D1.4-RE: Completion of the semi-automatic sample evaluation system

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Objectives of WP1 - Marine Microplastics sampling & processing

Our hypotheses are that highly-populated areas (via estuaries etc.) and Baltic Sea lanes are the most relevant sources for microplastics (MP), and that beaches and coastal sediments are the major sinks of MP. On the other hand, besides beaches, vertical flux of MP towards the sediments is supposed to be one of the essential sinks in the Baltic Sea. To test this, the aim of this WP is to identify MP distribution:
- vertically, in the water column and associated sedimentation rates.
- spatially, in water, biota, sediment and beaches of relevant estuaries, shorelines and the open seas in the Baltic Sea.

This deliverable is a contribution to Task 1.5 *Methodological development and evaluations*, *part 2 Identification of MP* and *Task 1.2 Extraction, identification and quantification of MP from field samples, part (C).* The focus of this task lies on the development of a (semi)-automated analysis and evaluation system to identify size, size distribution and composition of MP (< 500 µm) on filters using Raman and FTIR microspectroscopy. This includes three steps:

1. development of a contamination-free filtration system to apply purified MP samples on microspectroscopy substrates (sample extraction/purification is done by IOW, see Task 1.2 and 1.5 part 1)
2. development of a software-based optical particle identification in combination with automated Raman/FTIR measurement
3. development of automated software tools for the evaluation of Raman/FTIR data

Results

Summary

The sample processing and analysis procedures were optimized to allow for a clean, detailed and time-efficient analysis of large numbers of particles per sample. A dedicated MP-free laboratory was set up in order to avoid contamination by MP from the lab environment when MP are filtered and fractionated onto silicon filter substrates.
For maximizing the sample-throughput a (semi)-automated analysis and evaluation pipeline was developed. This includes optical particle identification and automated Raman measurement by GEPARD, and user-friendly, (semi)-automated data evaluation by WITec TrueMatch and GEPARD.
This optimized procedure heavily reduces the required analysis time, which is the key for being able to process all samples within the project.

1. Two-step sample filtration

The MP-free filtration laboratory was set up for avoiding any kind of MP contamination during the last step in the sample workup procedure. The filtration lab is equipped with an air-filter, a laminar flow box, as well as plastic-free lab ware and clothing.

A two-step glass filtration apparatus (Fig. 1) was designed and manufactured for fractionating the MP samples onto silicon filters with pore sizes of 10 and 50 µm, respectively.

Before filtration all equipment (glass devices, silicon filters, PTFE sealing) is carefully cleaned using appropriated media (H_2O_2, ethanol, MilliQ water, ultrasonication).

The workflow is routinely controlled by blank samples (MilliQ water, same volume and treatment as MP sample). The filtration procedure was optimized to reach very low MP contamination of less than 10 MP particles per liter of water.

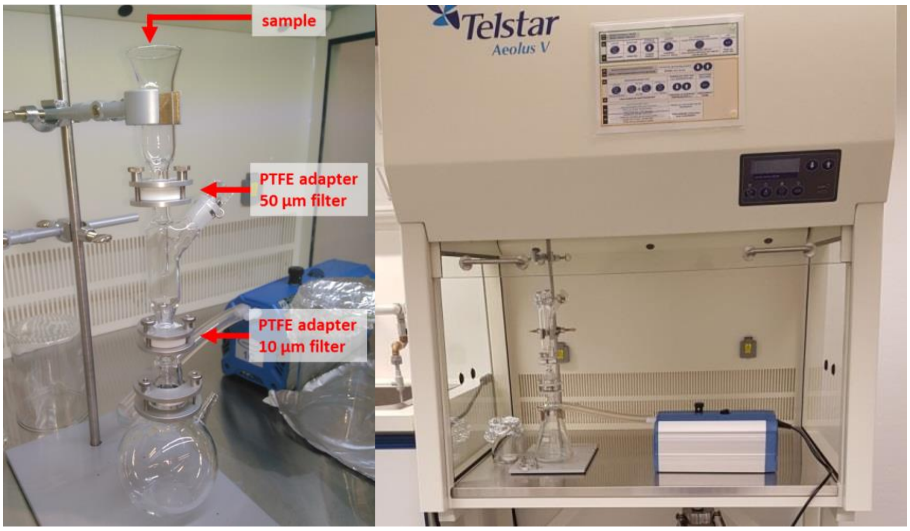

Fig. 1: Glass filter apparatus (left) for two-step filtration of MP samples in the Laminar Flow Box (right)

