Supplemental Material

High-Throughput Analyses of Microplastic Samples Using Fourier Transform Infrared and Raman Spectrometry

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Image Acquisition with GEPARD

Defining the Region of Interest (ROI):

The ROI is defined by moving the microscope stage to the desired positions on the filter, focusing on the background of the filter (not the particles) and pressing the corresponding "read" button to register the stage position (Figure S1). A level fit is performed on the defined *x*,*y*,*z*-positions to account for a tilted filter position. The level fit is ideal for flat filters, such as the silicon filters we use.[1] For non-flat surfaces, different modes of surface triangulation can be implemented with little effort as Python's SciPy library comes with several suitable functions.

Next, an either circular or rectangular pattern of scan tiles can be selected to cover the defined region. To optimize the coverage of the area of interest, the user can freely drag 20 position markers (the red circles in Figure S1). This optimization allows excluding, for instance, clamps for fixing the filter position, thereby decreasing the required time, and avoiding detection of particles at areas where no particles are expected.

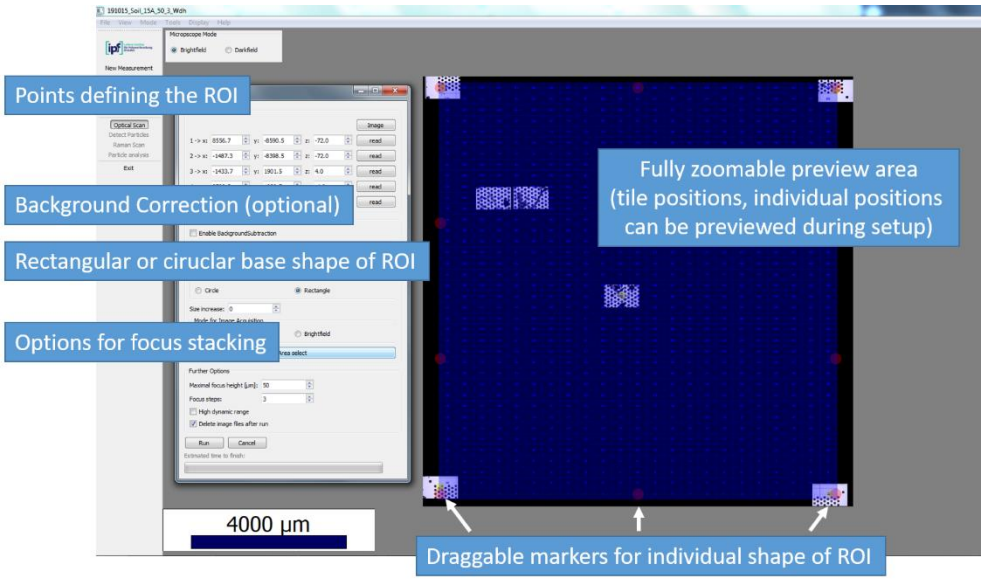

Figure S1. Defining the ”region of interest” (ROI) in GEPARD.

Background Removal. Gradients from inhomogeneous sample illumination lead to very pronounced tile borders in the stitched image and to difficulties in particle recognition in the subsequent step. To remove the gradients, up to six background images can be acquired at arbitrary, but empty positions on the sample. The images are averaged and blurred to remove small features (Figure S2).

On the sample, the particles of interest do not show strong contrast to the background filter, which makes their selection by thresholding impossible, if no background correction is performed.

