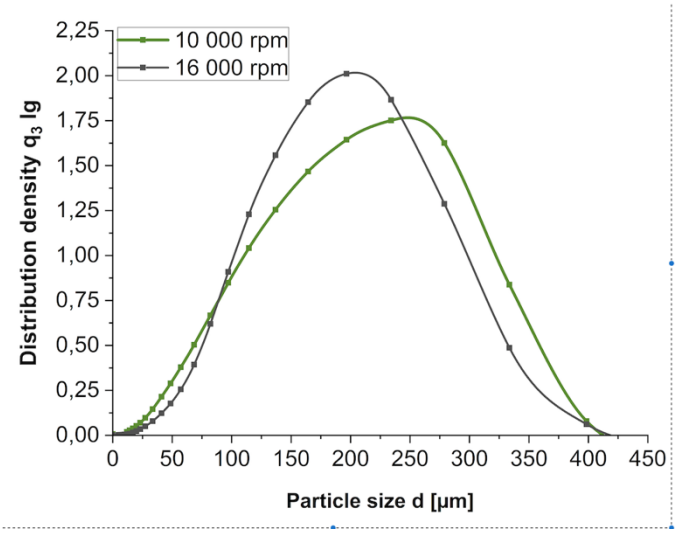


Q_3 %	Particle size* µm
D_{10}	78
	102
	146
D_{50}	167
	228
	260
D_{90}	274
	331
	344

*Average of 3 measurements, laser diffraction, dry dispersed (HELOS/BR+RODOS/L+ASPIROS, Sympatec)

Figure 4: Effect of different sieve mesh sizes according the particle size distribution of the produced particles

The velocity of the sieve within the centrifugal mill was also varied. As can be seen in Figure 5, higher velocities lead to a less polydisperse distribution.

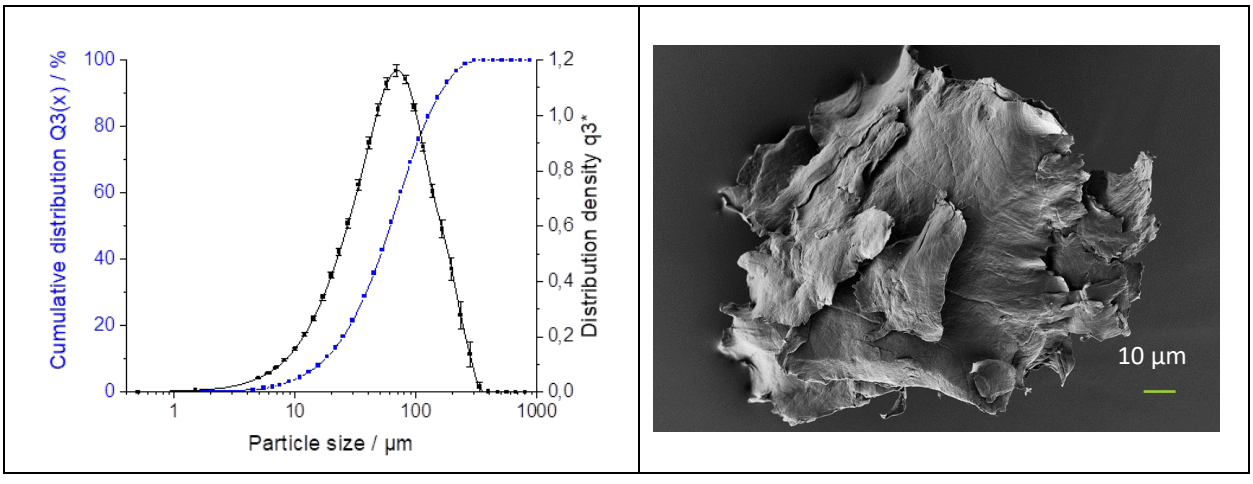


Q_3 %	Particle size* µm
D_{10}	62 78
D_{50}	168 167
D_{90}	293 274

*Average of 3 measurements, laser diffraction, dry dispersed (HELOS/BR+RODOS/L+ASPIROS, Sympatec)

Figure 5: Effect of different velocity, example at 16000 rpm (120 µm ring sieve)

The materials collected and distributed by BAM were well-characterized regarding particle size. Furthermore, a basic characterisation with ATR-FTIR, DSC and SEM was also done and will be included in a Technical Data Sheet that will be provided for each material and included in D1.2. The aim is to provide a physicochemical identity for each material, which will be used as a representative test material for the project and further studies. Particle sizes for MP particles are measured by laser diffraction, and for NP particles by DLS. Information on morphology and surface chemistry were also provided by electron microscopy (SEM), infrared spectroscopy (ATR-FTIR) and thermal analysis (DSC). Figure 6 shows an example of the aged PE with basic physicochemical characterisation performed.



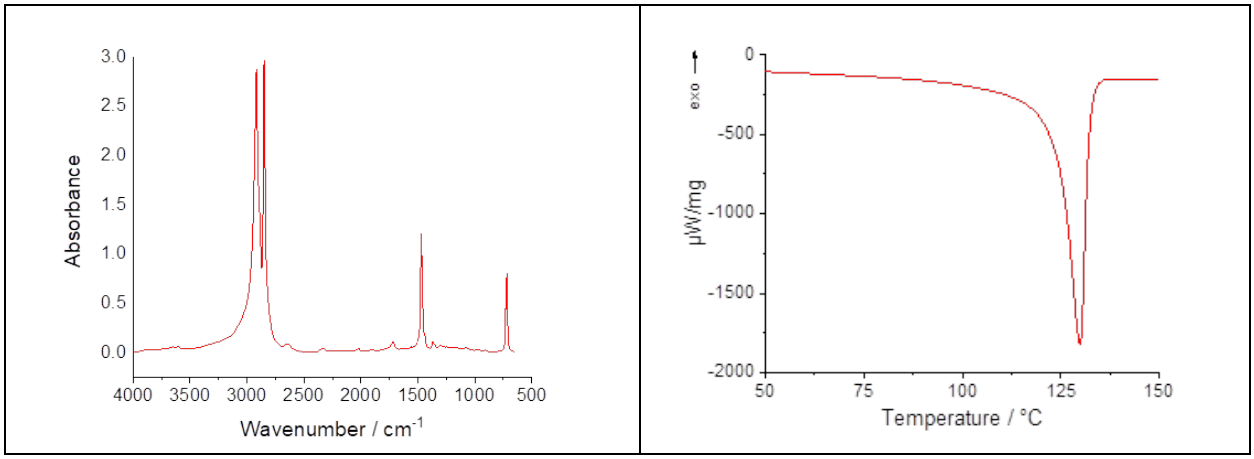


Figure 6: Example of basic characterisation of HDPE_21181_S-MP_W (left up: particle size distribution, left bottom: ATR-FTIR, right up: SEM, right bottom: DSC).

In parallel, CSIC performed cryomilling experiments, to assess the effects that milling treatments could have on MP/NP electrostatic charge, an important property to understand the behaviour that particles can have once dispersed in different environments. Electrostatic charging occurs when two materials with neutrally charged surfaces come into contact (<4 A °) and then separate. The materials, due to the contact with different surfaces can acquire so-called tribocharge, so it is a common process during powder handling.

The electrostatic charge of the different polymers was measured in both the received pellets and following cryo-blade grinding and/or high energy milling by alumina balls to produce smaller particles. The following table summarises the samples tested and results:

Table 3: Electrostatic charge of pristine (pellets) and milled plastics materials.

Material	Treatment	Size (D)	Q_{net} (nC/g)	$Q_{tribocharge}$ (nC/g)
PET	Pellets	3 mm	0,85 ± 0,28	0,82 ± 0,33
PET	Cryo-Blade grinder	< 500 µm	1,21 ± 0,19	7,03 ± 1,43
PET	High Energy Milling	< 5 µm	9,31 ± 0,29	17,34 ± 2,15
LDPE MFI 1	Pellets	4 mm	0,25 ± 0,08	0,20 ± 0,05
LDPE MFI 1	Micronized (industrial)	< 650 µm	1,20 ± 0,46	1,89 ± 0,56
LDPE MFI 7	Pellets	2,5-3 mm	0,35 ± 0,11	0,22 ± 0,10
PLA	Pellets	3 mm	2,03 ± 0,65	2,24 ± 0,31
PLA-PBAT (ECOVIO®)	Pellets	5 mm	2,43 ± 0,61	2,50 ± 0,34

It is worth mentioning that the intrinsic charge of both PET and PE microparticles increased as the particle size decreased. In particular, the net charge increased by more than one order of magnitude when particle size reduced to the submicronic range (High Energy Milling PET sample size below 5 µm with few particles in the micron range and most of the particles in the submicron range). This preliminary investigation showed that the increase of charge, due to tribocharge, is associated with

deagglomeration of small particles due to excess surface charge, and that polymer chemistry influences this electrostatic response.

Follow-up of material strategy

To complete the PlasticsFatE repository of target materials, BAM and CSIC worked to produce a second set of materials (Tier 2), including submicrometric and nano plastics (NP), obtained through chemical solubilisation and re-precipitation of resource plastics or through a cascade of grinding and sieving processes. The basic characterisation of these materials is described in D1.2. In addition, CSIC is developing strategies to obtain reproducible fibres of polylactic acid (PLA) that together with "modified" plastics, and with adsorbed contaminants or in presence of known additives, will be the subject of WP3 toxicological characterisation and included in related deliverables. The physico-chemical characterization of "modified" MNP for exposure, fate and hazard is addressed by Task 1.3, with the associated deliverable (D1.5) due in M36.

Specific Objective 3	To develop a standardized protocol for how to handle, characterize, store and distribute MP / NP.

The risk assessment of newly emerging contaminants (ECs) such as MP/NP necessarily includes simulated samples - laboratory tests in order to develop analytical methods for identifying, quantifying and assessing the effects that these contaminants may produce in real matrices. One of the first objectives addressed was the development and validation of protocols for sample dispersibility in real matrices, which include water-based environmental matrices and fluids simulating different human body compartments. As a follow-up of cross-WPs meetings and taking advantage of the experience gained from nano-safety research projects, it was agreed to adopt a two-step approach methodology inspired by the "Nanogenotox" dispersion protocols [1] that consists of the preparation of a general dispersion (stock dispersion) to be used and further diluted under various exposure conditions (work dispersions), in order to facilitate reproducibility and comparability of results between different tests and operators.

STABILITY OF STOCK DISPERSIONS

The dispersibility study for the optimisation of the stock dispersion protocol started with the preparation of dispersing medium, followed by the dispersion of powder in the sonicator bath (15 min) + 1 min Vortex before using. An ethanol (EtOH) pre-wetting step has been tested already. The following dispersing systems have been considered:

- Water
- Water + EtOH pre-wetting
- BSA (Bovin Serum Albumin) (0.25mg/ml)
- Tween 60 (0.25mg/ml)
- SS (Sodium Surfactin) (0.25mg/ml)

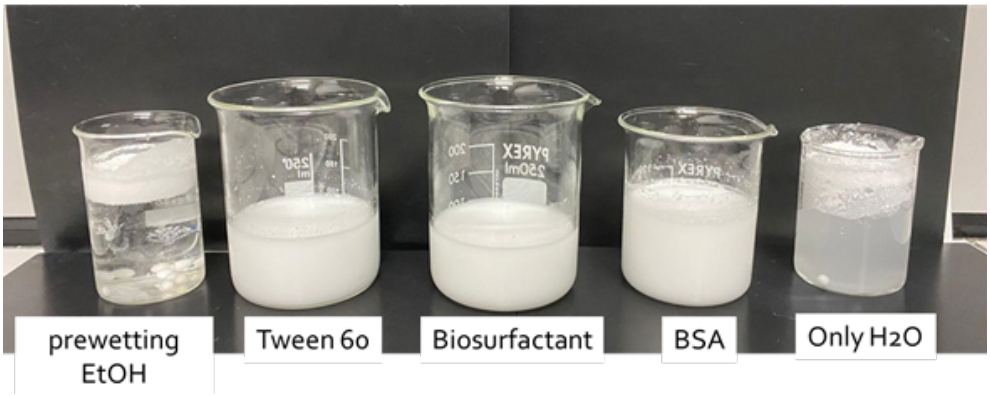
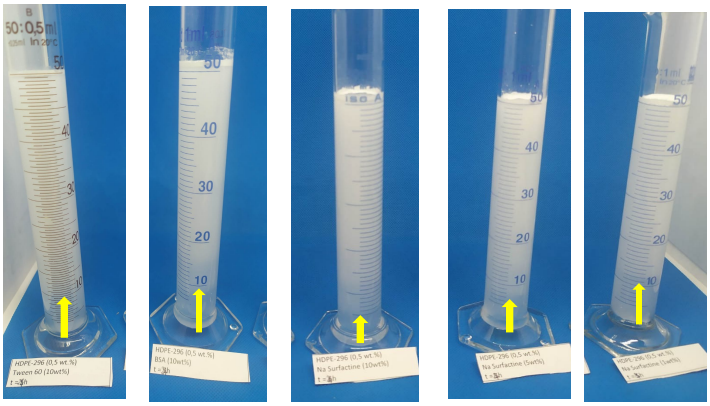


Figure 7: Dispersibility of HDPE_296 sample (10mg/ml) after 2h, dispersants (1mg/ml, 10% vs powder weight).

