Spotlight™ 400 FT-IR Microscope
with Spectrum 3™ FT-IR

Advancing Microplastics Analysis

The affects of microplastic pollution is growing every day. That's why we're dedicated to finding the right solutions to identify the risks and impacts of microplastics in our environment. Our Spotlight™ 400 FT-IR Microscope with Spectrum 3™ FT-IR helps you identify and quantitate microplastics and the organic pollutants hosted by them. From rapid, automated detection to high-quality spectral data, our complete solution gives you big insights from small samples.

To learn more visit www.perkinelmer.com/microplasticsanalysis

PerkinElmer®
For the Better

Special Issue: Microplastics

Applied Spectroscopy

Applied Spectroscopy
2020, Vol. 74(9) 1185–1197

DOI: 10.1177/0003702820932926
journals.sagepub.com/home/asp

SAGE

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

Josef Brandt[1,2], Lars Bittrich[1,3], Franziska Fischer[1], Elisavet Kanaki[1], Alexander Tagg[4], Robin Lenz[4], Matthias Labrenz[4], Elke Brandes[5], Dieter Fischer[1], and Klaus-Jochen Eichhorn[1]

Abstract

Determining microplastics in environmental samples quickly and reliably is a challenging task. With a largely automated combination of optical particle analysis, Fourier transform infrared (FT-IR), and Raman microscopy along with spectral database search, particle sizes, particle size distributions, and the type of polymer including particle color can be determined. We present a self-developed, open-source software package for realizing a particle analysis approach with both Raman and FT-IR microspectroscopy. Our software GEPARD (Gepard Enabled PARticle Detection) allows for acquiring an optical image, then detects particles and uses this information to steer the spectroscopic measurement. This ultimately results in a multitude of possibilities for efficiently reviewing, correcting, and reporting all obtained results.

Keywords

Microplastics, Raman, Fourier transform infrared, FT-IR, microspectroscopy, Open Source

Date received: 8 November 2019; accepted: 29 April 2020

Introduction

Microplastic particles (MP) have become a very intensively discussed and highly visible topic in both scientific and mainstream media.[1,2,3–10,11] Of the 8.3 billion tons of plastic produced worldwide by 2015, 6.3 billion tons of plastic waste produced are offset in the same period.[12] Plastics are estimated to remain in the environment for 100–500 years, depending on the type of plastic.[13] By 2015, only 9% of the plastic waste produced worldwide was recycled, just 12% of the plastic waste was incinerated and recycled for energy with 79% of the plastic waste was landfilled or emitted into the environment, mostly in the sea and seabed. According to estimates, approximately eight million tons of plastic waste find their way into the sea every year via rivers, wind, wastewater, etc. This waste accumulates in the environment, mostly in the sea and in the seabed.[13] These plastics can then fragment into MP by wind, waves, and solar radiation. Microplastics are solid and insoluble plastic particles smaller than 5 mm and larger than 1 μm.[14] To reduce the load of MP in the environment, there is a considerable need for research into the main sources, transport routes, and whereabouts, as well as their distribution in the environment. Decisive for future evaluation is the knowledge of how much MP, which MP types, and which sizes are present in different environmental compartments (e.g., oceans, sediments, rivers, soils, atmosphere).

A key issue of current MP studies is to gather greater and more comprehensive knowledge of MP occurrence in the environment and use this to reliably ascertain sources and sinks of MP, as well as to evaluate potential environmental impacts (i.e., effect on biota and humans).[15,16] The

[1]Leibniz-Institut für Polymerforschung Dresden e.V., Dresden, Germany
[2]Department of Marine Sciences, University of Gothenburg, Fiskebäckskil, Sweden
[3]Complex Fiber Structures GmbH, Dresden, Germany
[4]Leibniz-Institut für Ostseeforschung Warnemünde, Rostock, Germany
[5]Johann Heinrich von Thünen-Institut, Braunschweig, Germany

Corresponding Authors:
Josef Brandt, University of Gothenburg, Department of Marine Sciences, Kristineberg 566 SE-451 78 Fiskebäckskil, Sweden
Email: josef.brandt@gu.se
Dieter Fischer, Leibniz Institute for Polymer Research, Hohe Str. 6, Dresden, 01069, Germany
Email: fisch@ipfdd.de

currently employed methods for MP determination in environmental samples are still too time-consuming to be used as monitoring techniques. Hence, significant efforts have been made by numerous research groups to facilitate and harmonize MP analysis procedures.[5–7,17–19]

Determining the MP content of any environmental sample requires three steps: (i) a suitable procedure for actually obtaining the sample has to be found, depending whether a sample is to be taken from, e.g., air, water, biota, or soil.[1,4,20–22] (ii) An adequate protocol for sample processing has to be applied. Depending on the type of analysis, this can be, e.g., sample homogenization or removing as much organic and/or inorganic matter as possible. In particle-based analyses, increasing the MP to non-MP particles ratio significantly reduces analysis time, as a lower number of potential MP particles has to be measured. Commonly employed procedures entail density separation to remove inorganic compounds,[23,24] and Fenton's reagent ($H_2O_2 + Fe^{2+}$)[25] or enzymatic digestion to remove organic residues.[26] (iii) The previously purified MP sample has to be analyzed in detail by an appropriate analysis technique. All three steps are equally important and have to be carried out with extreme care in order to avoid contamination from MP from either equipment used or simply the laboratory air itself. Taking and evaluating blind samples (i.e., samples without any MP that undergo the exact same procedure) for assessing the level of contamination is critical.