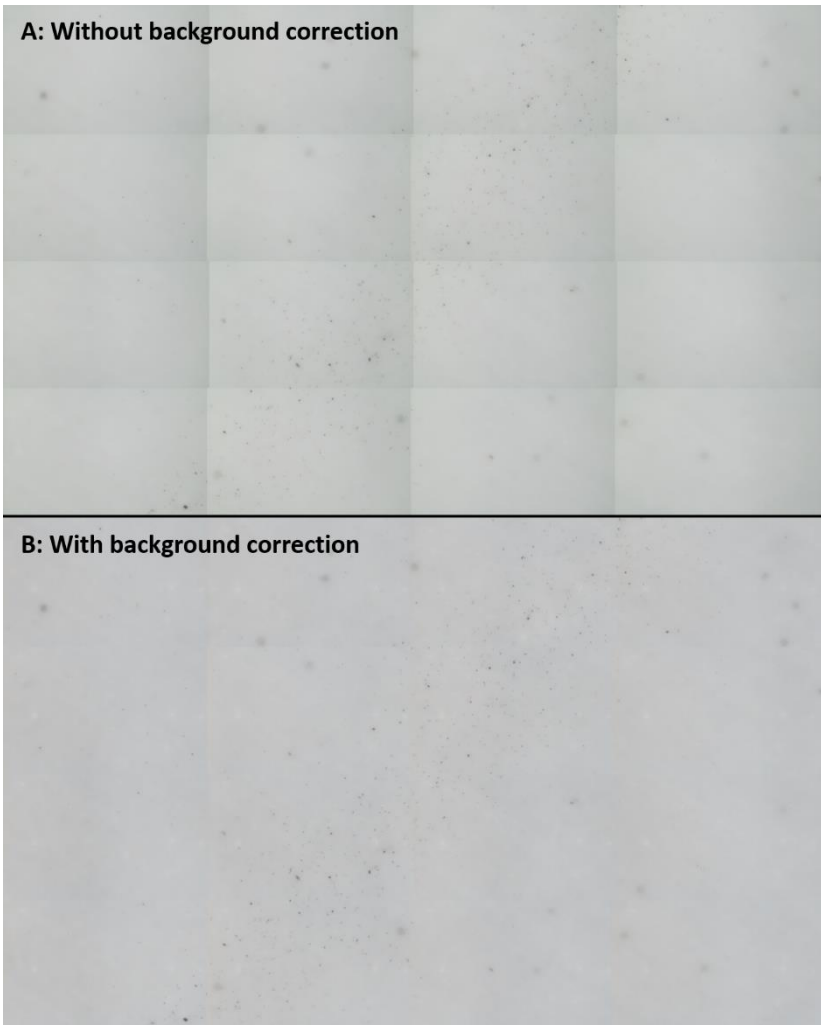

Figure S2. Effect of background correction during optical scan. Each tile represents an area of approx. 500 x 320 µm. Image A clearly shows the distinct shading artifacts from the inhomogeneous illumination of the sample. The artifacts were removed in Image B, making the subsequent particle recognition much more effective.

Particle Detection with GEPARD

We developed a watershed segmentation pipeline using the manifold methods of the image processing packages OpenCV and skimage, both readily integrated into Python. A set of parameters is exposed to the user in a dedicated particle-detection window (Figure S3). Each step can be interactively visualized in a small preview window (1000x1000 px), which can display any sector of the full image. Its center position is set by clicking into the full image window or dragging the mouse in the preview window itself. The segmentation in the preview window takes well below one second to process, thus allowing for an interactive tuning of the parameter set, which then can be applied to the full image. On a regular office PC, the

segmentation of a 17000 x 17000 px image takes about five to fifteen minutes, strongly depending on the total number of particles present.

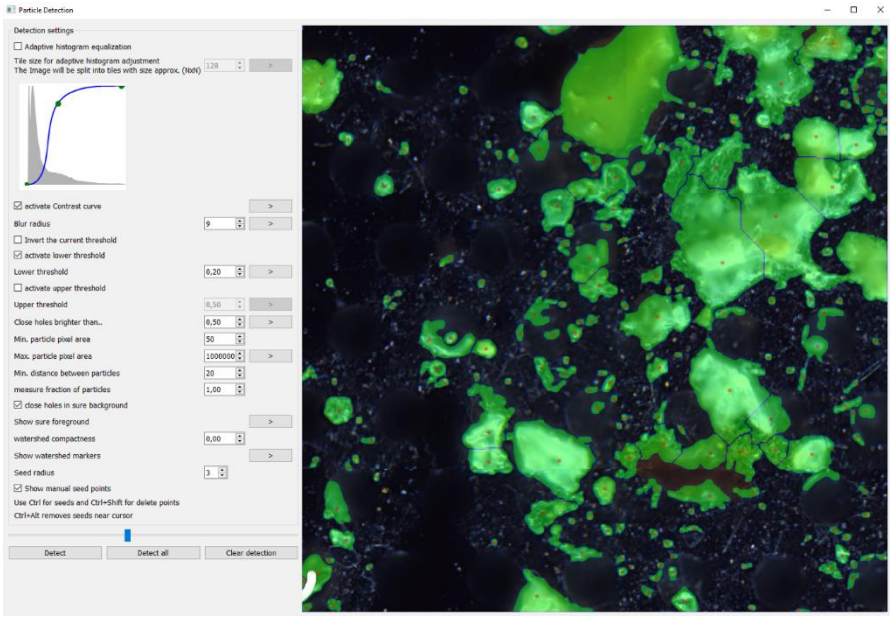

Figure S3. Tuning the watershed segmentation in the preview-window.

Reviewing Particle Measurement Results

The available modification actions include combination of over-segmented particles, drawing of particle contours, and overwriting of chemical classification, color and/or shape. Figure S4 shows a screenshot from within GEPARD. Pressing the right mouse button on any particle (or a selection of multiple particles) opens a context-menu, which gives access to the different modification actions.

Figure S4. Reviewing and correcting automated results.

Investigation of a Soil Sample

The soil sample was taken from the test field “Rinkenbergerhof” at the Agricultural Investigation and Research Institute (LUFA Speyer, Germany) where 190 tons dry matter (DM) per hectare of wastewater sludge had been applied since 1981. Soil cores from the top 30 cm were taken along multiple transects and combined to produce a ≥1 kg sample using plastic-free sampling equipment. This was homogenized by 5 minutes of constant stirring and subsampled down to 2 x 500 g sample, which were freeze-dried and sieved into fractions (> 1mm, 1 mm - 0.1 mm and <0.1 mm). The 1mm – 0.1 mm fraction of 1 x 500g sample was then transferred to a static separator (KWS; Hamos GmbH, Germany). The sample was processed maintaining a monolayer at all times (1–12% vibration) for a total of eight cycles (parameters = 20 kV, drum speed of 4 %, flap position 19.5o). Following static separation, the sample was processed according to Enders et al.[2]

Detailed Methodology. Following static separation, the sample was then combined with sodium polytungstate (SPT; 1.8 g L^{-1}) in a custom-made 1000 ml separation funnel (Squibb form) with extra wide opening (50 mm) and valve outlet (10 mm) (Hellbach Glasbläserei, Germany). This mixture was then mechanically stirred for 1 h before being left to settle for ⩾16h. Settled material was drained off via the valve outlet, remaining material was re-suspended and left again to settle for ⩾16h. This material was vacuum-filtered and then transferred to a 30% hydrogen peroxide (H_2O_2) exposure treatment. The volume and duration of the H_2O_2 treatment was dependent on sample reactivity, where sufficient H_2O_2 was added to stimulate a reaction and to totally cover all sample material and the reaction was allowed to continue until no signs of reactivity were visually discernible, with a minimum exposure time of 24 h. To ensure total non-target particle removal and thus maximizing efficiency of surface chemistry approaches, a second round of both SPT density separation and 30% H_2O_2 treatment (identical to that described above except for the absence of mechanical stirring) were applied, before the final sample was suspended in MilliQ (UHQ) water. All liquids used (for methods or for cleaning purposes) were pre-filtered and all glassware cleaned. All procedures downstream of static separation where the sample (or any material coming into contact with the sample) was exposed to air occurred in a plastic-free laminar flow cabinet (Safe 2020, Thermo Fisher Scientific, USA). Filters with a pore size of 50 µm were used.