2. Software-based Particle Identification and automated Raman measurement - GEPARD

For the (semi)-automated analysis of up to 40,000 particles on the sample filters by Raman microspectroscopy the software GEPARD (**G**epard-**E**nabled **PAR**ticle **D**etection) was developed by the partner IPF Dresden. Key features of the software are:

- Acquisition of an optical overview, high-resolution darkfield image of the entire filter area (11 mm x 11 mm) in several z-levels. Images at different z-levels are used for calculating an image of lowest possible depth-of-field and for estimating particle height (important for the subsequent focusing of the Raman laser). An example of this darkfield image is shown in Fig. 2a.
- Automated particle identification by watershed image segmentation. A set of parameters driving the algorithm is presented to the user to tailor the algorithm to each sample. Furthermore, manual editing of single particles (e.g.

separation/combination/addition/removal of particles, correction of fibers) is included. It is shown in Fig. 2b.

- per particle, information from image segmentation are:
    - location (x,y,z) -> required to drive the microscope to each measurement spot
    - short and long size of particle (from ellipse fit)

The software is published open source under GPL and can be used free of charge (https://gitlab.ipfdd.de/gepard/gepard). Sharing the code with other groups allows optimizing the program further.

The high-performance optical particle identification and subsequent automated Raman measurement by GEPARD was implemented for two Raman systems - *alpha 300R* (WITec, Germany), equipped with two lasers 532 or 758 nm, and *inVia Qontor* (Renishaw, UK), operating at 532, 633 and 758 nm.

For comprehensive analysis of MP filters a *Spotlight 400* FTIR-microscope (Perkin-Elmer, UK) with an integrated particle analysis module was installed. Due to unforeseen technical and software issues, not caused by the partner IPF, the system is useable just since December 2018. To make the instrument ready for high-performance particle analysis of larger particle fractions, an extension of the GEPARD software is planned in the first three months of 2019.

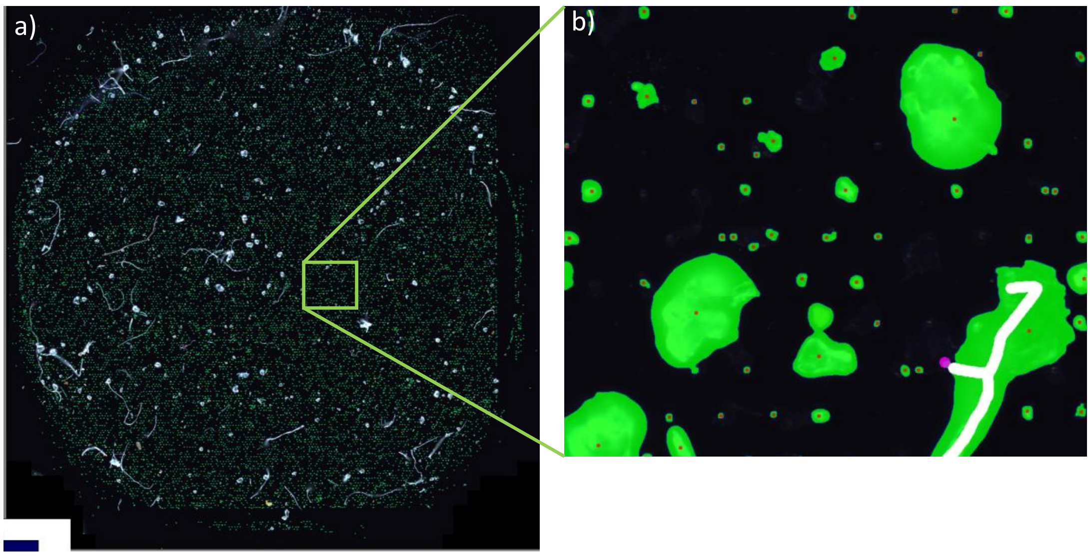

Fig. 2: a) Optical overview image (darkfield) of a sample filter b) Detail of particle identification via GEPARD, green: detected particles, red points: measurement position, white lines: manual corrections

3. Semi-automated Data Evaluation by WITec TrueMatch and GEPARD

For evaluation of both, the FTIR and the Raman data, commercial polymer databases are used in conjunction with specialized self-acquired spectral databases of common industrial polymers, additives (such as dyes and pigments) and natural substances.

Automatization of the evaluation process is provided using a commercial database search program, TrueMatch (WITec, Germany) in combination with GEPARD. TrueMatch enables the database search of the whole data set (up to 40 000 spectra) with the option of manual correction of uncertain results.