Our work focuses on the third part of the aforementioned procedure, the chemical analysis of the purified sample. Numerous works can be found throughout literature that suggest different procedures for MP assessment in environmental samples.[19,27] Notable examples are: Selecting single particles under a microscope for Fourier transform infrared (FT-IR) measurements in attenuated total reflection (ATR),[28–30] transmission[31–33] or reflection,[34–36] FT-IR imaging,[6,31,37,38] Raman microspectroscopy,[7,37,39] pyrolysis–gas chromatography–mass spectrometry (py-GC-MS),[40–42] or thermoextraction and desorption coupled with gas chromatography–mass spectroscopy (TED-GC-MS).[41,43–45] The current key challenge is to streamline suitable methods into easy to apply tools that do not require a team of scientists to be performed. The future of MP analyses lies in scaling up the number of samples, thereby obtaining more comprehensive datasets. Only by significantly expanding campaigns to sample environments at higher frequency both spatially and temporally, effective measures against MP pollution can be derived. Thus, the methods we develop have to be fast, affordable, and easy to learn and use, while not sacrificing information, accuracy, or reliability.

In general, the MP content of any sample can be determined in terms of mass or number of MP. Mass-based techniques, such as py-GC-MS or TED-GC-MS, report total mass but cannot infer from how many individual MP this mass is derived. However, when using a particle-based approach, mass ratios (e.g., milligrams MP per kilogram dry weight of sample) can only be assumed by estimating particle volumes and applying bulk density values.[5] On the other hand, particle-based techniques measure each particle individually to obtain both the chemical composition and size, thereby providing more detailed information. The most frequently employed techniques for that approach employ FT-IR or Raman microspectroscopy.[17,37] Therein, MP samples are filtered onto suitable substrates and then analyzed spectroscopically to determine size and chemical nature of each particle.

Both analytical approaches, thermoanalysis and vibrational spectroscopy, come with their advantages and caveats. The huge advantage of a mass-based technique is its high sample throughput. For example, state of the art py-GC-MS or TED-GC-MS takes only a few hours to process 100 mg of an environmental sample that does not need specific treatment other than careful homogenization.[41,45] This makes these techniques very promising candidates for widespread environmental monitoring in routine operation. In contrast, the spectroscopic techniques require elaborate sample purification and can take significantly longer for measurement and data processing. Their main benefit, however, is that the analysis happens on the particle level, i.e., each particle is registered individually with information on shape, size, chemical classification and, in most cases, even color. This information is currently of high relevance for elucidating sources and sinks of MP particles. Additionally, knowledge of polymer type and particle size/shape is also critical for modeling pathways of MP,[46–48] as well as for assessing potential MP impacts.[15,16] Given the importance of this information, spectroscopic particle analysis is currently widely preferred for MP studies, despite the lack of efficiency compared to mass spectrometry. Therefore, improving the efficiency of these approaches is of paramount importance for the future of MP research.

This study contributes significantly to this issue by enabling the microspectroscopic particle analyses to be employed far more efficiently.

Microplastic Analysis Using FT-IR and Raman Microscopy: State of the Art

Both methods of vibrational spectroscopy can generally be used in two inherently different modes (Fig. 1). The first one is the imaging approach, which entails complete spectroscopic measurement of the entire sample surface in order to obtain an individual spectrum for each "pixel". After evaluation of all spectra, a chemical image is obtained in which each pixel is assigned to a certain material class. Neighboring pixels of the same class are then considered one particle. In contrast to that, the second approach is to first acquire an optical image of the entire filter and to run an automated particle recognition algorithm that identifies all present particles. Spectra then need only to be acquired where particles were found.