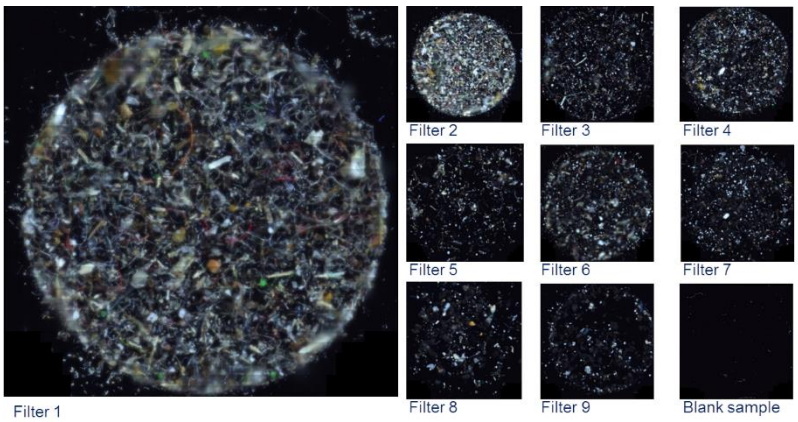

Figure S5. Overview over the filters of a soil sample.

The first two filters are full, which makes the particle recognition a challenging task. The acquired optical image is not always sharp. An external light microscope could overcome that issue but was not accessible at that time. Also, the particles are largely aggregated. To obtain a reasonable particle recognition it was necessary to manually draw very prominent

particles and fibers by hand to reduce over-segmentation. As discussed in the main manuscript, having an overly segmented particle recognition to start the particle measurement with Raman does only results in more than one measurement point per particle (in average). Given such inhomogeneous samples that might actually be desired to have a higher chance to collect a good spectrum from each particle or fiber. GEPARD allows to readily review particles with polymer spectra (typically < 1 % of all particles) and to correct any over-segmentation or incorrectly determined shape.

Representative examples from Filter 1 and 2 are shown in Figure S6 and Figure S7.

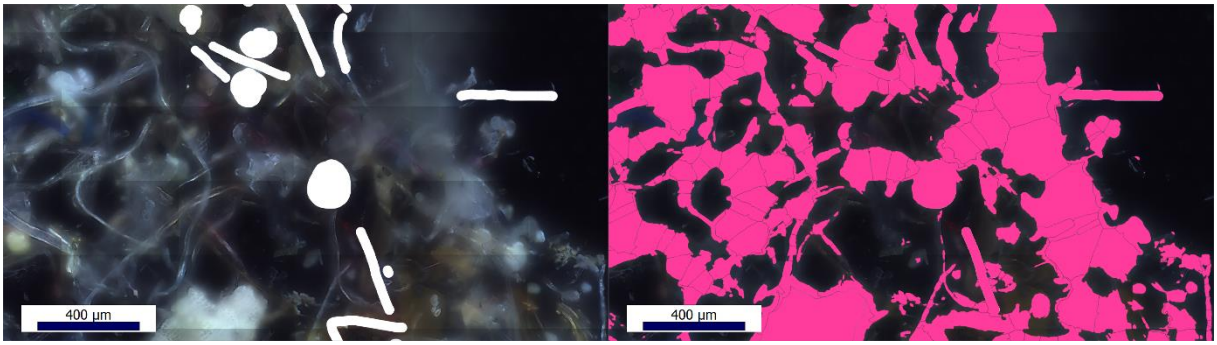

Figure S6: Detail of particle recognition of Filter 1. The white lines and blobs are drawn by hand to guide the watershed algorithm.

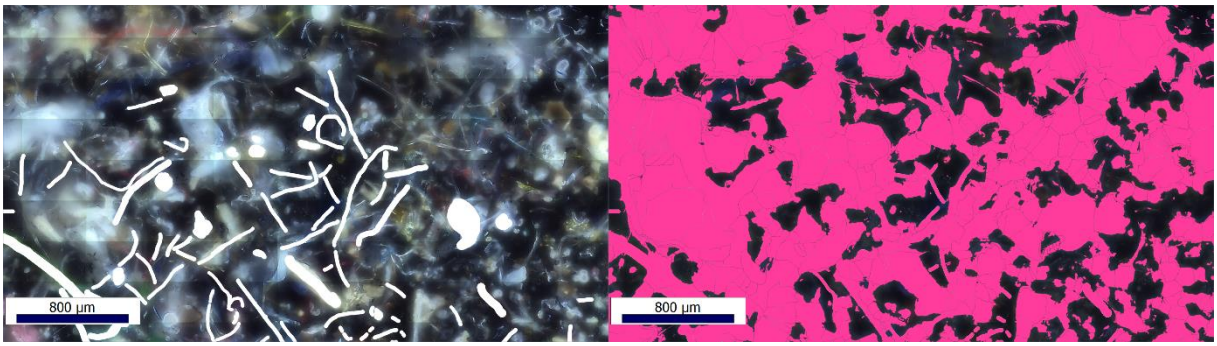

Figure S7: Detail of particle recognition of Filter 2. The white lines and blobs are drawn by hand to guide the watershed algorithm.