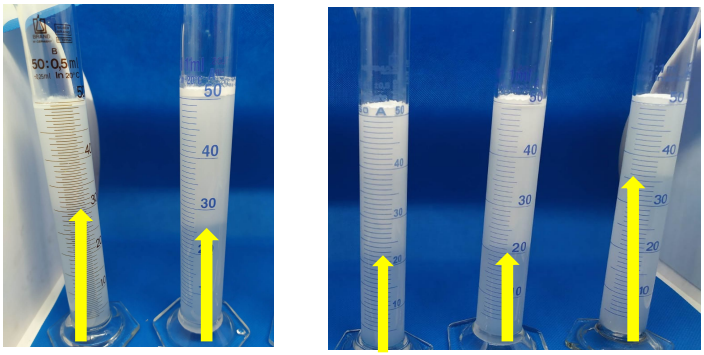
In Figure 7, we report some results of experiments made to test the different dispersing systems. We note that, due to the lower density of PE to water, that samples dispersed in water and water plus ethanol float, while using the dispersants (BSA, Tween 60 and SS) the powder appears homogeneously dispersed. Pre-wetting treatment does not seem to affect dispersibility at all, so it was not considered further.

We repeated the dispersion tests vs time in order to investigate stability and found that at 24h the suspension of HDPE_296 with dispersants (BSA, Tween 60 and SS) was quite stable, with Sodium Surfactin appearing to be best, as can be observed in Figure 8. Based on these results we decided to investigate particle dispersibility in Sodium Surfactin, before moving to very well investigated dispersants that are suitable for toxicological studies, such as bovine serum albumin (BSA).



24 h

Tween 6o (0,5mg/ml) | BSA (0,5mg/ml) | (0,5mg/ml) | Na Surfactin (0,25mg/ml) | (0,05mg/ml)

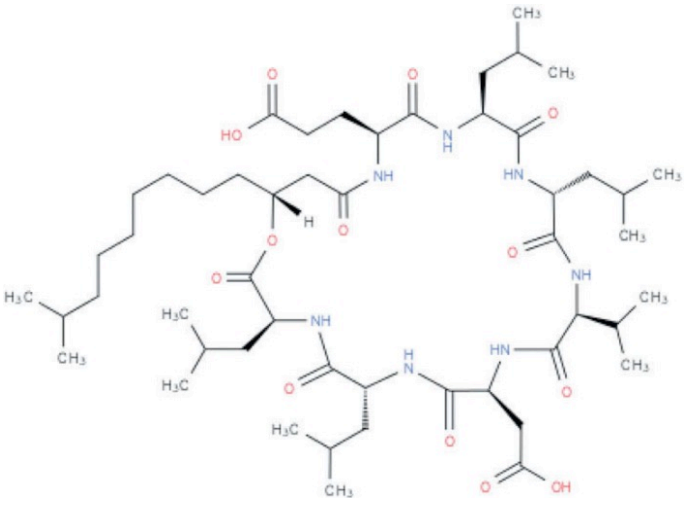


72 h

Tween 6o (0,5mg/ml) | BSA (0,5mg/ml) | (0,5mg/ml) | Na Surfactin (0,25mg/ml) | (0,05mg/ml)

Figure 8: Dispersibility of HDPE_296 sample (5mg/ml) after 24h and 72h with different dispersing systems.

1) Dispersability of microplastics with Na Surfactin (SS) dispersant



Sodium Surfactin (SS) is a bio-surfactant, belonging to the group of Lipopeptides (LPs), mainly from Bacillus sp. Different isoforms of surfactin occur naturally, but overall its amphiphilic structure[2] makes it a very good surfactant with a critical micelle concentration (CMC) in water (23 mg/L) and approximately two orders of magnitude less than the majority of other surfactants. Due to the recognized bioactivity (anti-microbial, anti-cancer) various investigators examined the cytotoxic effects of surfactin against both non-tumor and tumor cell lines, and determined an IC50-48 h of 105.4 and 93.8 µg/ml, by means of in vitro tests with normal cell lines BRL and HEK293[2]. In PlasticsFatE we evaluated the dispersion ability of this surfactant as a potentially more effective alternative to BSA (dispersant used in Nanogentox protocol) or to dispersants suggested by plastics producers (Tween series). This is additional work (not described in

the GA) that was undertaken to overcome the significant challenge of obtaining stable and reproducible dispersions for simulated laboratory tests.
From a visual observation of PlasticsFatE's samples dispersed in SS (Figure 10), it can be noted that only PE samples (LDPE_16242; HDPE_296; UHMWPE_16190 and 16186) showed a uniform opalescent white colour, that remained so in the first minutes after their dispersion.

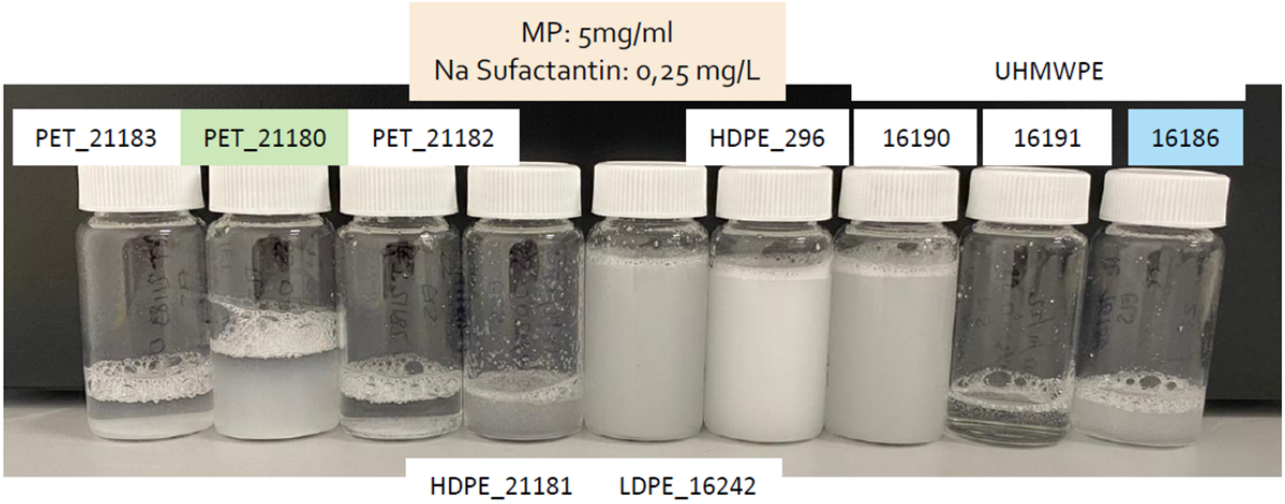


Figure 10: Dispersibility of PlasticsFatE's fresh prepared samples (t = 1 minute) in SS: good stability observed only for PE samples and PET_21180.

A morphological (optical microscope) and colloidal characterization of the stock dispersions in SS is reported in the following.

Morphological characterization
The morphology of microplastics was investigated by dropping stock dispersions, aged for over 10 days, on a glass slide and drying in an oven at 80°C for 15 minutes. The samples were analysed by optical microscopy and the size dimension was evaluated by software ImageJ, over a count of more than 100 particles for each sample.

Polyethylene plastics

Comparing the PE microplastic samples, we observed a high agglomeration of samples PE_ 16190 and PE _21181, that did not allow a size distribution to be determined (Fig. 11 and 12). As reported in Table 2 the samples present an irregular surface.

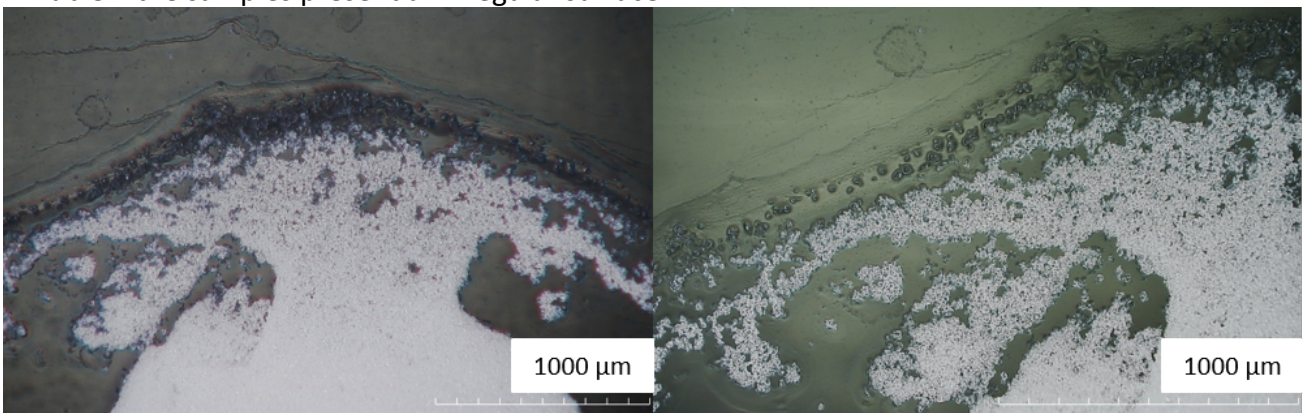


Figure 11: Optical microscope image of sample PE_BAM_16190.

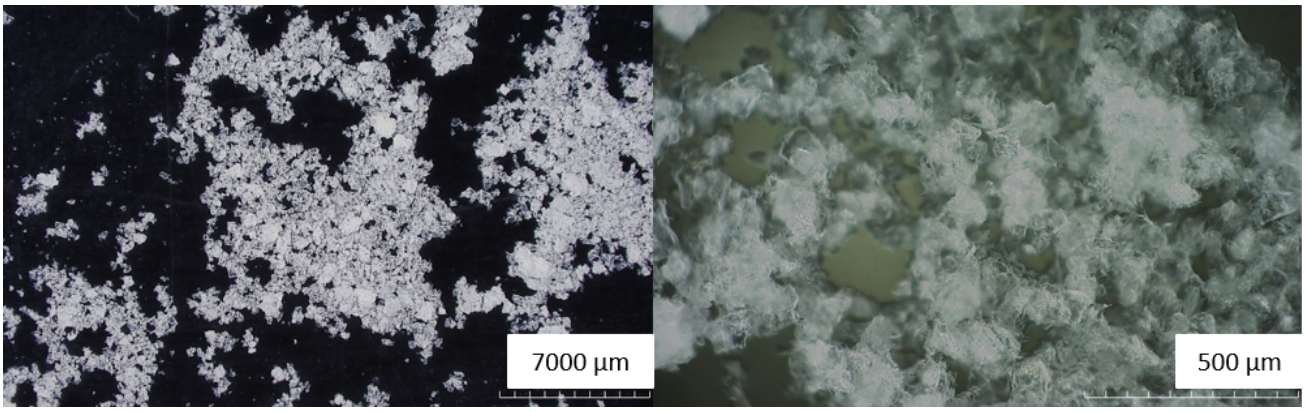


Figure 12: Optical microscope image of sample PE_BAM_21181.

Fig. 13 shows morphology and size distribution of sample PE_ 16191, that confirmed the irregular 'cloud' shape reported in Table 2. However, the size dimension detected ca. 82 µm, is under the value measured by laser diffraction and reported in the Table, that was around 145 µm.

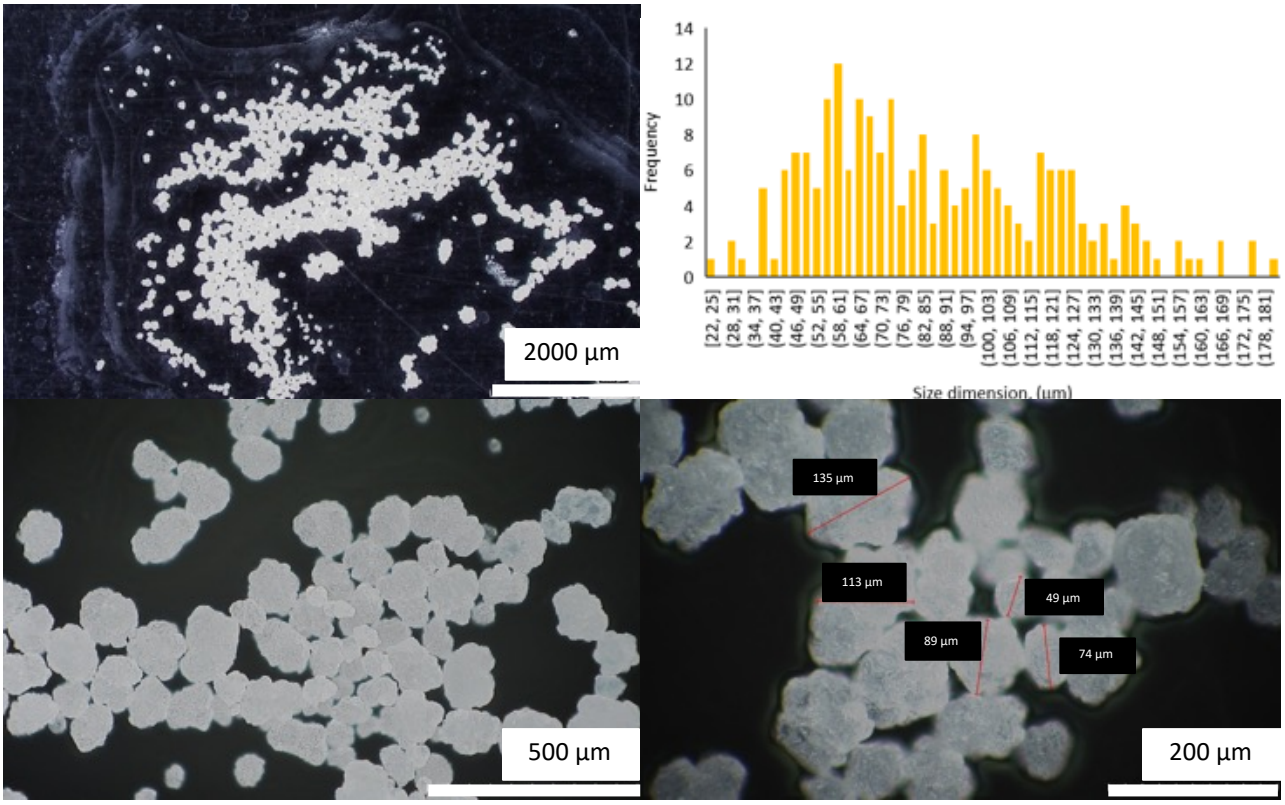


Figure 13: Optical microscope image of sample PE_ 16191 and statistical measurements of size distribution (mean 81.7 ± 33.8 µm, mode 41.1 mm).