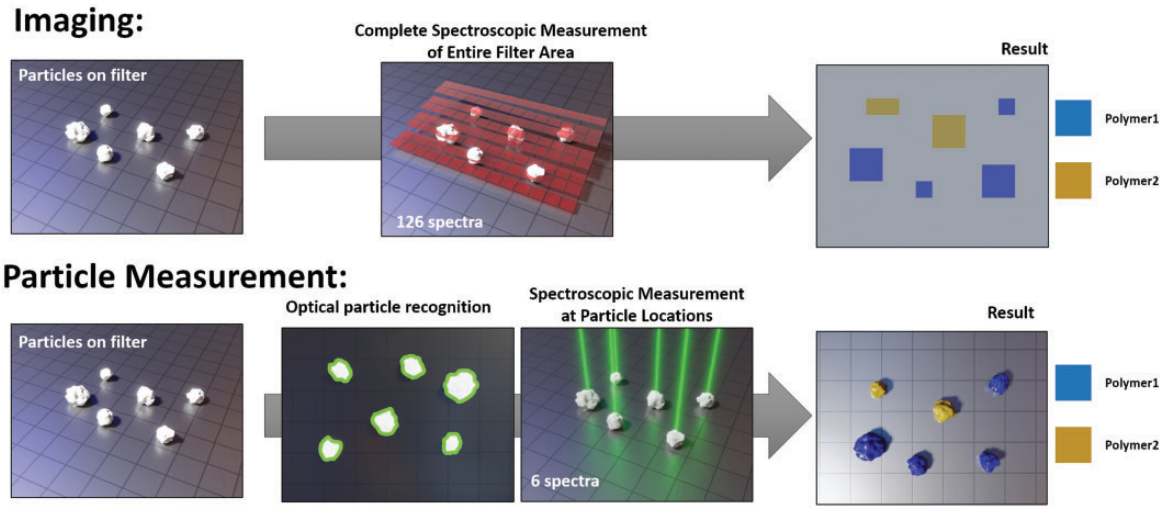

Figure 1. Imaging and particle measurement as two different approaches for analyzing particles on a filter substrate.

The first and most obvious difference between both approaches is the number of acquired spectra. Considering 10 000 particles on a filter of 10 mm × 10 mm, the imaging approach requires acquiring 10^6 spectra in a resolution of 10 μm, whereas only 10^4 have to be taken for the particle recognition approach. Requiring 100 times more spectra for imaging renders both data acquisition and evaluation, a particular challenge. Spectra acquisition can only be done in reasonable time scales, when array detectors are used, such as the focal plane array (FPA) detector, which is capable of acquiring up to 128 × 128 (=16 384) spectra simultaneously.[49] Unfortunately, such detectors are only available for FT-IR spectroscopy, which makes Raman spectroscopy not feasible for high-resolution imaging of larger surfaces. The imaging approach is also more challenging regarding data evaluation.[6,50] Conventional correlation of the measured spectra to databases is becoming either very slow with very high spectra numbers or requires very high computational power. Chemometric tools, such as classification by random decision forest, can significantly speed up analysis, but are far from trivial to set up and are limited in the number of substance classes they can distinguish.[18]

In contrast, the approach using optical image recognition to identify particles requires less sophisticated hardware and allows for a higher flexibility in choosing the evaluation strategy. Both chemometric classification and "conventional" database evaluation are readily applicable. Furthermore, FT-IR and/or Raman microscopy can be used similarly to perform the chemical identification, which is advantageous as Raman, unlike FT-IR, can analyze particles below 10 μm. The practically achievable lower limit strongly depends on instrumental setup and type of sample, the theoretically possible value is 0.4 μm. On our routine setup (20 × objective), we can confidently work down to a size of 2–3 μm. The main challenge remains the particle recognition step, as finding and tuning a suitable image segmentation algorithm is anything but trivial. However, once a reliable particle recognition has been achieved, additional information about particle color or morphology can be derived from the optical image.

To draw a conclusion from this short comparison of imaging and particle recognition approaches, the initial perspective of making MP analysis techniques more suitable for large-scale monitoring strategies has again to be considered. Considering this perspective, the imaging approach has distinct disadvantages, namely costly equipment (FPA detector), less flexible spectra evaluation, and finally the practical limitation to FT-IR, thus excluding Raman measurements for MP particles < 10 μm. Thus, we believe that pushing forward the particle recognition approach is the strategy of choice for greatly expanding the scale in which MP analyses are performed, not only by highly trained MP analysis teams but also by a broader community.

As stated in the Introduction, the key challenge is to decrease the required measurement time for a given particle analysis. Considering the complex nature of the environmental samples on the one side, but also of the respective spectroscopic workflows on the other side, clearly shows that there cannot be a universally valid formula to decrease measurement time. Instead, an analysis procedure should offer the user the highest possible freedom in designing and performing the particle analysis, respecting the constraints coming from the nature of the samples and the accessible laboratory equipment.

Herein, we present our realization for MP analysis using the optical particle detection approach that can be combined with both FT-IR and Raman spectroscopy. We developed a new software for bundling all steps in the analysis pipeline in an easy to use package while providing the highest possible flexibility. Gepard Enabled PARticle Detection

(GEPARD) is an open-source project under the GNU General Public License. It can be downloaded from a GitLab repository and used freely.[51] Moreover, the software is designed in a modular way and can be modified and extended by others for further increasing its usability in the field. In this manuscript, we describe the application of GEPARD in combination with Raman and FT-IR microspectroscopy and present first results in the field of MP analysis.