The best way to reduce the amount of manual work and, simultaneously, increase the quality of the particle recognition, is to avoid overloading of the filters. In case of particle-rich samples coming from soil or sludge we therefore split the sample on multiple filters (nine in the illustrated example). Again, compromises have to be made between improving measurement quality and increasing measurement times due to a higher number of filters to process.

Table S1 summarizes the experiment times for the individual filters. The optical image was acquired on the WITec microscope at 20x magnification and six focus steps per tile

position (distributed over 250 µm). The Raman scan was done with four accumulations to 0.4 s integration time, each (except filter 6, there five accumulations of 0.5 s were used). The last column gives an estimate about additional manual time that was used for reviewing, checking, and potentially correcting the automatically determined results. That reviewing time very strongly depends on the sample type and desired level of confidence of the results. Correctly recognizing fibers is still challenging for the watershed image-segmentation and, thus, samples with a high fiber count need to be reviewed more critically. As the shown soil samples are complex in their composition, we dedicated substantial time to the reviewing process. Easier samples (e.g., water samples) usually require significantly less time.

Table S1. Overview over measurement times of all filters from the soil sample.

Filter	Image capture time (h)	Setting up particle detection (h)	Number of detected particles	Raman scantime (h)	Total time (h)	Time for manual review of results (estimated h)
1	1.7	1.0	5194	5.2	6.9	2.3
2	1.7	1.0	4925	5.0	6.6	1.3
4	1.7	0.5	5155	5.2	6.8	3.8
5	1.7	0.3	1786	1.8	3.5	2.0
3	1.7	0.5	7017	7.0	8.7	2.8
6	1.7	0.3	896	1.1	2.8	2.2
7	1.7	0.3	4862	4.9	6.5	3.3
8	1.7	0.3	1052	1.1	2.7	0.9
9	1.7	0.3	1323	1.3	3.3	1.4

The below images show recorded spectra (blue), together with information about chemical classification, hit quality index (HQI) and overlaid reference database spectra (red), all were acquired on Filter 7.

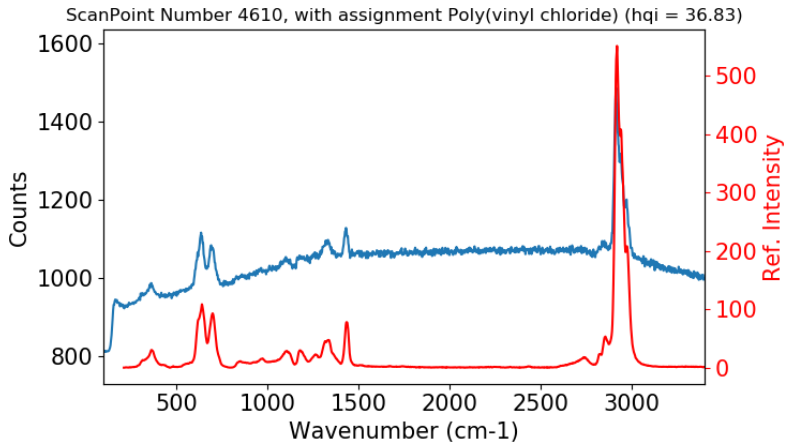

Figure S8. A spectrum of Poly(vinyl chloride) in the environmental sample (blue) together with the reference spectrum from the database (red).

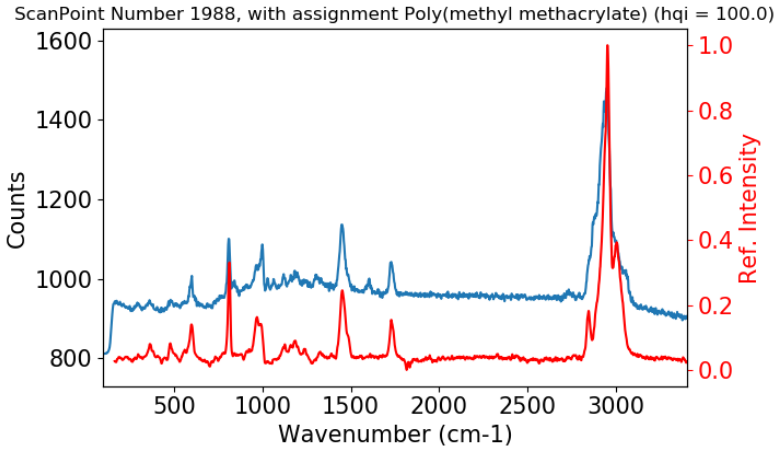

Figure S9. A spectrum of Poly(methyl methacrylate) in the environmental sample (blue) together with the reference spectrum from the database (red).

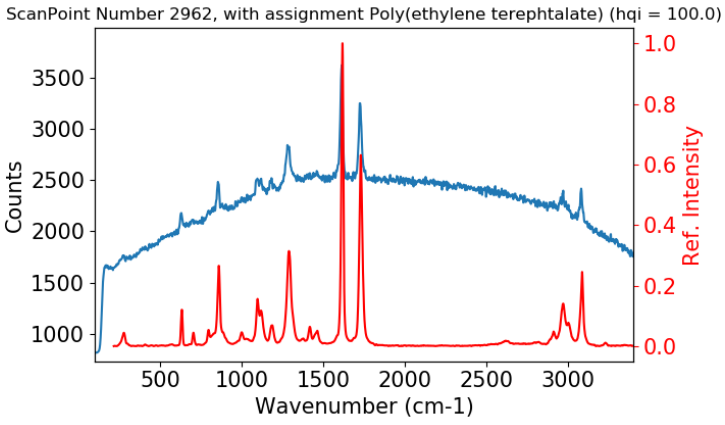

Figure S10. A spectrum of Poly(ethylene terephthalate) in the environmental sample (blue) together with the reference spectrum from the database (red).