Fig. 14 shows the morphology and size distribution of sample PE_BAM_16186, that presented an irregular 'potato' shape as reported in Table 2. The reported size of 57 µm, is in agreement with the calculated size of ca. 52µm.

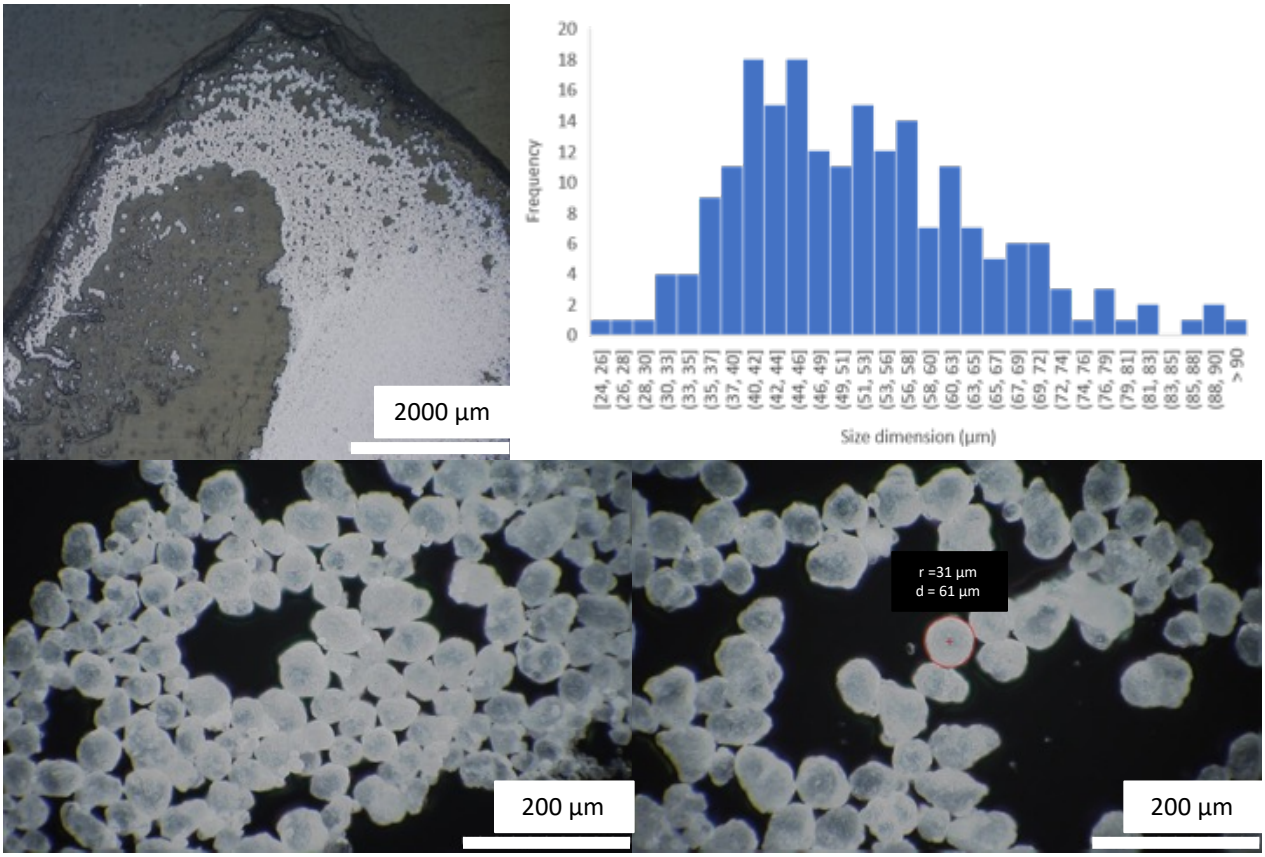


Figure 14: Optical microscope image of sample PE_BAM_16186 and statistical measurements of size distribution (mean 51.8 ± 12.8 µm, mode 37 µm).

Fig. 15 shows morphology and size distribution of sample PE_BAM_16242, that presents a round shape as reported in Table 2. However, the size dimension detected ca. 22 µm is below the value reported and measured by laser diffraction (< 75 µm).

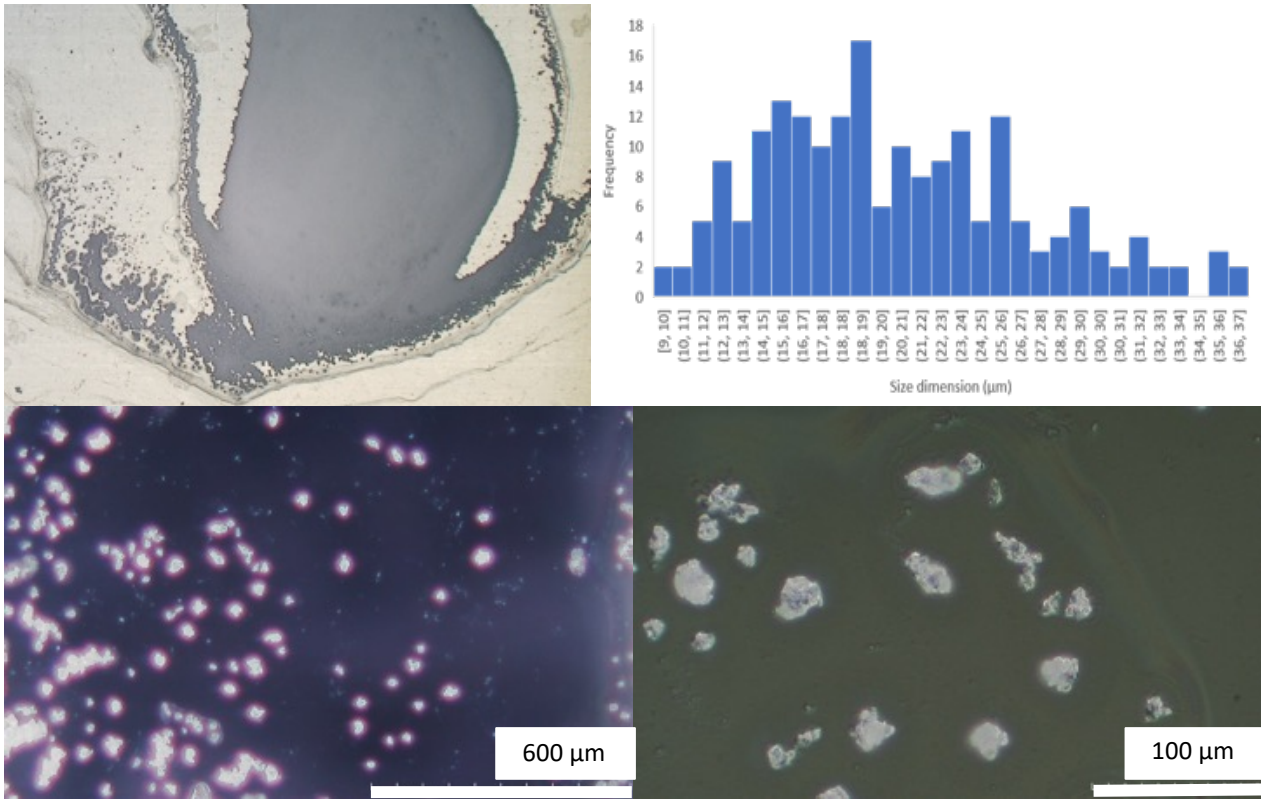


Figure 15: Optical microscope image of sample PE_BAM_16242 and statistical measurements of size distribution (mean 22 ± 13.7 µm, mode 14.1 µm).

Fig. 16 shows morphology and size distribution of sample PE_Ceridust_296. The size dimension declared is 5 µm which is in agreement with the calculated size of ca. 5 µm.

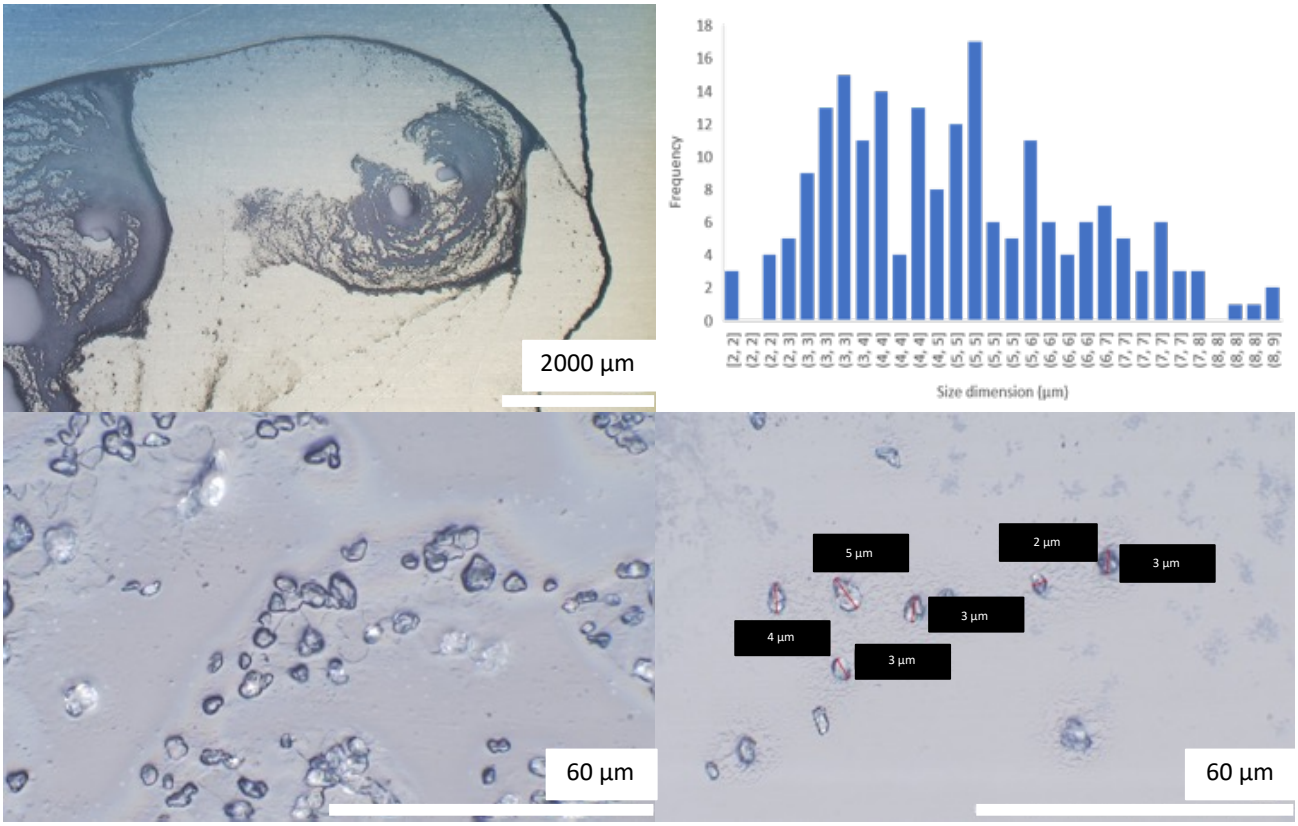


Figure 16: Optical microscope image of sample HDPE_296 and statistical measurements of size distribution obtained by ImageJ software over 100 particles (mean 4.7 ± 1.6 µm, mode 4.6 µm).

PET plastics

Concerning the PET samples reported in Figures 17-19, all samples appeared homogenously dispersed (no aggregates present), with an irregular shape, as reported in Table 2. We detected in all samples, a variety of fragments with different shapes and sizes, probably produced during the cryo-milling process. The size dimension calculated by ImageJ is almost in agreement with that referred to in Table 2.