Materials and Methods

Instruments

The Raman imaging microscope (WITec alpha 300R) was equipped with 532 and 785 nm lasers together with dedicated detectors. Spectra are acquired with gratings of 600 or 1200 l/mm. For typical instruments, we use the 532 nm laser on the 600 l/mm grating, which allows acquiring the entire spectral range (150–3600 cm^{-1}) with one shot, at a spectral resolution of ~3.5 cm^{-1}. Image acquisition can be done in brightfield (BF) and darkfield (DF) at magnifications of 5, 20, 50, and 100×. The step size of the motorized stage is 100 nm. The entire microscope can be remote-controlled through a component object model (COM) interface (interprocess communication developed by Microsoft) to the WITec control software.

The Raman microscope (Renishaw inVia Qontor) can irradiate with lasers of wavelengths of 532, 633, and 785 nm. The switching of lasers and gratings (600 l/mm, 1200 l/mm, or 1800 l/mm) is fully automated. By default, the combination of 532 nm laser and 600 l/mm grating is used, resulting in a spectral range from 150 to 3600 cm^{-1} at a spectral resolution of approx. 7 cm^{-1}. Optical image acquisition works in both DF and BF at magnifications of 5, 20, 50, and 100×. The automated stage has a step size of 100 nm. The liveTrack feature allows for an automated focus tracking in real time. The microscope can be remote controlled via a network interface.

The FT-IR microscope (PerkinElmer Spotlight 400) features a mercury–cadmium–telluride (MCT) line-detector for imaging and an MCT single-detector. Spectra can be acquired in transmission, reflection, or micro-ATR with visual autofocus. The microscope cannot be remote controlled, but allows importing seed-point lists for predefined measuring layouts. The seed-lists contain information about location (*x,y,z*) and aperture configuration (with, height, rotation angle) of each particle. In default operation, we acquire spectra with eight scans per acquisition at a spectral resolution of 4 cm^{-1}.

The optical microscope (Zeiss Imager.Z2m) allows rapid acquisition of optical images at very high fidelity at magnifications of 5, 10, or 20× in either BF or DF. Multiple *z*-levels can be acquired for obtaining an image, with optimal depth-of-field and height map being extracted. Both images can be imported into GEPARD to create a project file that can be used for subsequent particle recognition and spectral measurement. The Zeiss software itself features manifold image segmentation and processing modules.

MP Analysis Pipeline with GEPARD

GEPARD is designed to cover all steps in the analysis pipeline from optical image acquisition over spectra acquisition, combination of particle and chemical data to generation of final reports, both visually and in spreadsheet form. The software currently supports importing images from Zeiss microscopes, controlling Raman microscopes from WITec and Renishaw to allow for image acquisition and particle measurement. Furthermore, particle position data can be exported to FT-IR microscopes from PerkinElmer. Each step is developed to minimize the need for human adjustments, thus keeping human bias and required time at the lowest possible limit.

GEPARD is designed around a particular analysis workflow, as summarized in Table I. First, a full image of the filter is acquired during an optical scan. That image is used to run a particle recognition for identifying particles, determining their size, shape, and color and to set the coordinates for the subsequent spectroscopic measurement. In the spectroscopic scan, a spectrum is acquired for each particle. All acquired spectra are then evaluated to obtain a chemical classification (i.e., particle type) to each spectrum. All spectroscopic classifications are then combined with the already determined particle characteristics. The results can then be reviewed and edited, if necessary. Finally, the results can be displayed graphically and exported into different formats.

Table I. Individual steps of the analysis pipeline using GEPARD.

Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
Image acquisition	Particle recognition	Spectroscopic measurement	Spectra evaluation	Review/edit results	Data output
GEPARD, Zeiss	GEPARD	WITec, Renishaw, PerkinElmer	TrueMatch (refer to the Spectra Evaluation section)	GEPARD	GEPARD

The lower row in Table 1 indicates that most tasks are directly integrated into GEPARD. The following sections will review the individual steps in detail.

Image Acquisition/Optical Scan

The first step is to acquire an optical image of the filter. In the case of a WITec or Renishaw instrument, the optical scan can be done either with the Raman microscope or with a dedicated optical microscope. In the case of the PerkinElmer FT-IR microscope, the optical scan must be performed on an external microscope, as there is no technical possibility to control the instrument from an external software. By using a dedicated optical microscope, such as the Zeiss Imager.Z2m, this allows for reducing image acquisition time while providing a higher fidelity of the acquired image. The use of an external microscope requires using a sample tray with fixed position markers to transfer the coordinate systems from the optical to the Raman/FT-IR microscope. The mathematical error of the coordinate transfer can be at 1 μm for each marker or even less. However, when bringing a sample from one instrument to another, also the precision of the microscopy stages has to be considered, which can be about a few μm. Further deviations can occur when the filter is not fully fixed on the microscope tray or if particles can move on the table. Having image acquisition and particle measurement on different instruments requires very careful sample handling and should only be considered for larger particles (>10 μm) and only after thorough testing.