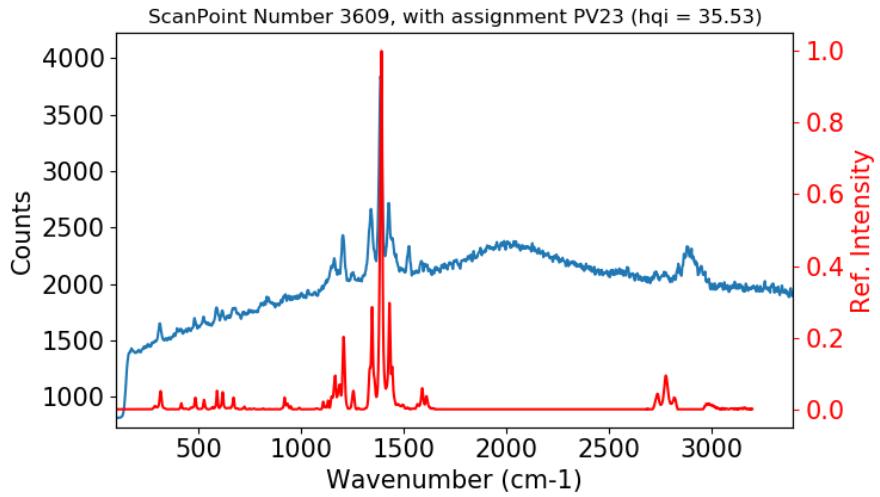

Figure S11. A spectrum of C. I. Pigment Violet 23 in a matrix of polypropylene in the environmental sample (blue) together with the pigment reference spectrum from the database (red).

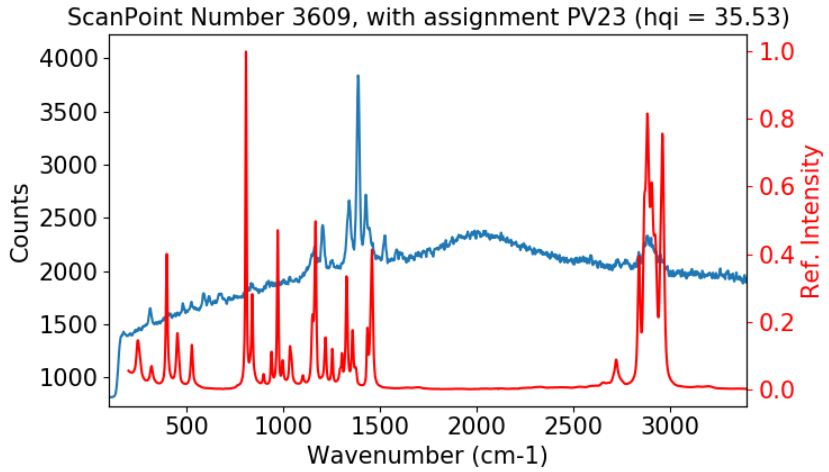

Figure S12. A spectrum of C. I. Pigment Violet 23 in a matrix of polypropylene in the environmental sample (blue) together with the polymer reference spectrum from the database (red).

Investigation of a River Water Sample

We obtained an MP sample from river water on 20 March 2018 from the Kösterbeck river in northern Germany. The river is a tributary to the larger river Warnow, which is one of the major discharges to the Baltic Sea from Mecklenburg–Western Pomerania. The point of sampling was located approximately 700 m downstream of a highway crossing at Lat. 54.055778 and Lon. 12.187055. Other anthropogenic influences on the sampled river system

include intense agricultural land use, municipal waste waters and surface drainage. We conducted the sampling by using an encapsulated flow through device, which has been designed and built for the purpose of sampling small-sized MP down to 10 µm.[3] The sampling device draws river water (upper 10 cm surface layer) through a sampling hose with PTFE inner lining and retains suspended matter on stainless steel cartridge filters inside an enclosed environment. The operator removes and seals the filter cartridges after sampling for further processing inside a laboratory laminar flow bench. The total water volume for this sample was 117 L.

Prior to analysis we treated the sample in a step wise purification process as described by Enders et al.[2] The detailed steps applied were:

(i) Recovery of the sample material from the filter cartridges (incl. 48 h soaking in 5% H_2O_2)

(ii) Freeze-drying (60 h)

(iii) Digestion in 200 ml 30% H_2O_2

(iv) Density separation (sodium polytungstate solution at pH 3.0 and 1.8 g / ml for 15 h)

(v) Washing of particles in a vacuum filtration using microplastic-free ultrapurified water (Milli-Q).

We then submitted the purified particles from this sample in aqueous suspension to a pre-analysis filtration, whereby particles were retained on the porous etched Si wafer which served as the spectroscopic substrate (see Filtration of Silicon Filters section below).

In the following, the analysis of the water sample is shown to present an example where particle sizes down to 10 µm where of interest. Overview images of the acquired filters are shown in Figure S12, a zoom into the first 10 µm filter is depicted in Figure S13.