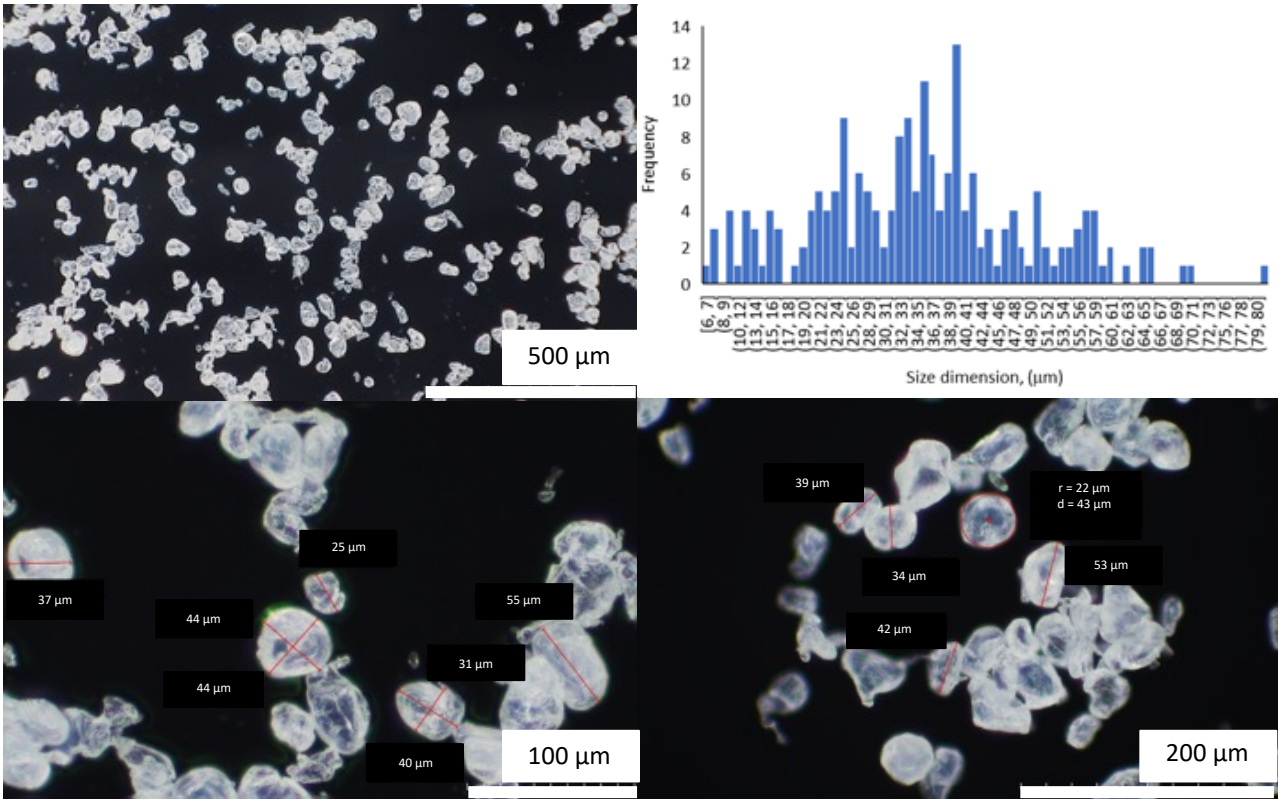


Figure 17: Optical microscope image of sample PET_BAM_21180 and statistical measurements of size distribution (mean 35.2 ± 14.4 µm, mode 39.9 µm).

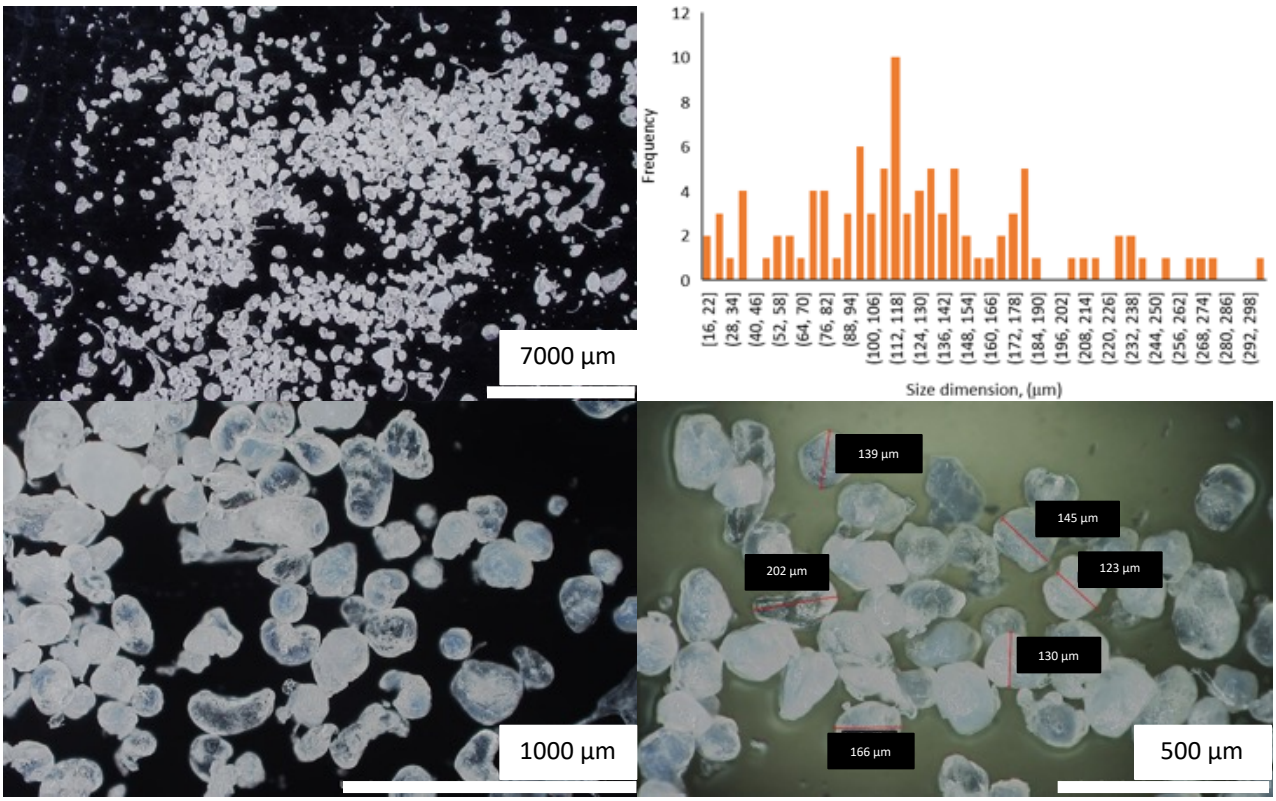


Figure 18: Optical microscope image of sample PET_BAM_21182 and statistical measurements of size distribution (mean 126.4 ± 62.2 µm, mode 81.2 µm).

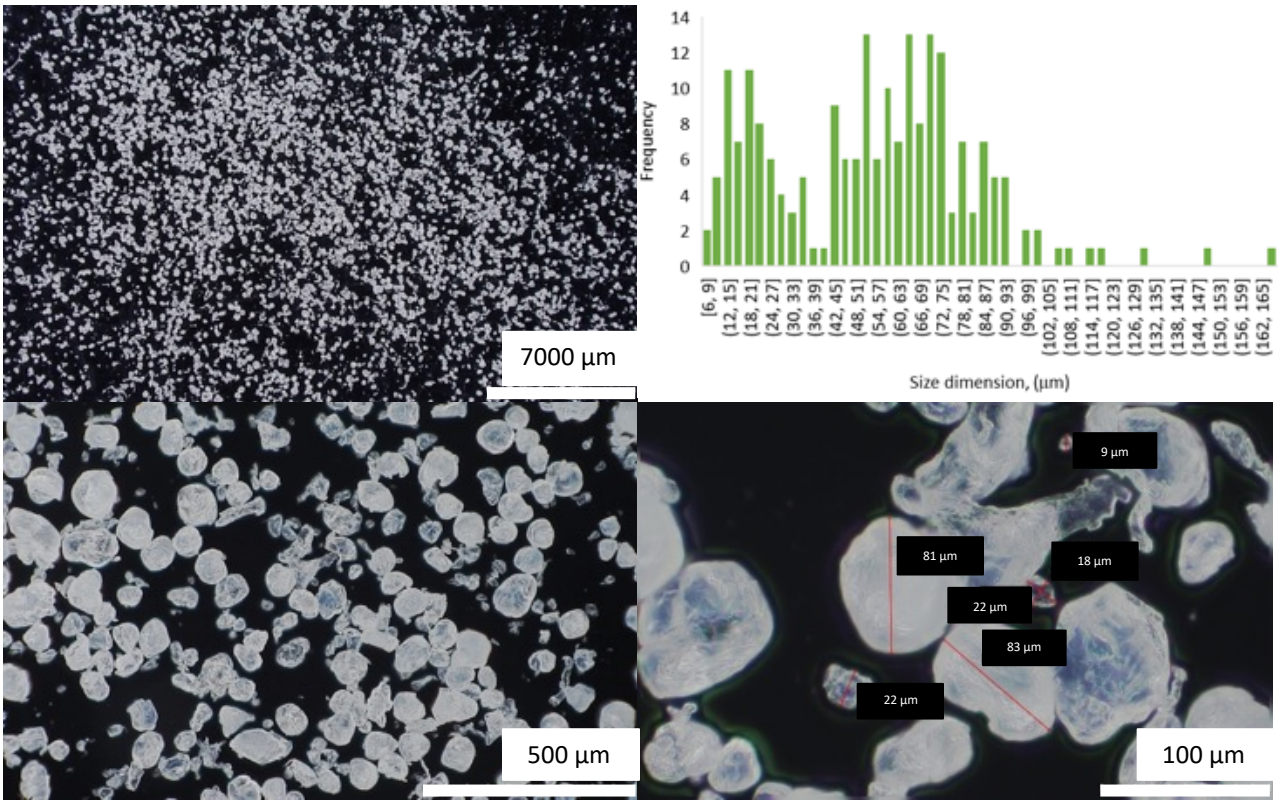


Figure 19: Optical microscope image of sample PET_BAM_21183 and statistical measurements of size distribution (mean 54.9 ± 28.2 µm, mode 11.7 µm).

Overall, it can be concluded that the low stability of PET samples is not due to aggregation phenomena but due to the large size dimensions and >1 density, in comparison to PE samples. As can be seen in Fig. 10, only PE samples were stable over the observed time period, apart from PET_BAM_21180 that was quite stable due to its lower particle size.

Colloidal Characterisation

The stock samples without dilution were analyzed with Zetasizer-Nano (Malvern) in order to measure Zeta Potential (ZP), by electrophoretic light scattering (ELS) technique (Table 4).

Table 4: ZP of micro plastics 5mg/ml in SS dispersant solution.

	Material	CODE	Z-potential (mV) ± dev.st. ; pH
	SS		-45 ± 2 ; 7
Primary	UHMW-PE	UHMWPE_16191	-37 ± 5 ; 8
	UHMW-PE	UHMWPE_16186	-87 ± 6 ; 7,6
	UHMW-PE	UHMWPE_16190	-106 ± 4 ; 7,8
	LD-PE	LDPE_16242	-85,4 ± 5 ; 7,4
	HDPE	HDPE_296	-66 ± 2 ; 7
Secondary	HDPE	HDPE_21181	-75,4 ± 3 ; 8
	PET	PET_21180	-60,1 ± 20 ; 8
	PET	PET_21182	-53,6 ± 10 ; 8
	PET	PET_21183	-67,9 ± 12 ; 7,8

All the samples showed high negative Zeta Potential consistent with the formation of SS surface coating.
The presence of SS coating was confirmed by comparing ZP vs pH curves of stock dispersions with that of SS alone (Figures 20 and 21).