The GEPARD interface offers a variety of parameters for controlling the image acquisition procedure. The user can set three to five positions that define the border of the region of interest, in which images will be acquired. The *z*-focus of all initial positions is adjusted by the user to define a surface tilt for the filter in the microscope frame. A background-removal option is integrated to account for brightness gradients within each tile, resulting from non-constant illumination of the respective filter area. Next, a number of *z*-levels for a so-called focus stacking approach are defined. The number of *z*-levels and the maximum focus height relative to the tilted filter determine how many focus steps are done and at what *z*-levels. The focus stacking gives two advantages: First, an image with highest possible depth of field is obtained, which is beneficial for the particle recognition step. Second, a height map of the filter and particles is calculated and stored that allows for focusing the laser during the Raman scan on the surface of the particles. A detailed breakdown on the image acquisition options is given in the supporting information (Image Acquisition with GEPARD section, Supplemental Material).

The optical scan procedure is tailored to minimize the required image acquisition time but also to retrieve height information of the sample. Consequently, elsewhere-described procedures for artificially pressing fibers on the filters to force them into the focal plane are not necessary.[6] The image is contained in full resolution and can be interactively reviewed within the GEPARD sample view.

Particle Recognition

After having obtained the full optical image, the actual particle recognition step has to be performed. Effective and reliable image segmentation is highly challenging, and the employed algorithms have to be chosen and configured carefully. The challenge for GEPARD is to process images of very different natures (i.e., MP samples from drinking water are much cleaner than MP samples from wastewater treatment sludge, even after sample treatment). As all images are stored and processed in full resolution, typical image sizes for 10×10 mm filter acquired with $20\times$ magnification are in the range of $17\,000 \times 17\,000$ pixels. Significant progress was achieved throughout the past years using semantic image segmentation (i.e., using machine learning) on challenging images to achieve very convenient segmentation results.[52–54] Despite their high capability in recognizing complex particle patterns, semantic image segmentation techniques require training procedures that are both difficult to set up and computationally expensive. Consequently, they are usually restricted to images of rather low-resolution images, with 8192×4096 pixels are already considered very large and need to be handled with particular approaches.[55] Conversely, simple segmentation by thresholding the image into fore- and background is very fast but only works well for non-overlapping particles, which is often not the case.

We decided to implement an interactively controllable watershed segmentation to find a compromise between quality of segmentation and computation time/expense. Watershed segmentation allows separating adjacent or overlapping particles and works by flooding the image from marker points that represent particle centers. Particle borders are defined by adjacent water basins that meet during the flooding process.[56] As already demonstrated in a recently published article, watershed segmentation can easily lead to strong over-segmentation, i.e., many particles are fragmented into numerous small particles.[56–58]

A detailed description of all parameters can be found in the Particle Detection with GEPARD section in the Supplemental Material, and shall be skipped here. Instead, the most important features are briefly summarized in the following list:

(i) Contrast adjustments of the acquired image by a user-defined contrast curve or adaptive histogram equalization (contrast limited adaptive histogram equalization, or CLAHE, for compensating local inhomogeneity of image brightness).

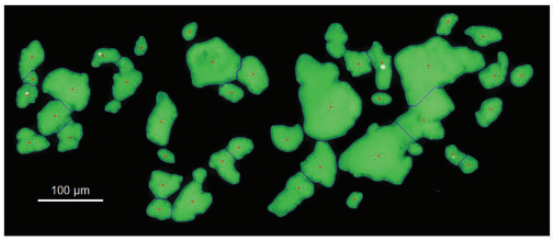

Figure 2. Example results of particle recognition on cryo-milled polymer particles. The necessity and potential of watershed segmentation is demonstrated by touching particle boundaries in several cases. Red spots mark Raman measurement spots, white points are user-defined seed points for guiding the watershed algorithm.

(ii) Thresholding to define particles that are above or below a certain brightness, but also in between two set brightness values or vice versa.
(iii) Size filtering to exclude very small and/or very large particles.
(iv) Manual corrections to split particles or to recombine over-segmented particles.

Our particle recognition leads to good results in a broad variety of cases, as shown in Fig. 2. However, the method frequently leads to over-segmentation, which results in more than one measurement per particle, thus increasing measurement time. However, having more than one spectrum per particle increases the confidence in the spectroscopic identification of the respective particle. Segmented particles that are too heavily segmented can be recombined in the final particle evaluation step (Particle Evaluation section) to obtain reliable particle size information. These manual corrections only have to be done for the relevant MP particles (usually about 1% of the particles), which makes the post-processing possible in little time. Particle count and size distributions of all other particles, though, will be biased by over- or under-segmentation. However, the interpretation of size and count of non-MP particles is highly questionable anyways, as already the previous sample workup steps (e.g., density separation or digestion) make these values not representative for the environmental sample.