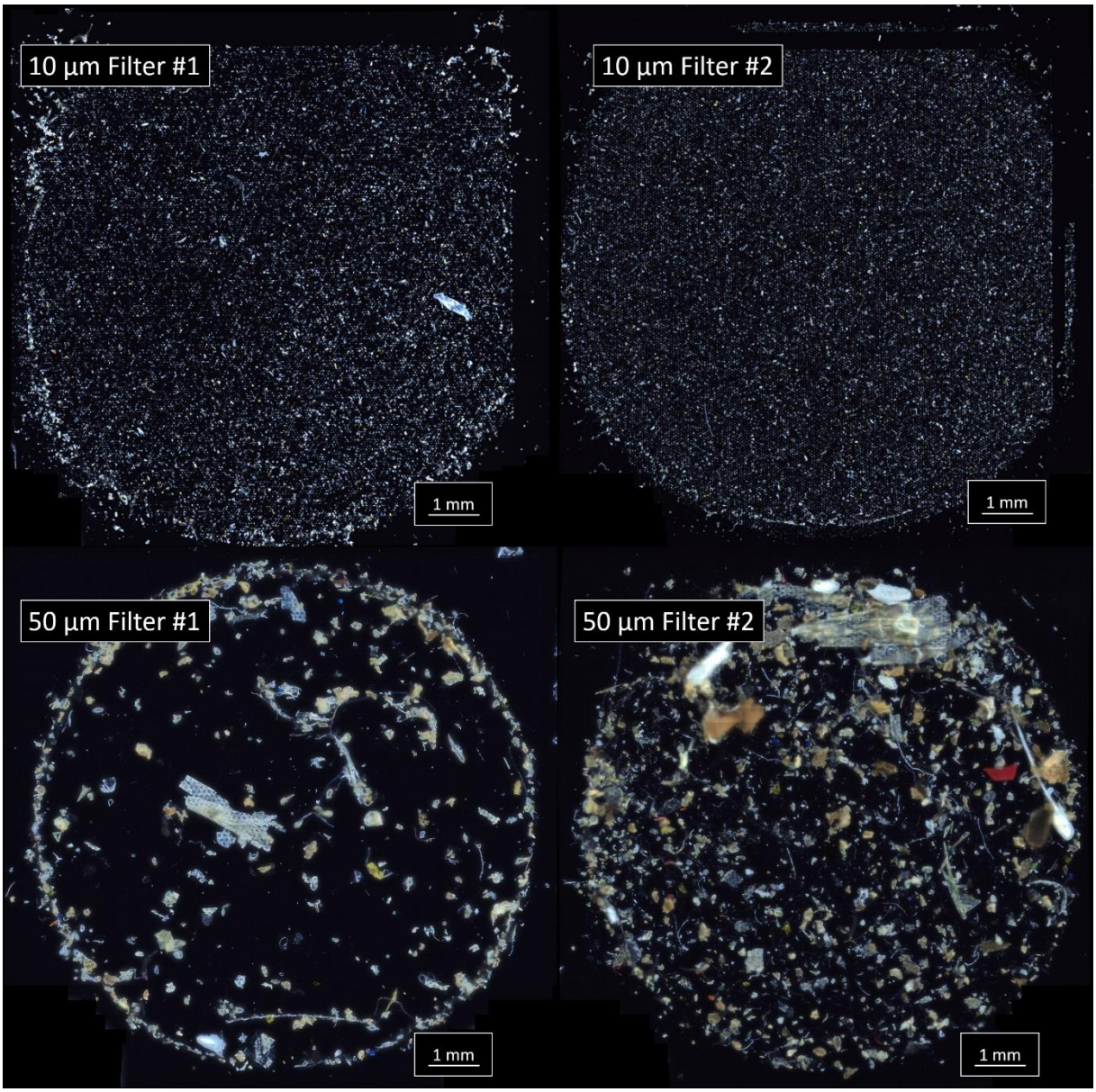

Figure S13. Overview over the filters of a water sample.

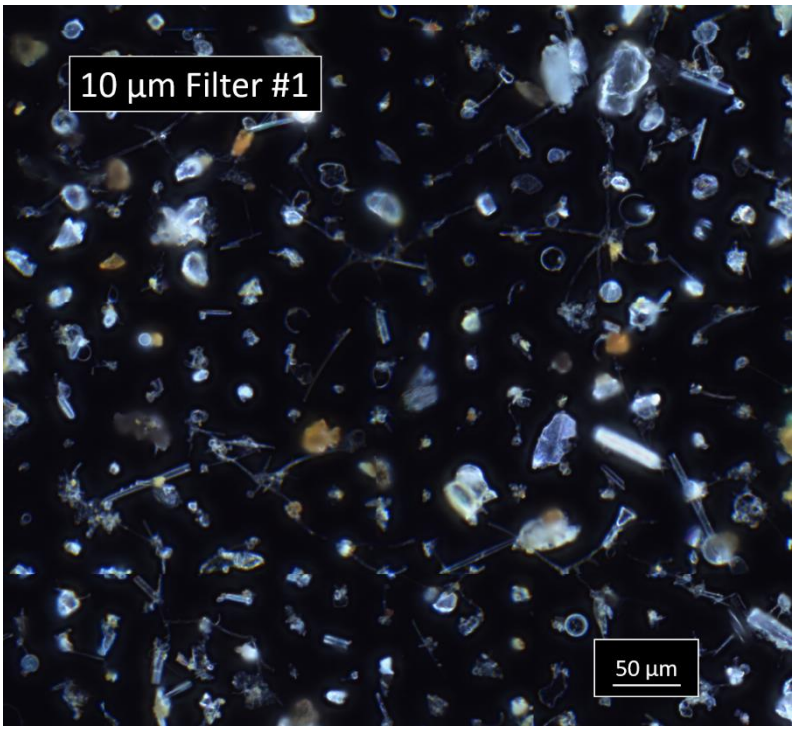

Figure S14. Zoom into Filter 1.