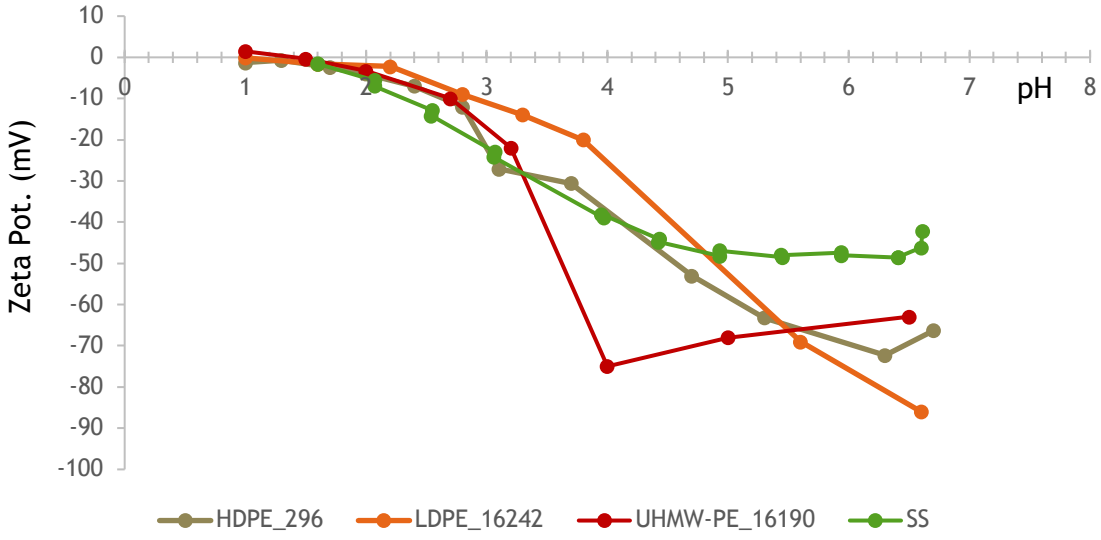


Figure 20: ZP vs pH curves of different PE samples (green, yellow and red). In orange SS titration curve. (PE stock suspension: 5mg/ml diluted 1:100)

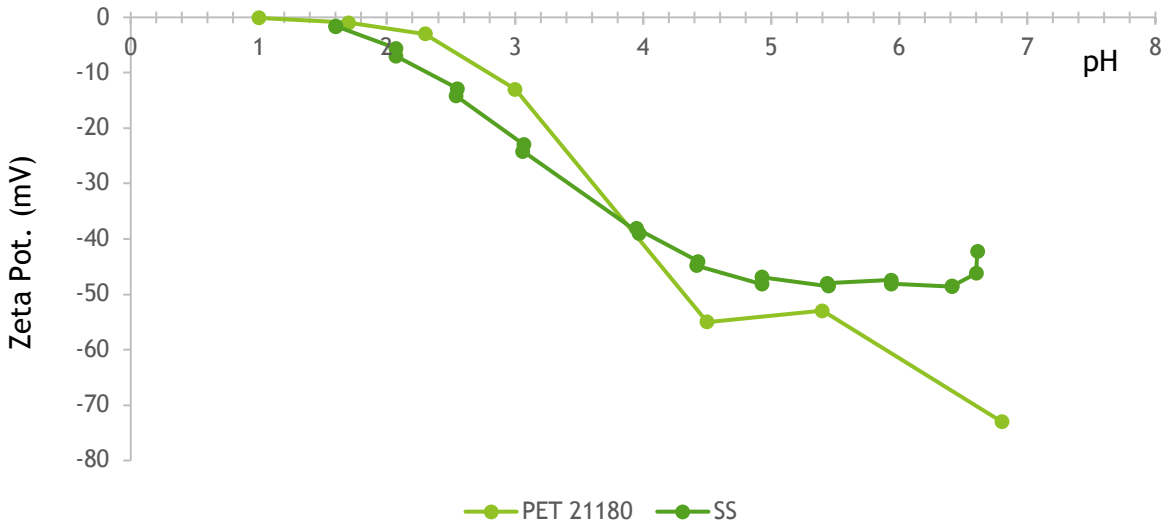


Figure 21: ZP vs pH curves of PET sample (light green). In green SS titration curve.
(PET stock suspension: 5mg/ml diluted 1:100)

From these observations it can be concluded that in all cases there is a good overlap between PE and PET curves and that of SS indicating an effective coating of plastics surface by this surfactant.

2) Dispersability of microplastics with BSA dispersant

Bovine serum albumin (BSA), having 76 % homology with human serum albumin (HSA), has been regarded as a promising material in nanomedicine for its low cost, biodegradability, nontoxicity, non-immunogenicity, water solubility, high ligand-binding properties, intrinsic fluorescence, stability to pH and temperature, wide acceptance in pharmaceutical industry[3]. For the purpose of increasing the colloidal stability of dispersions suitable for toxicological studies, the use of serum albumin is thought to be “bio-mimicking” and relevant due to its presence or compatibility in the most relevant primary exposure organs (skin, lung, and circulation). The serum-albumin-enhanced stabilization of colloidal suspensions may be due to different stabilization mechanisms. Stable dispersions can be achieved in three different ways: 1) steric, 2) depletion (or polymeric) or 3) charge-stabilization. The steric (depletion) stabilization is due to e.g., proteins or polymers, which separate particles and inhibit their agglomeration, whereas repulsive surface charges are the cause of charge stabilisation (Figure 22)[1].

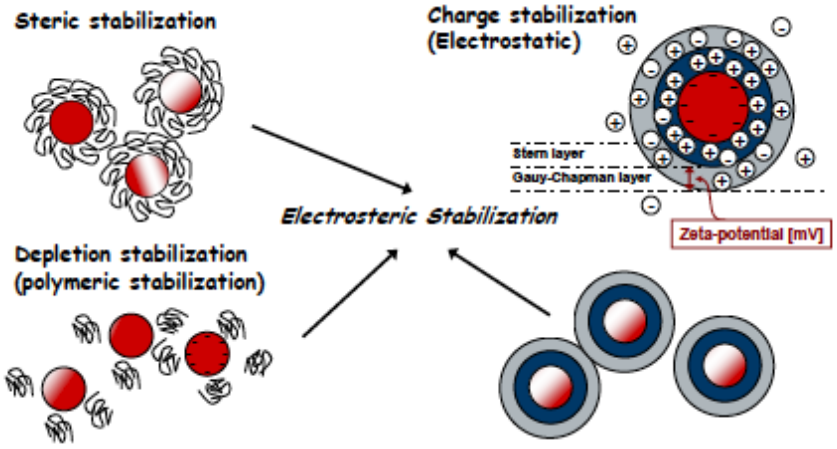


Figure 22: Mechanisms for enhanced stability of BSA stabilized colloidal dispersions [2]

The stability of the PlasticsFatE MP test materials dispersed in BSA (conc. 5mg/ml) was evaluated by a visual observation (Figures 23-28). From this preliminary observation, we noticed that already after 5 minutes the majority of stocks settle down to the bottom (PET) or floated to the top (PE) of the vials. As already observed with SS, only the HDPE_296 sample (size lower than 10μm) showed a good degree of dispersion over time and was easily re-dispersable, by a gentle shaking of the sample.
The floating kinetic of the HDPE_296 sample over time was investigated by the use of a turbidimeter. The turbidity, expressed as Nephelometric Turbidity Unit (NTU), halved in the first hour and then slowly decreased over 72 hours (Figure 30), indicating the presence of dispersed particles also after 24h.

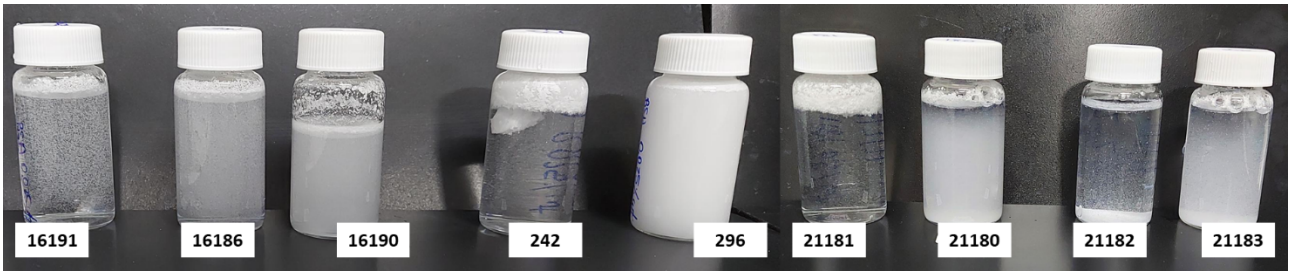


Figure 23: Dispersibility of UHMWPE_16191, UHMWPE_16186, UHMWPE_16190, LDPE_16242, HDPE_296, HDPE_21181, PET_21180, PET_21182, PET_21183 (from left to right) in BSA after 30 second of exposure.

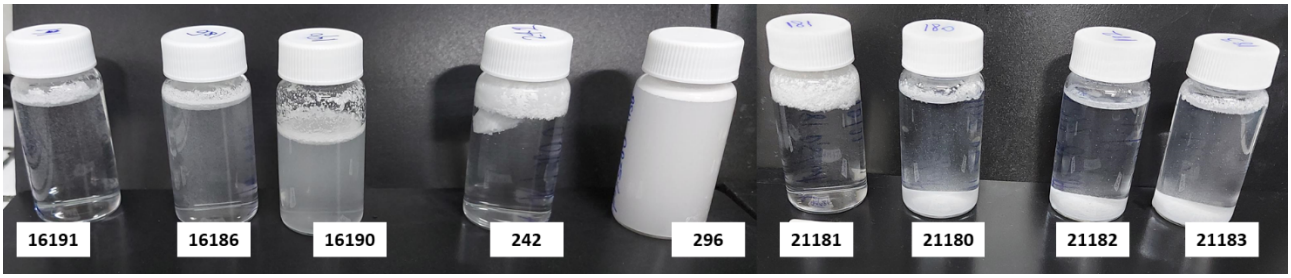


Figure 24: Dispersibility of UHMWPE_16191, UHMWPE_16186, UHMWPE_16190, LDPE_16242, HDPE_296, HDPE_21181, PET_21180, PET_21182, PET_21183 (from left to right) in BSA after 5 minutes of exposure.

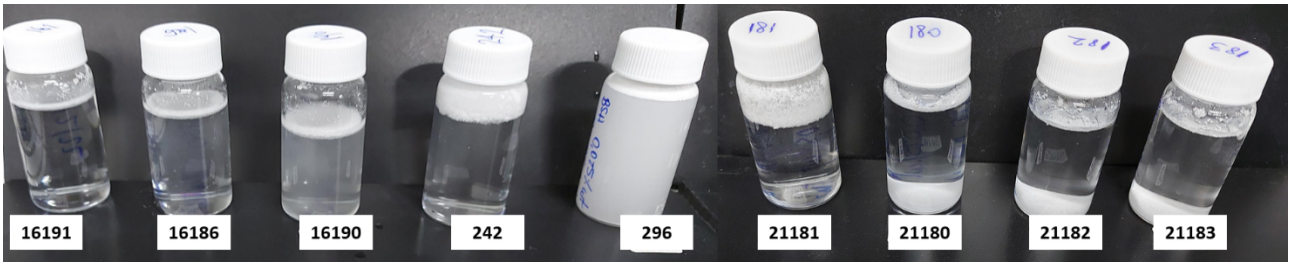


Figure 25: Dispersibility of UHMWPE_16191, UHMWPE_16186, UHMWPE_16190, LDPE_16242, HDPE_296, HDPE_21181, PET_21180, PET_21182, PET_21183 (from left to right) in BSA after 30 minutes of exposure.

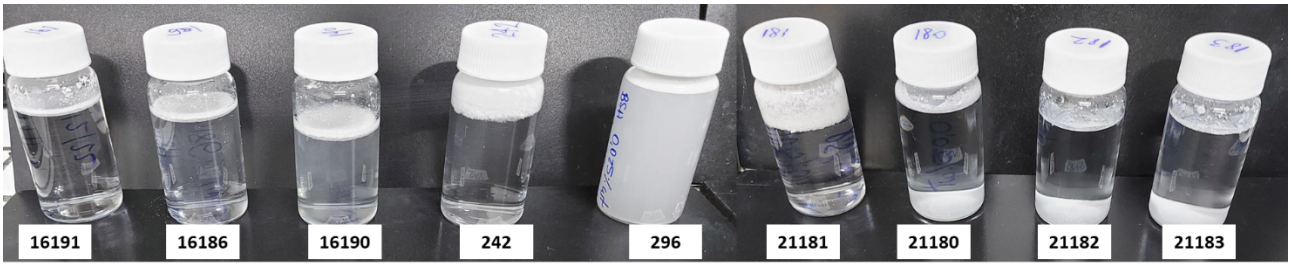


Figure 26: Dispersibility of UHMWPE_16191, UHMWPE_16186, UHMWPE_16190, LDPE_16242, HDPE_296, HDPE_21181, PET_21180, PET_21182, PET_21183 (from left to right) in BSA after 2 hours of exposure.

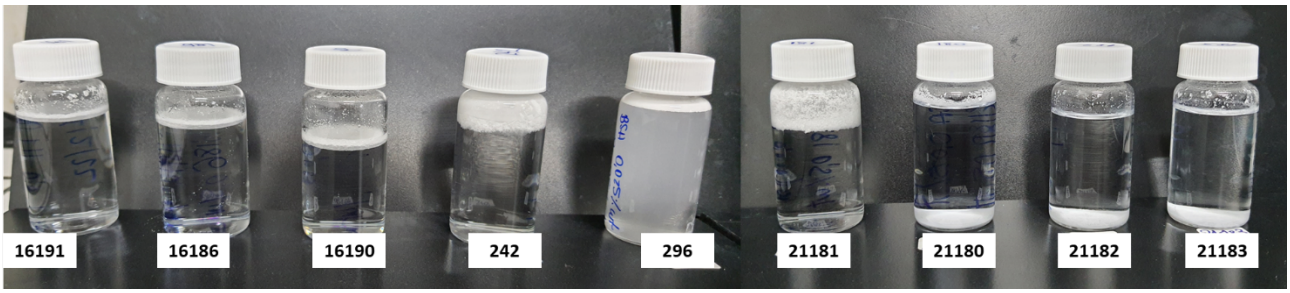


Figure 27: Dispersibility of UHMWPE_16191, UHMWPE_16186, UHMWPE_16190, LDPE_16242, HDPE_296, HDPE_21181, PET_21180, PET_21182, PET_21183 (from left to right) in BSA after 24 hours of exposure.