Spectroscopic Measurement

After completing the particle detection, the actual spectroscopic scan can be initiated. GEPARD allows for controlling the measurement directly through the instrument interface to the Raman microscopes. A device-specific window prompts measurement parameters, such as number of accumulations, integration time, or laser power. The order in which all particles are measured is optimized by solving the traveling salesman problem through a simulated annealing approach,[59] thus reducing the distance the stage has to move by approx. 20% compared to an intuitive meandering line scan technique. For measuring particle amounts in the thousands to ten of thousands, we chose relatively short Raman scan times per particle, such as five accumulations with 0.5 s integration time. Both an increasing number of accumulations or longer integration times would improve the signal-to-noise ratios of the acquired spectra at the cost of accordingly longer experiment time and, thus, less sample throughput. Balancing out spectrum quality and sample throughput is a delicate endeavor and has to be done carefully for each analytical task. A way to improve the spectral quality is to increase the number of z-levels during the optical scan for increasing the accuracy of the z-focus during measurement. Alternatively, instrument specific hardware options for tracking focus height (i.e., Renishaw Qontor Live Track) can be a great benefit for increasing focus accuracy without significantly impacting the overall measurement time.

We currently measure the entire filter surface with all particles and do not make use of any aliquotation approach. Measuring only a fraction of the filter decreases overall measurement time, thus increasing sample throughput. Alternatively, keeping the overall measurement time constant, the acquisition time for each spectrum can be increased for this fraction of the filter, thus increasing the quality of the performed measurements. GEPARD only allows for the definition of a fraction of particles to be measured currently. Any other subsampling approach can be integrated on demand, due to the open-source nature of GEPARD.

The workflow for using GEPARD for FT-IR measurements on the Perkin Elmer instrument is currently implemented in a workflow that requires acquisition of the optical image on the Zeiss light microscope (LM). A sample tray with position markers in the micrometer size is used to transfer the coordinates to the FT-IR instrument. GEPARD needs to import three files from the LM: (i) the color image, (ii) the height map, and (iii) an extensible markup language (XML) file with all metadata about the sample, such as the exact coordinates of the position markers. Before importing these files into GEPARD, the sample tray has to be mounted into the FT-IR microscope and the coordinates of all position markers have to be determined manually. When importing the LM files into GEPARD, the user is prompted to input the position marker coordinates, which allows GEPARD to transfer the coordinate system from LM to FT-IR microscope. After having performed the particle recognition as described in the previous step, a script is run to determine the FT-IR aperture configurations for all detected particles. Therefore, an algorithm finds the largest rectangle fitting on each particle without leaving its respective boundary. Ultimately, a *.slf-file is generated and exported that can be imported from the FT-IR microscope's Spectrum software to set up a measurement with all detected particles (Figs. 6a and 6b show an example of that setup). The FT-IR measurement is entirely configured