In total, 67766 particles and fibers were measured, taking approximately 3.8 days of measurement time, which translates to approximately 5 s per particle (stage movement + Raman measurement). Raman conditions were five accumulations with 0.5 s integration time, each (exception: Filter 10 µm #2: 5 accumulations with 0.4 s integration time, each). The final MP particle distribution is given in Figure S15.

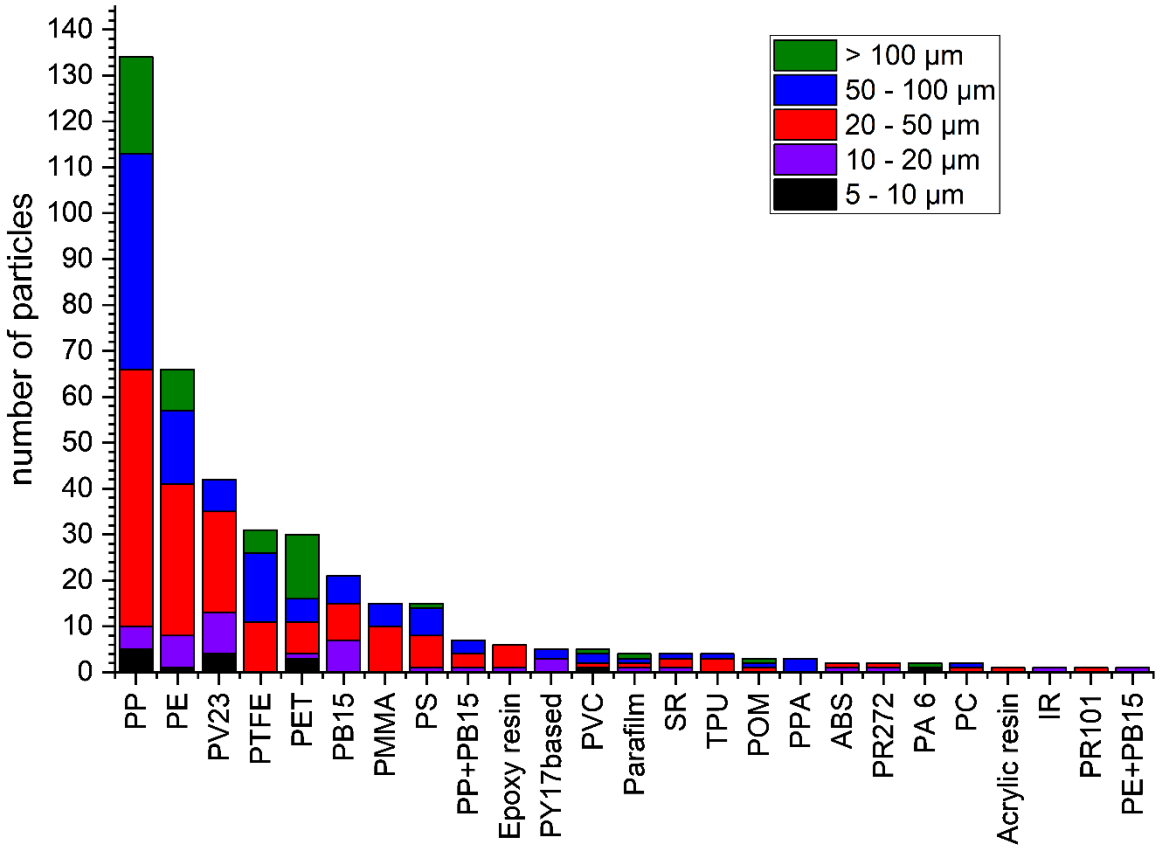

Figure S15. Overview over found MP particle distribution. PP = polypropylene, PE = polyethylene, PTFE = poly (tetrafluorethylene), PET = poly (ethylene terephthalate), PS = polystyrene, PVC = poly (vinyl chloride), SR = silicone rubber, TPU = Thermoplastic urethane, POM = polyoxymethylene, PPA = polyphthalamide, ABS = acrylonitrile butadiene styrene, PA = polyamide, PC = polycarbonate, IR = isoprene rubber, PB = pigment blue, PV = pigment violet, PY = pigment yellow, PR = pigment red.

Filtration onto Silicon Filters

We developed a tailor-made filtration apparatus for filtering samples from aqueous dispersion onto silicon filters.[1] As shown in Figure S16 the device is built in a modular design allowing to do a fractionated filtration onto two filters with different pore sizes, or just on one filter by leaving out the middle glass part.