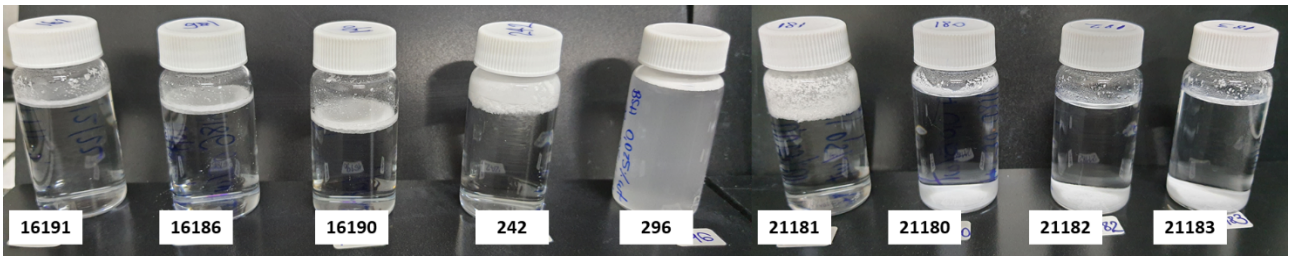


Figure 28: Dispersibility of UHMWPE_16191, UHMWPE_16186, UHMWPE_16190, LDPE_16242, HDPE_296, HDPE_21181, PET_21180, PET_21182, PET_21183 (from left to right) in BSA after 48 hours of exposure.

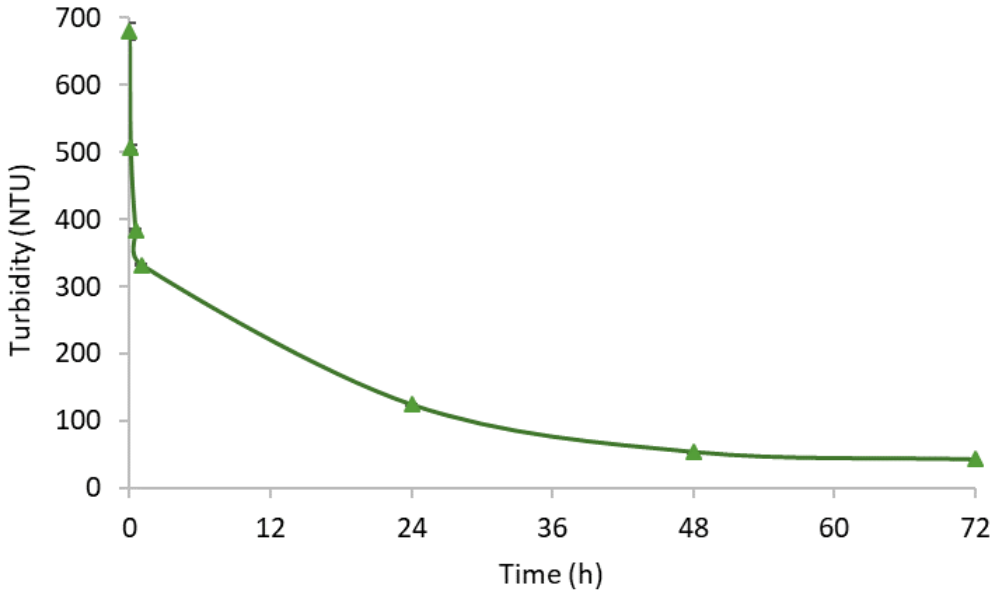


Figure 29: Turbidity of HDPE_296 sample 0-72 hours of exposure in BSA solution.

The dispersibility study with BSA confirmed that PE and PET particles with a size larger than a few microns tend to separate from the liquid very quickly, and sediment or float, depending on whether their density is higher or lower than water. Nevertheless, BSA was also considered as the selected dispersant for the preparation of stock dispersion, also in view of the fact that 1) stock dispersions can be gently shaken before use, guaranteeing a good homogeneity for the time needed (few seconds) before further diluting in simulant fluids and that 2) the Tier 2 list of PlasticsFatE samples will be enriched by lower size samples ≤ 10 microns, so with definitively higher success of handling stable dispersions (see results with HDPE-296).

STABILITY OF WORK DISPERSIONS

The selected surfactants proposed and investigated for simulating real exposure and/or testing conditions are reported in Figure 30.

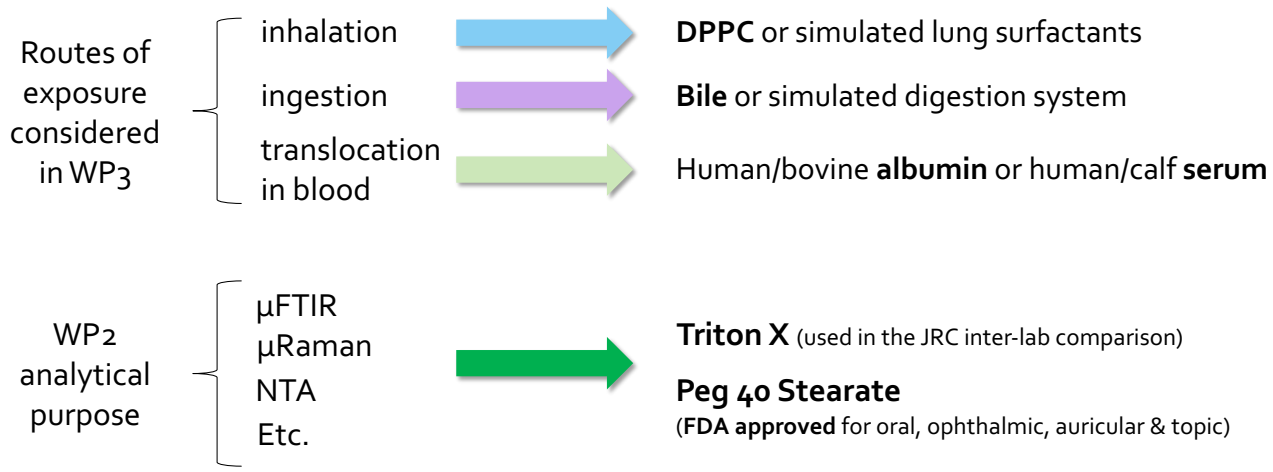


Figure 30: Proposed surfactants/stabilizer for mimicking exposure and /or testing conditions

UNITO investigated the dispersibility of secondary micro PET_21180_S-MP_F (PET), HDPE_21181_S-MP_W (aged HDPE) produced by BAM and primary HDPE_296_P-MP_P (Ceridust) purchased from Clariant, in order to provide protocols for the preparation of work dispersions, to be investigated for different exposure scenarios. PET and HDPE are highly hydrophobic polymers, and this property was maintained even when these materials were micronized (Figure 31A). This suggests that the milling process did not substantially modify the surface chemistry of the material, which is mainly responsible for the MPs dispersibility in aqueous media. For this reason, surfactants or dispersant macromolecules were necessary to bring MPs into aqueous media, forming MPs dispersions (Figure 31A). For the *in vitro* tests (in WP3), different natural surfactants/dispersants, related to the route of exposure, were chosen. The routes of exposure considered by WP3 partners are inhalation, ingestion and translocation in blood, therefore lung surfactants, bovine bile and foetal bovine serum have been proposed and tested, respectively. While for assessing the exposure level in drinking water (in WP2), synthetic surfactants (Triton X 100 and PEG-40-Stearate) have been tested.

Images in Figure 31A, clearly show the ability of bovine bile (that contain strong bile surfactants, i.e. bile salts) to bring (disperse) MPs into water, with similar results obtained with Triton X 100 and PEG-40-Stearate; while work to disperse MPs with lung surfactants and FBS is ongoing.

Effective tested dispersion protocols:

MPs (mg/mL)	Bovine Bile (mg/mL)	Triton X 100 (mg/mL)	PEG-40-Stearate (mg/mL)
Up at least to 5	6 (filtered at 0.2 µm)	≥ 0.2	≥ 0.5

	Bath sonication (30 min, 305W, ice)	Bath sonication (30 min, 305W, ice)	Bath sonication (30 min, 305W, ice)

Flow particles imaging analysis (FPIA) was used to characterize the obtained aqueous dispersions. With this technique it is possible to measure MPs size distribution, concentration and circularity. It is important to consider that this technique, depending on its setting, can measure a size range between 2-100 µm and 0.8-100 µm. Figure 31B shows the FPIA profiles (size *vs* MPs concentration) of PET, aged HDPE and Ceridust all dispersed with Triton X. In particles concentration, PET presents two populations with mean sizes at 40 µm and 3 µm, respectively (very similar results were obtained dispersing PET with bovine bile and PEG-40-Stearate). Aged HDPE presents a broad size distribution ranging from 2 to 80 µm, with the majority of the particles between 2 and 20 µm (very similar results were obtained dispersing aged HDPE with bovine bile and PEG-40-Stearate). Ceridust presents a size distribution ranging from 0.8 to 10 µm. Unlike the other tested materials, once dispersed, Ceridust presents a different size distribution depending on the surfactant used (Figure 32A). Bovine bile and, to a lesser degree, PEG-40-Stearate increased the percentage of smaller particles compared to Triton X, suggesting the presence of MP aggregates that the first two surfactants disaggregate more effectively. The effect of sonication on the size distribution of Ceridust dispersed with the different surfactants was studied. Figure 32B shows the effect of Ceridust dispersed with Triton X, evidencing a strong effect of sonication and the power of sonication. This result is in good agreement with the hypothesis of having microscopic aggregates (<10 µm) in the dispersion. In support of this hypothesis, optical microscopy images (representative of a larger number of particles) taken by FPIA (Figure 32C) show that part of the dispersed MPs in the range of 5-10 µm are actually aggregates. The difference in MPs concentration between just vortexing the dispersion and sonicating it by bath sonication is due also to the ability of sonication to bring into dispersion a higher amount of Ceridust powder, since just by vortex large wettable millimetric aggregates were observed (data not shown).
Sedimentation and flotation kinetics of PET and aged HDPE were measured following the decrease of the dispersions' absorption at 580 nm by a UV-Vis spectrophotometer (Figure 33). PET dispersed with Triton X seems to manifest two-step sedimentation kinetics, a rapid step in the first 10 minutes where a decrease of around 95% of the absorbance was observed and a slower one leading to absorbance equal to ~ 0 after 115-120 minutes. Clearly, the absorbance value depends on the MPs size and concentration but also on other MP properties, thus a direct correlation with FPIA results is not possible. However, kinetic results are in good agreement with the observed size distribution measured by FPIA, since the large MPs are expected to rapidly sediment while the small population needs more time. Instead, aged HDPE exhibit just a 40% of absorbance decrease after 10 minutes, 67% decrease after 50 minutes, 69% after 120 minutes and 95% after 1080 minutes (18h). An initial rapid flotation kinetic, followed by a second slower step and a third very slow step is again in good agreement with the broad size distribution observed by FPIA of aged HDPE. Studies on the flotation kinetics of Ceridust are under investigation.

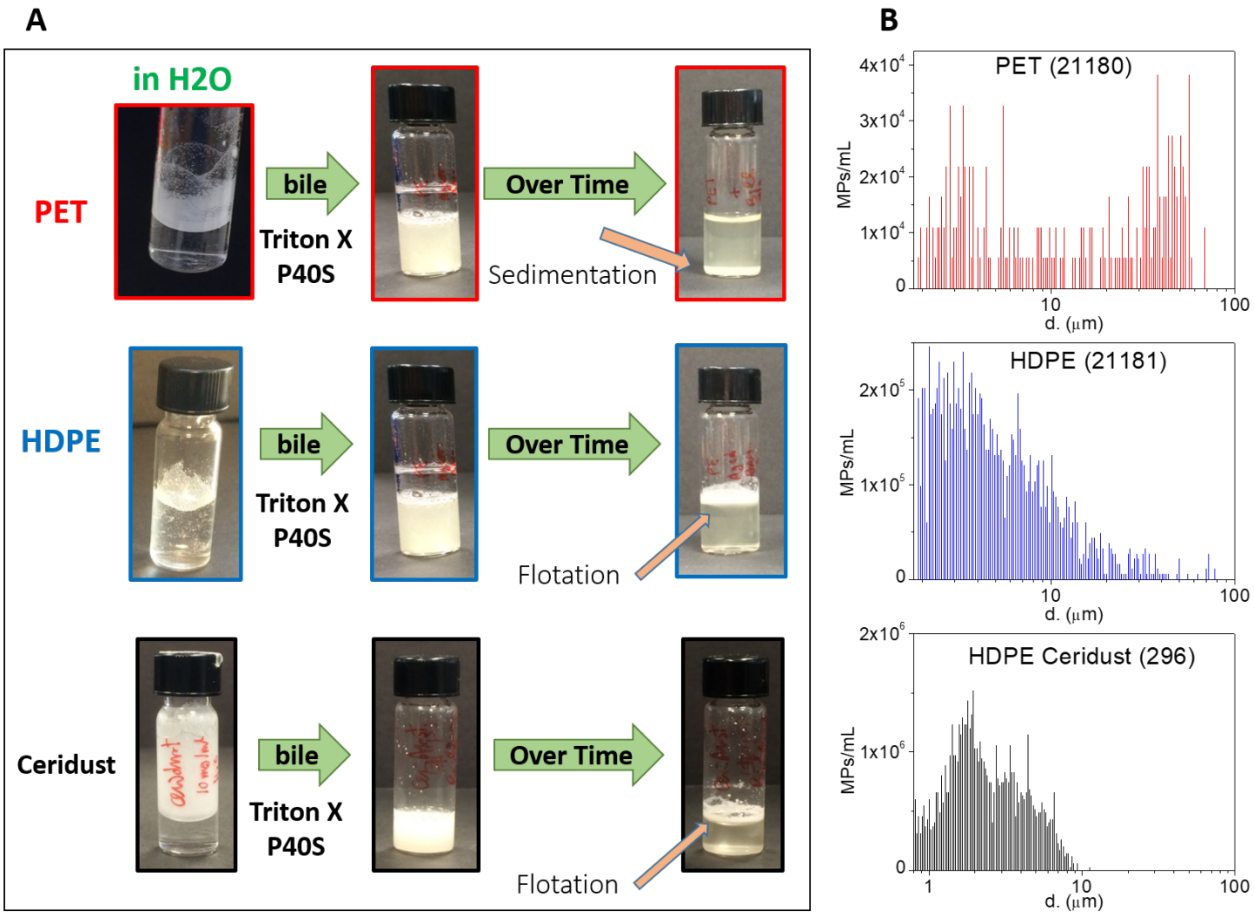


Figure 31: (A) Dispersion and stability of PET, HDPE_21181, HDPE_296 Ceridust by bovine bile, triton X and PEG-40-stearate (P40S). (B) FPIA profiles of PET, HDPE, Ceridust MPs (1mg/mL) dispersed by triton X (1mg/mL).