The results are reimported into GEPARD to connect and summarize all information of the whole analysis process, like image of all particles of the whole sample filter, particle ID, particle size, particle image, particle spectrum, size distribution. This is shown for an example in Fig. 3a and 3b. All information can be summarized and exported as excel sheets (per particle information plus distribution information of all detected polymer types). The evaluation pipeline also comprises automated data transfer to the final MP SQL-database, which was developed at Klaipeda University (see also task 1.4 and D 1.2).

With the program GEPARD a user-friendly and high throughput data evaluation tool is provided.

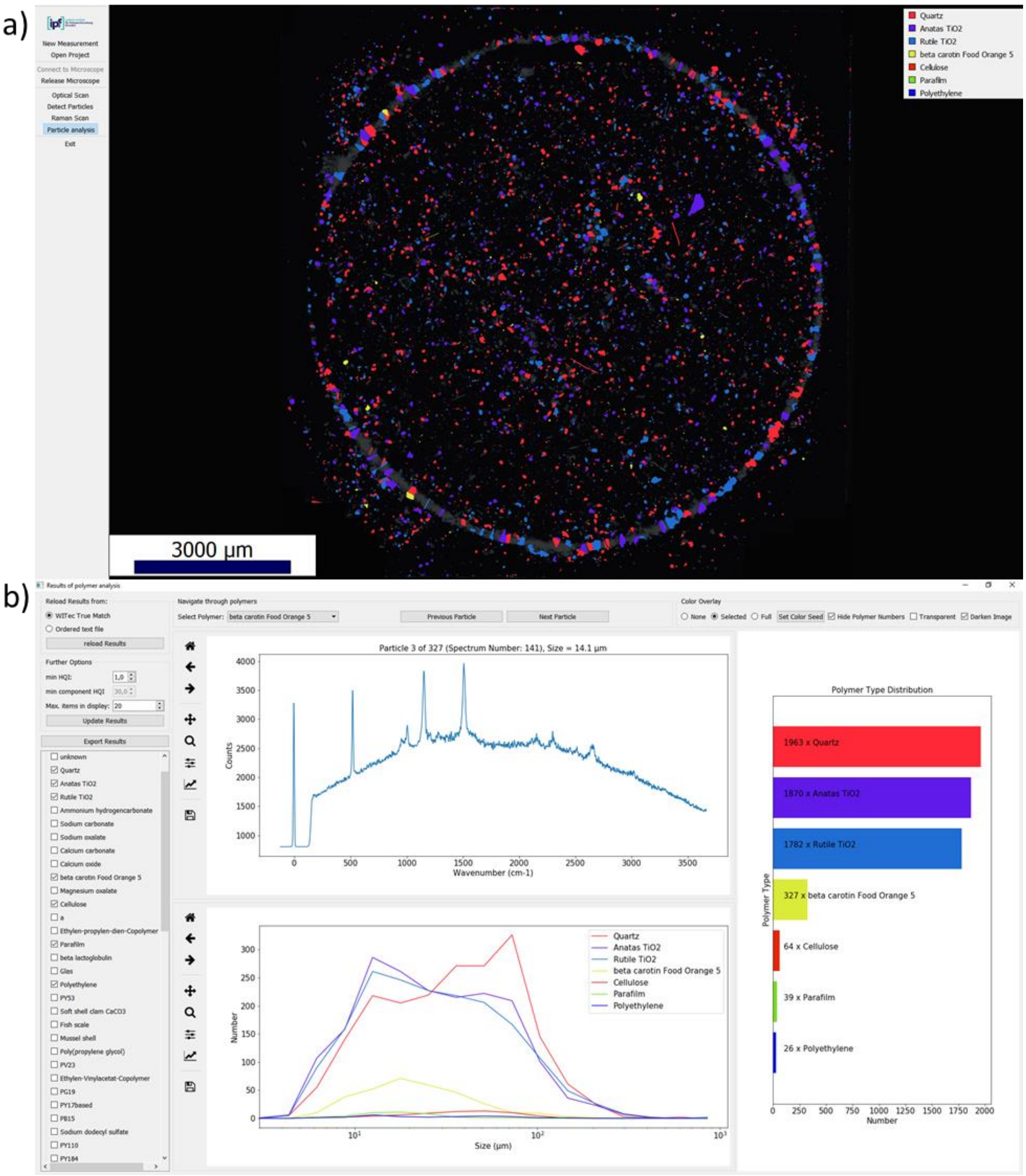

Fig. 3: Example of data summary of GEPARD for a BONUS MICROPOLL MP sample with about 6000 particles.

a) Overview of the whole sample filter, particles are colored corresponding to their identity.

b) Details of sample data (e.g. particle size, size distribution, particle spectra)