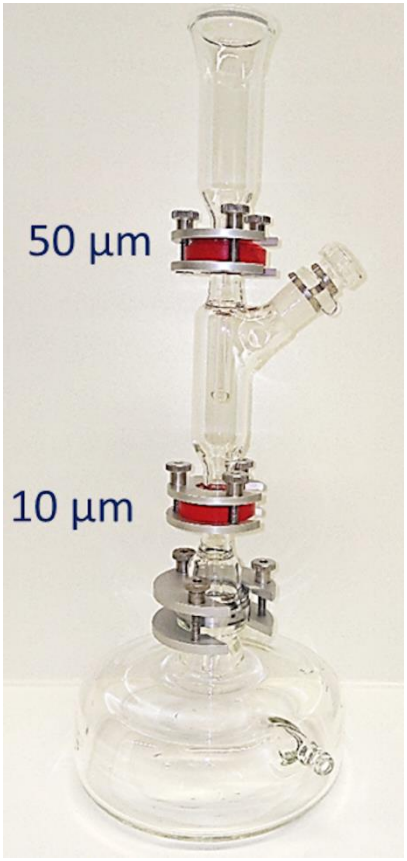

Figure S16. Glass filtration apparatus for realizing a fractionated filtration.
To hold the Si filters, the filtration system contains colored PTFE seals, which are the only polymer in contact with the sample in this device. For the red seals shown here, the color results in fluorescence in the Raman spectrum acquired with parameters used for sample particles that completely masks PTFE signals. Thus, contaminations from the filtration device are not classified as polymer.

For filtering the soil sample onto filters with 50 µm pore size and 100 µm pitch (see Figure S5), the filtration apparatus was used with one filter only, i.e. without the middle glass part and one PTFE seal only. All glass parts of the device were thoroughly cleaned by incubating them in 3 % H_2O_2 and sonicating them multiple times in MilliQ water. The Si filters were cleaned using 30 % H_2O_2 and multiple sonication steps in MilliQ and absolute ethanol. The filtration system was assembled in a laminar flow box (Telstar Aeolus V) and rinsed with MilliQ water. Then, a blind sample is prepared. For that purpose, a clean filter with 10 µm pore size is placed in the PTFE seal and a volume of MilliQ water that equals the sample volume plus the assumed volume of rinsing water is filtered applying vacuum suction. Next, a clean filter with 50 µm pore size is placed in the PTFE seal. A part of the sample volume is poured into the filter system and vacuum is applied until the built-in filter appears covered with a monolayer or the filtration speed is reduced considerably. The full filter is

replaced by a clean one and the sample filtration is continued. This is repeated until the whole sample is filtered.

Estimating the Experiment Time

The required time for an entire MP analysis is highly dependent on a number of different factors. As pointed out in the introduction of the main manuscript, GEPARD does not allow to per-se increase analysis speed. Instead, the operator is offered a number of options to tune the workflow to the requirements for each specific analysis task.

$$t_{total} = t_{opt.image} + t_{part.rec.} + t_{part.meas} + t_{spec.eval}$$

with t_{total} being the total analysis time (in hours), $t_{opt.image}$ the time for acquiring the optical image (in hours), $t_{part.rec}$ the time for the particle recognition (in hours), $t_{part,meas}$ the time required for measuring all particles (in hours) and $t_{spec.eval}$ the time for evaluating all spectra (in hours). The individual contributions can be estimated using the following equations:

$$t_{opt.image} = \frac{filterSize_x}{tileSize_x} * \frac{filterSize_y}{tileSize_y} * numZLevels * \frac{SecondsPerImage}{3600} * CoverageFactor$$

where filterSize is the size of the entire filter (in mm) to scan in *x* and *y*, respectively, tileSize is the size of an individual tile image (camera snapshot, in mm) in *x* and *y*, respectively, numZLevels the number of height levels for the focus stacking and SecondsPerImage the time (in seconds) it takes to acquire one image, including the time for the stage to move the image position. The CoverageFactor gives the percent of the area of the filter that has to be scanned and is 1.0 for scanning the entire filter or approx. 0.79 when only a circular area has to be scanned.

Estimating $t_{part.rec.}$ is not easy, as it highly depends on the resolution of the final image and the particle count on the filter, as well as the PC that runs the algorithm and the algorithm's exact configuration. Typical values range between 5–10 minutes and one hour. If the process takes too long due to large image size, GEPARD offers an option to internally scale down the image to increase the speed at the cost of a lower pixel resolution of the detection. Also, 15–30 minutes of manual work should be added for optimizing the detection parameters and manually correcting difficult sections of the image.

$$t_{part.meas.} = numParticles * numAccumulations * \frac{secondsPerAccumulation}{3600}$$

where numParticles is the total number of particles detected, numAccumulations the number of accumulations (or scans in FT-IR) and secondsPerAccumulation the time (in seconds) for each accumulation (or scan in FT-IR). Intuitively, the stage needs a certain time to travel in between the particles, but the exact time is hard to predict and should in general not be of significant impact.

Finally, also the spectra evaluation time with a database tool such as WITec TrueMatch is difficult to predict and highly depends on particle count, databases to use and preprocessing steps, such as baseline correction. However, it typically does not exceed 10–20 minutes. More important is the amount of manual work that is put in to revisit the automatically generated results and potentially overwrite them.

References

1. A. Käppler, F. Windrich, M.G.J. Löder, M. Malanin, D. Fischer, M. Labrenz, et al. "Identification of Microplastics by FTIR and Raman Microscopy: a Novel Silicon Filter Substrate Opens the Important Spectral Range Below 1300 cm^{-1} for FTIR Transmission Measurements". Anal. Bioanal. Chem. 2015. 407(22): 6791–6801. 10.1007/s00216-015-8850-8.
2. K. Enders, R. Lenz, J.A. Ivar do Sul, A.S. Tagg, M. Labrenz. "When Every Particle Matters: a QuEChERS Approach to Extract Microplastics from Environmental Samples". MethodsX. 2020. 7: 100784. 10.1016/J.MEX.2020.100784.
3. R. Lenz, M. Labrenz. "Small Microplastic Sampling in Water: Development of an Encapsulated Filtration Device". Water. 2018. 10(8): 1055. 10.3390/w10081055.