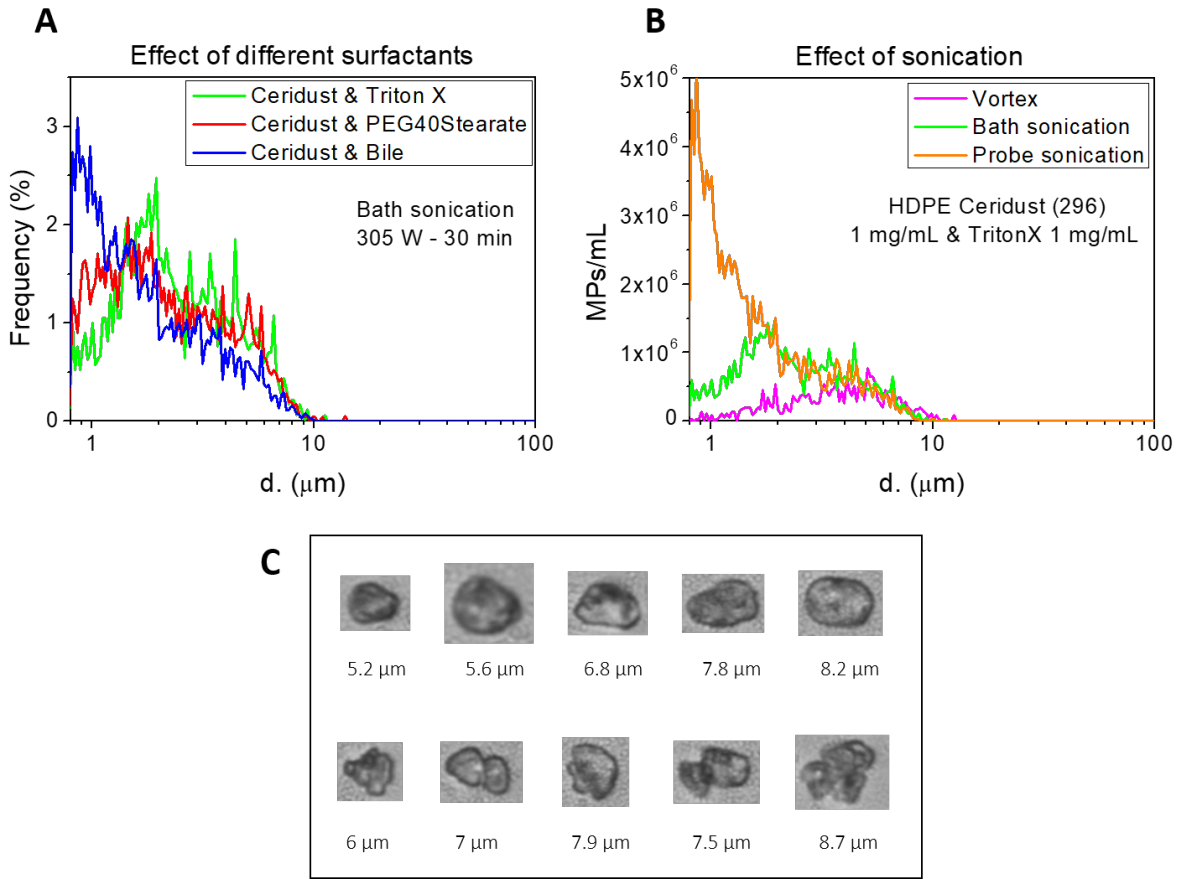


Figure 32: (A) FPIA profiles of HDPE_296 Ceridust dispersed by Triton X, PEG-40-Stearate and bovine bile. (B) FPIA profiles of Ceridust dispersed by Triton X; vortexed; sonicated by bath sonication (30 min -305W) and by probe sonication (15 min - 400W). (C) FPIA optical images of MPs in dispersion.

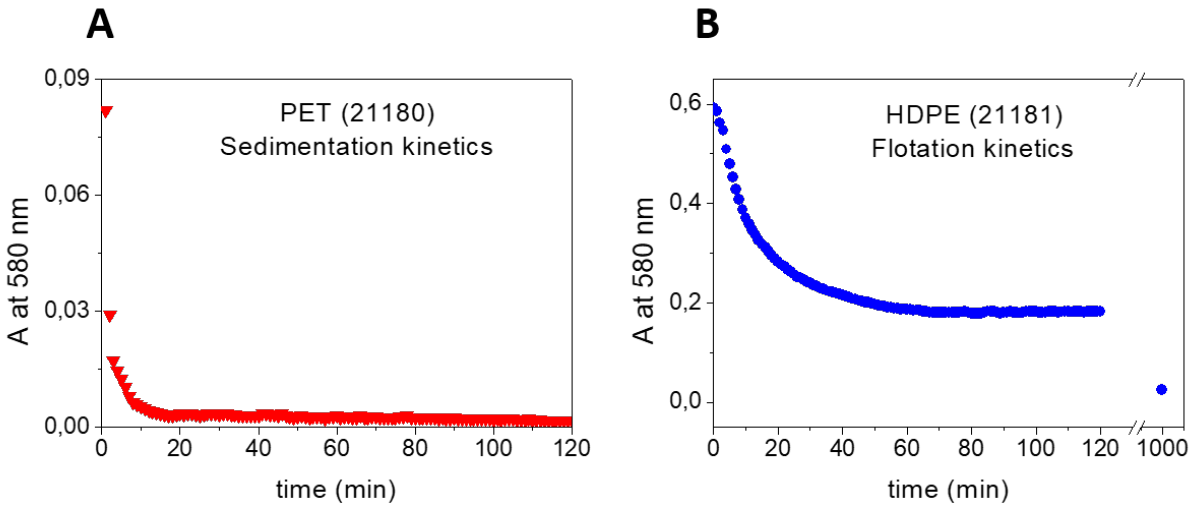


Figure 33: (A) Sedimentation kinetics of PET (1mg/mL) dispersed by Triton X (1mg/mL) followed by UV-Vis spectroscopy (Absorption values at 580 nm). (B) Flotation kinetics of aged HDPE (1mg/mL) dispersed by Triton X (1mg/mL) followed by UV-Vis spectroscopy (Absorption values at 580 nm).

Specific Objective 4	To facilitate particles labelling, traceability and detection by means of fluorescent markers.

In ISTEC, we investigated the state of the art of existing strategies for plastics labeling with fluorescent molecules, from organic/biological sources and inorganic (rare earth metal complexes). Nile red is used as a reference for the quantification of primary and secondary microplastics[4]–[6]. However, other organic and inorganic dyes are under evaluation to find more selective and efficient luminescent markers[7]–[9]. In this field, in ISTEC, we attempted to couple two different types of microplastics, PU and LDPE MPs, with BSA protein and luminescent complexes. BSA can be attached by Re(I) luminescent complexes [10], thus, BSA could possibly transfer the luminescent ability to plastics coated by BSA. Nevertheless, the results, obtained so far, showed a weak affinity between BSA and microplastics, which does not promote the formation of a stable and luminescent protein corona requiring further optimization of the system. However, we identified other possible alternatives to Nile red dyes such as some essential oils: citronella (Cymbopogon winterianus), Japanese mint (Mentha arvensis), clove bud (Syzygium aromaticum), and bergamot (Citrus bergamia)[11].

RAMAN-LUMINESCENCE MAPPING

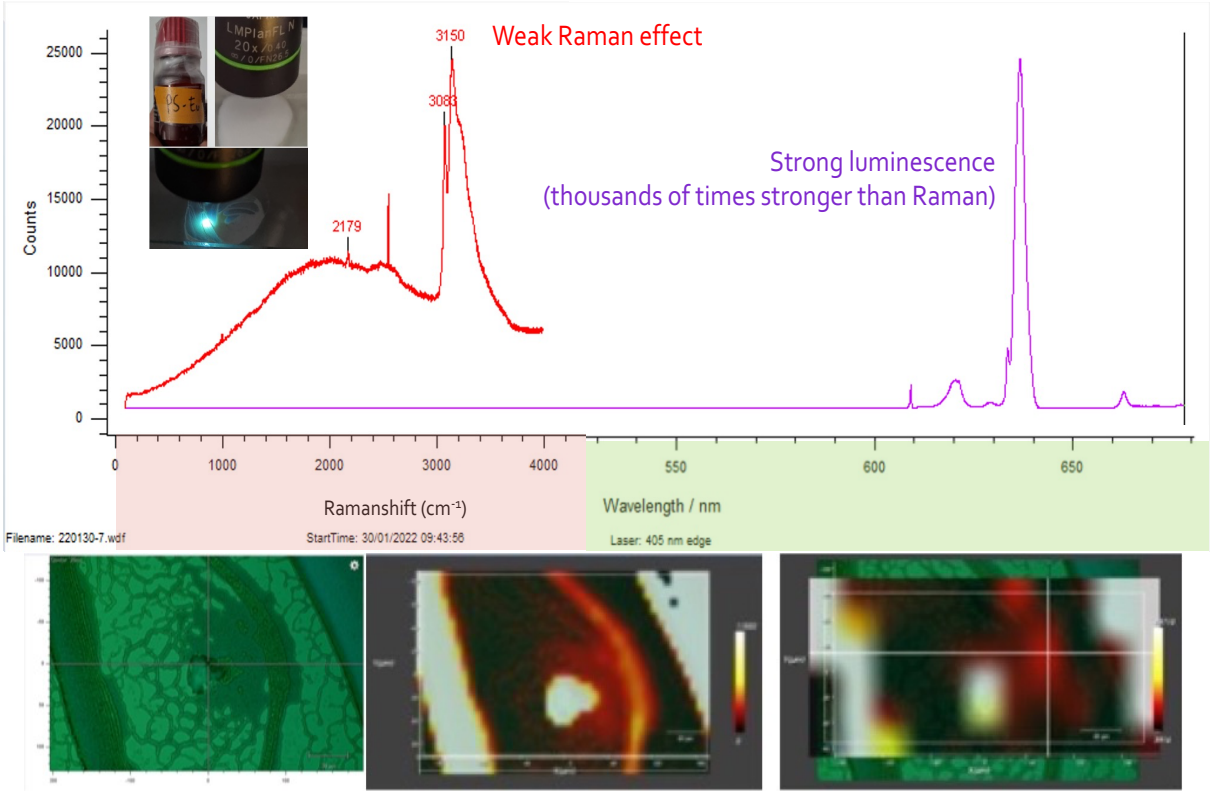


Figure 34: (TOP)Representative same-spot Raman/luminescence (red/magenta traces) spectra on same spot of a dried Eu-PS solution drop. (BOTTOM) (left) white light 400x250 µm2 area used to map the dried drop; (middle), 1200-spot fast luminescence mapping of the intensity of the 618 nm emission of europium line in such area (6 minutes); (right), 66-spot slow Raman mapping of polystyrene C-H vibration mode intensity in same area (more than 1 hour)

The team at CSIC used Raman spectroscopy combined with luminescence spectroscopy in the same equipment and tested the methodology with Eu-doped polystyrene (Eu-PS) particles. This combined Raman system recorded luminescence with a high intensity as compared to Raman alone. Thus, fast detailed luminescence mapping was performed to locate where the Eu-PS particles were. Raman mapping was then collected at the locations where luminescence "beacons" polystyrene particles were detected.