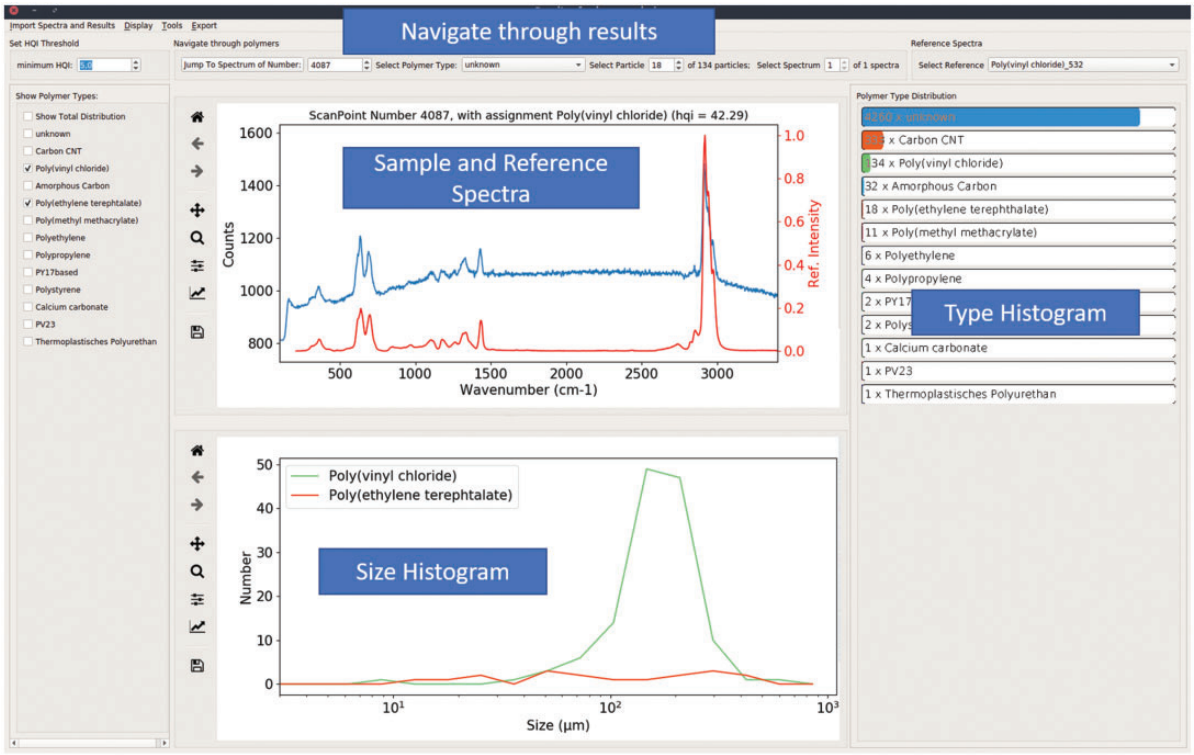

Figure 3. Overview of the particle analysis interface using a Raman microscope. The sample is a soil sample (see Figure 5 and Supplemental Material). All spectra with a hit quality index (HQI) below a given threshold (min HQI = 5 in this example) are labeled as "unknown".

in the Spectrum software. After finishing the measurement, the acquired spectra are exported into a *.csv file to be processed in the following spectra evaluation step.

Spectra Evaluation

All spectra have to be evaluated to determine each particle's chemical nature. The spectra evaluation is the only step, which is currently not yet implemented in GEPARD. Instead, we use the commercial software TrueMatch (WITec) for batch spectra correlation and import the results back to GEPARD. We created dedicated databases containing relevant selections of standard polymers, additives, and other substances that are likely to be found in environmental samples (e.g., fish scales, mussel shells). Each database contains 50 to 100 spectra, thus making the spectra correlation very quick and restricting the results to reasonable entries. We perform background subtraction and take the first derivative of the spectra.

The main challenge is to handle spectra that were acquired in short time and, consequently, are characterized by a low signal-to-noise ratio. As every spectra correlation process can lead to wrong classifications, all automatically obtained results can be revised and checked. The TrueMatch software allows quickly browsing through the results and setting flags to each spectrum. By such an approach, the automatic results can be overwritten, thus increasing the overall result confidence.

Choosing appropriate settings for the database search is critical for obtaining meaningful results. In most cases, we use the build in shape background subtraction with a shape size of 200 points, use the first derivative for correlation and set the hit quality index (HQI) to represent the correlation coefficient (ranging from 0 to 100). After sorting the search results according to their HQI, we manually revisit all spectra down to an HQI of 5 to exclude false classifications. That threshold parameter strongly depends on all chosen database search parameters and, for reproducibility of results, keeping these fixed is important. The list of results and their respective HQIs is then saved to a text file which can be opened in GEPARD. There, the final HQI threshold can be set to determine from which HQI on a particle is considered as "unknown". We usually keep this threshold at an HQI of 5 as well.

Particle Evaluation

GEPARD offers rich possibilities to review and edit particle results after having imported the spectra results. Each detected particle is assigned with a specific color for easy visual identification of particles on the filter surface. A second additional window displays statistics about found

particle types and sizes. Figure 3 shows the analysis window that readily allows for the extracting information about:

Particle type histogram: How many MP particles were found of which polymer type?
Particle size histogram: What is the size distribution of these MP particles?
For each individual particle: Size, classification, color, shape and spectrum or spectra of each MP particle.
Each MP particle spectrum can be overlaid with reference polymer spectra that can be imported from ASCII formatted text files.

The analysis window is linked to the particle image of the main window. Clicking on any particle in the main window displays the corresponding particle information in the analysis window and vice versa, selecting a particular particle in the analysis window makes the main window focus on the corresponding particle.

GEPARD's analysis interface is designed with the same philosophy as the previously described particle detection. We implemented algorithms that process all particles automatically and derive parameters, such as size, shape, and color. However, given the vast diversity of particles in environmental samples, these algorithms can lead to wrong results in some cases. The user has not only the opportunity to review all results but also to check and override them in detail accordingly.

It is highly recommended to perform a critical review of the relevant results. The term "relevant" relates to actual MP particles in our scope. Given that MP usually amount to less than 1% of all particles, this greatly reduces the number of particles that have to be reviewed, which makes the post-processing relatively quick.

The wide array of reviewing options of GEPARD (refer to the Reviewing Particle Measurement Results section; Supplemental Material) is a major strength of our particle analysis approach. We combine and display information from the optical image, particle recognition and spectral analysis. Making it intuitive and quick to review "relevant particles" (here: MP) greatly increases the confidence of the generated results, thus improving their scientific quality.

GEPARD currently offers two ways of exporting the particle results. First, an excel spreadsheet can be generated, containing the information of each individual particle, together with a summarized table listing the number of particles in certain size classes of each particle type. Second, an interface to connect to an SQL database is included. Uploading all particles to a central database is beneficial for making results accessible to all project partners and allows storing all required metadata, which assists in tracking the history of each individual particle from sampling to final analysis.

Assessment of Measurement Quality

Confirming the reliability of any MP analysis is an important, yet challenging step. Preparing a sample of known MP content is still a major challenge for MP round robin test, where the performance across different methodologies and laboratories is compared.