Sequential same-spot Raman/luminescence spectra are reported in Figure 34. At the bottom are shown the white-light image of the mapped area (left), and the corresponding intensity maps of luminescence (middle) and Raman (right) signals. Microscopy spatial resolution is the same for luminescence or Raman spectroscopy. The higher spatial resolution in the luminescence map is due to the very short acquisition time (0.3 sec) which allows measuring 1200 spots in reasonable time. The lower spatial resolution of Raman mapping is due to much longer acquisition time (60 sec). These preliminary results confirm the advantage of combined luminescence/Raman mapping that allows a fast high-resolution mapping of selected areas to spot with a detailed Raman study, at the exact coordinates where europium-doped polystyrene particles are located.

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3. Deviations from the Work plan

In order to address a hot topic in the start-up period, which is the definition of suitable dispersion protocols that allow a sound comparison and link between data acquired by different partners using different characterisation endpoints, it was agreed to move some PMs / efforts of UNITO from Task T2.3.3 to T1.1. This will, however, not compromise the work planned in T2.3.3 (the respective work performed is summarised in the section Stability of Work Dispersions).

4. Performance of the partners

CNR-ISTEC as WP1 leader is taking care of PlasticsFatE's material strategy reported in the present deliverable and contributed to the development of the stock dispersion protocol.
BAM produced the main samples included in the present portfolio of PlasticsFatE's test materials and took care of samples distribution.
UNITO contributed to the development of the work dispersion protocols.
CSIC investigated plastics traceability by Raman-luminescence mapping.

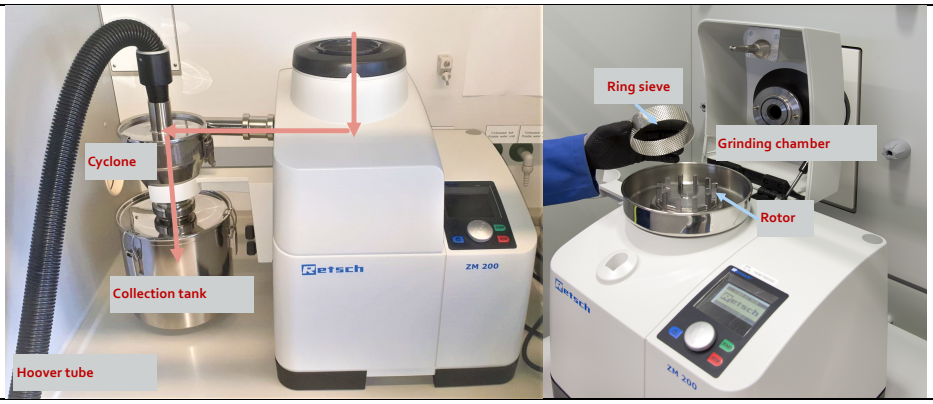
5. Conclusions

The Steering Board deems this deliverable to be fulfilled satisfactory.

Annex

METHOD DOCUMENTATION

Technique	Methodology
Weathering chamber	To produce HDPE_21181_S-MP_W 70 g of a PE-foil delivered by Lyondellbasell (Rotterdam, Netherlands) was irradiated at 75 °C under constant UV-A radiation and 15% r.H. for 100 h in a climate chamber.
Cryomilling	Two ways of cryomilling were applied to different plastic materials. Aged PE foil was cryogenic ground in the Retsch CryoMill with five stainless steel balls (diameter 12 mm each) in a 25 ml grinding vessel. The pre-cooling time was 15 min (5 Hz). Milling occurred by 6 cycles each 5 min (25 Hz). The time between the individual cycles was 2 min (5 Hz). For plastic granulates e.g., of PET, the granulates have been pre-cooled for 15 min in liquid nitrogen before milling in the centrifugal mill Retsch ZM200 with a 120 µm ring sieve at a rotor speed of 10000 rpm.

	
Particle size distribution by laser diffraction	The HELOS BR (Sympatec GmbH, Clausthal-Zellerfeld, Deutschland) was used. According to the expected particle size range a fitting lens was chosen R5 (4.5-875 µm), R3(0.9-175 µm). All measurements were processed with the supplied software PAQXOS 4.1 (Sympatec GmbH) using Fraunhofer approximation. For dry particles the RODOS dry dispersion system with an injector width of 4 mm and pre-pressure of 2.5 bar in combination with the micro dosing unit ASPIROS (transportation sleigh speed 100 mm/s) was applied. 50 to 100 mg of sample powder were put in a device-specific glass vial. The measurement time was 10 s.
Electrophoretic Light Scattering	Zeta potential of MPs dispersed at 0.5 wt% in DI water and dispersing system, were evaluated by using a Zetasizer instrument (Malvern Instruments, Zetasizer Nano-ZS, Malvern, UK) based on the electrophoretic light scattering (ELS) techniques. The zeta potential was measured on 700 µL of the sample at 25 °C, setting the measurement time, the attenuator position, and the applied voltage to automatic. After a 2 min temperature equilibration step, the samples were measured three times, and the data were obtained by averaging the three measurements. The data of zeta potential (mV) are derived by electrophoretic mobility using Smoluchowski's formula. The reliability of the measurements was controlled by check the phase plot graph. The same instrument coupled with an automating titrating system was used to create zeta potential vs. pH curve (sample: HDPE – PF – P; UHMWPE – PF – PC; LDPE – PF; PET – PF – L suspended in dispersing system and DI water) to identify the pH at which the zeta potential sets to zero, namely the isoelectric point (pHi.e.p.). The titrants used were 0.1M KOH solution and 0.1M HCl solution.
Optical microscopy	The morphology and shape of MPs was investigated by dropping stock dispersions, on a glass slide and drying it on an oven at 80°C for 15 minutes. We analyzed the samples at optical microscope (Hirox 3D digital microscope, RH200). The size dimension was evaluated by software ImageJ, over a count of more than 100 particles for each sample.
Scanning electron microscopy	Images of the particles were recorded with a Scanning Electron Microscope of type Zeiss Supra 40 (Zeiss, Oberkochen, Germany) equipped with a high-resolution cathode (Schottky field emitter), an Everhart–Thornley SE detector. The particle powders were transferred onto carbon tape and the loosely bound particles were boosted with an air jet. Images have been

	recorded at an acceleration voltage of 1 kV, WD of ca. 5 mm and at different magnifications.
FTIR Spectroscopy	A Nicolet 6700 equipped with a DTGS detector was used. ATR technique using SmartOrbit Diamond module was applied if larger amounts of material are available. Small amounts were measured in transmission mode embedded in KBr pellets. The spectra were recorded from 4000 to 500 cm-1 averaging 32 scans with a resolution of 4 cm^{-1}. The obtained data were processed with the OMNIC 9 software (Thermo Fischer Scientific, Karlsruhe, Germany). By IR spectroscopy it is possible to identify common plastics according to the characteristic absorption bands. It also provides indicators to identify aging.
Differential scanning calorimetry	DSC was performed with a DSC 7020 (Seiko, THASS, Germany). The temperature program with a heating rate of 10 K/min was chosen according to the expected glass transition temperature T_g, melting point T_m and decomposition temperature T_{dec}. The lower limit was either room temperature or at least 30 K below the expected T_g. The upper limit was 30 K above the T_m but below T_{dec}. By DSC measurements significant changes in the morphology of the polymers become visible.
Electrostatic charge	Electrical charge of powder was measured by a double Faraday cup connected to an electrometer (Keithley 6517A, Cleveland, OH, USA).The double Faraday cup consisted of two stainless steel cylindrical cages, of different sizes, concentrically set and isolated to each other by a Teflon plate. The device is connected to an electrometer which has a particularly high input impedance of 1014 Ohm. Intrinsic or net electrostatic charge was determined by direct dropping of the polymer powder into the inner cage of the Faraday's cup. Tribocharge was determined by sliding the dry powders by a 50 cm long quartz tube with an inclination of 45°. The experimental setup included an electronic balance to determine the powder weight; thus, it was possible to normalize the measured charge by the polymer powder mass. The process was repeated 10 times for each powder by using new samples of 2 g each time. All experiments were carried out at room temperature and under a relative humidity, R.H., of 55–65%. These environmental conditions were the normal working condition used during tests and materials handling.
Flow Particle Imaging Analysis	FPIA was used to measure particles size distribution and concentration in aqueous dispersion; particles circularity was also determined. FPIA was performed by using a Sysmex FPIA3000 analyser equipped with the high magnification objective lens unit. Low and high-power field (2× secondary lens) was applied, which allows to measure particles from 0.8 to 100 µm. Dispersions were kept in agitation by mixing motor set at 650 rpm without any sonication.
UV-vis Spectrophotometer	MPs sedimentation/flotation kinetics were performed measuring the Absorbance of MPs dispersion, recorded at 580 nm with a UVICON 930 UV-Vis spectrophotometer (Kontron Instruments, Basel, Switzerland).
Raman-luminescence mapping	The measurements were made with a Renishaw inVia Qontor Raman microscope, using an x50 long distance objective. The sample was a drop of Eu-PS solution, which was left to dry in ambient conditions (Figure 1, top,

	insert). The excitation laser line was 405 nm, 0,3 second acquisition time for luminescence spectra and 60 seconds for Raman spectra.
Preparation of dispersant solutions	The following stepwise procedure is followed to prepare a water dispersant solution of Tween 60 or Na Surfactin a) Add from pipette 50 mL MilliQ water to a 100 mL mixing flask. b) Weigh out 25mg Dispersant (powder) in a weighing boat and pour it into the flask with 50 mL water, rinse the weighing boat into the mixing bottle with MilliQ water to retrieve as much Dispersant as possible into the mixing flask. c) Fill MilliQ water into the mixing flask up until 100 mL to reach a 0.25 mg/ml water solution. d) Gently stir or swirl the Dispersant-solution for a few minutes (avoid foam by not using agitated stirring) and leave the mixing flask in the refrigerator over-night for the complete dissolution of the Dispersant. The following stepwise procedure is followed to prepare a water dispersant solution of BSA ***1) Preparation of 5mg/ml BSA water stock solution:*** a) Add from pipette 50 mL MilliQ water to a 100 mL mixing flask. b) Weigh out 0.5 g BSA (powder) in a weighing boat and pour it into the flask with 50 mL water, rinse the weighing boat into the mixing bottle with MilliQ water to retrieve as much BSA as possible into the mixing flask. c) Fill MilliQ water into the mixing flask up until 100 mL to reach 5mg/ml BSA- water solution. d) Gently stir or swirl the BSA-solution for a few minutes (avoid foam by not using agitated stirring) and leave the mixing flask in the refrigerator over-night for complete dissolution of the BSA. e) Sterile filter the solution into a new flask through a 0.2 µm sterile disposable filter ware. Sterile filtration causes about 28% loss of BSA resulting in a true BSA concentration of about 3,6mg/ml (as determined by a Pierce BCA protein Assay Kit for microplate reading in Nanogenotox protocol). ***2) Preparation of 0.25mg/ml BSA-water solution (0.25 mg/mL):*** f) The 0.25mg/ml BSA solution is achieved by simple dilution of the sterile-filtered 3,6mg/ml BSA batch solution. Example: 2 mL of BSA solution (about 3,6mg/ml BSA) is diluted with 28 mL ultrapure or MilliQ water (final volume = 30ml) (Dilution factor = 15x) to reach a batch solution of about 0.25 mg/ml.
Preparation of STOCK DISPERSION	The suggested concentration for (eco) tox studies is 5 mg/mL stock dispersion (5000 ppm) a) Turn on the weighing box, glove box, fume-hood 15-30 minutes before use. b) Ensure wearing appropriate work dress and that all material to be used for weighing and storage is present before commencing the work.

	c) Open a clean empty vial for preparation of the stock dispersion and place it on the weigh. d) Tara the weigh. e) Open the vial without shaking it f) Remove the electrostatic charge on the vial using a neutralizer and **weigh out the required mass** with a spatula in steel or glass and close the lids g) Add the require volume of water + dispersant (W+ disp.) solution: C (*mg/mL*) = m (*mg*) / V (*mL*) e.g.**250 mg** for **50 mL** of W + disp. (C = 5mg/mL) (BIG STOCK VOLUME) e.g. **30** mg for **6** mL of W + disp. (C = 5mg/mL) (Nanogenotox VOLUME) *Sonication / Storage* a) Place the glass vial containing the stock dispersion in the US bath for 15 min, using a sonicator bath (e.g. 80W per 100% amplitude). b) Store the close vial containing stock dispersion at 4°C and vortex 1 min before using.
Preparation of MPs dispersion with Bile; Triton X & PEG-40-Stearate	**5 mg/mL stock dispersion (5000 ppm)** 1) Prepare the dispersion solution: - Bovine Bile: 6 mg/mL; filter it with a 0.2 µm RC filter - Triton X: ≥ 0.2 mg/mL - PEG-40-Stearate: ≥ 0.2 mg/mL 2) Weight the MP powder in a glass vial 3) Add the dispersion solution to the MP powder 4) Well vortex the dispersion Place the dispersion in US bath filled with water and ice for 30 min (305W)
Turbidity measurements	The measurements were performed by a Turbidimeter (AL225 T-IR MICRO USB 0.01-1100 NTU). The NTU unit measures the scattered light at an angle of 90°, as stipulated in EN ISO 27 027. The instrument is calibrated in the range of < 0.1 to 800 NTU. Samples were prepared by diluting the stock dispersion 4 times. The vial was put in the turbidimetry hole and left there, in order to follow the sedimentation or floatation kinetic during the experiment time.