We decided to perform a simple experiment with artificially prepared samples for asserting correct function of our method. We used commercially available spherical particles of polyethylene (PE) and poly(methyl methacrylate) (PMMA) with narrow size distribution and different average sizes. For PE, two batches were used with sphere diameter ranging from 10 to 27 μm and from 106 to 125 μm, respectively. The size of the PMMA particles was indicated to be in between 53 and 63 μm. Particles of each of the three batches were put on a silicon filter and the filter was subjected to the herein described particle analysis procedure using GEPARD with the Renishaw inVia Raman microscope. Figures 4a and 4b show the outcome of the measurement. The size-distribution plot clearly shows that the originally employed particle types and sizes are reproduced correctly. The photo of the filter with false-color overlay shows that also aggregated particles of different types are distinguished correctly.

GEPARD in Routine Operation

The herein proposed analysis procedure has been used by the microplastics group of IPF Dresden in routine operation for more than a year, on over 80 environmental samples; originating from air, water, sediment, beaches, or soil. Having routine operation and method development run in parallel allows for effectively identifying tasks that can be improved and/or accelerated by automatization. The diverse nature of the samples we process confronts us with new challenges in all aspects of not just image acquisition, but also particle detection, spectra evaluation, revision, and final compilation of results.

Figure 5 shows the result of a soil sample from an agricultural area close to Speyer, Germany. Details about sampling and sample workup can be found in the Investigation of a Soil Sample section (Supplemental Material) together with example spectra. The final filtration was done on silicon filters with 50 μm pore size. Due to the relatively large pitch of 100 μm, also smaller particles can be found on the filter, but their number cannot be considered to be quantitative. Image acquisition was done directly within the Raman microscope and took approximately 1.7 h per filter. On the filter shown in Fig. 5, a total of 4862 particles was detected and the Raman measurement took 4.9 h. In addition, approximately 4 h of manual work was required for setting up the measurement and thoroughly reviewing the results as described in the previous sections. The presented sample is a complex soil sample with many fibers but also particle agglomerations. Both are a particular challenge

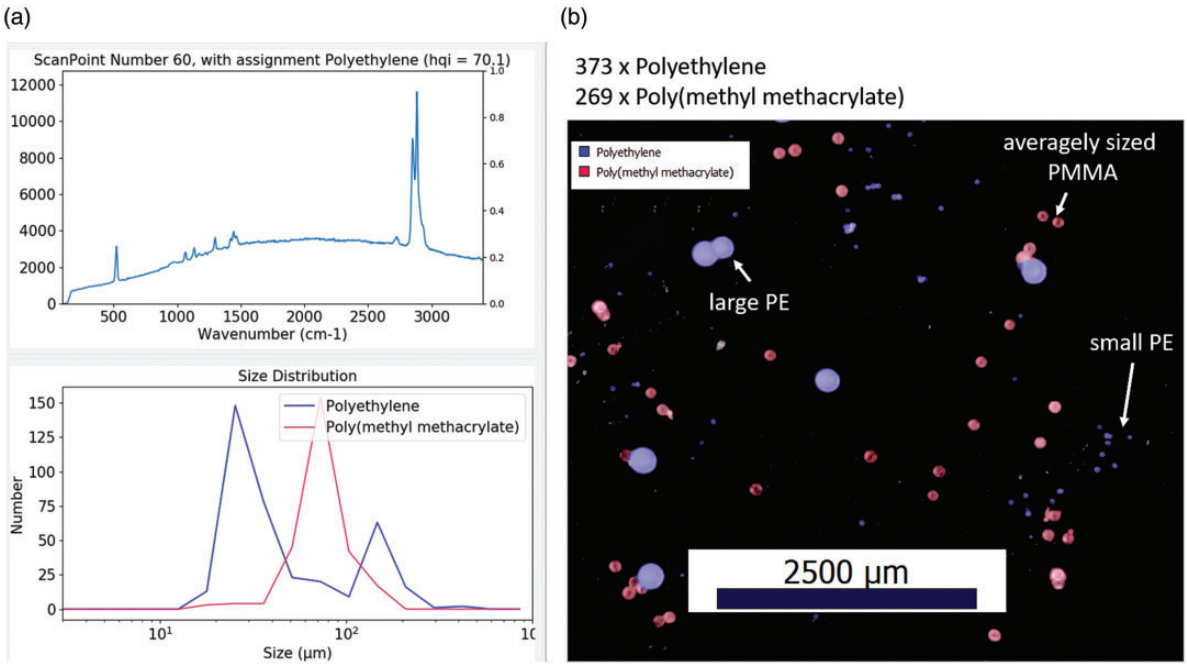

Figure 4. Evaluation of the test sample, clearly showing the two different size distributions of polyethylene and the average sized poly(methyl methacrylate). Image acquisition and Raman scan were performed on the Renishaw inVia microscope. (a) Example spectrum and size distributions. (b) Representative section of the filter with color assignment of PE (blue) and PMMA (purple).

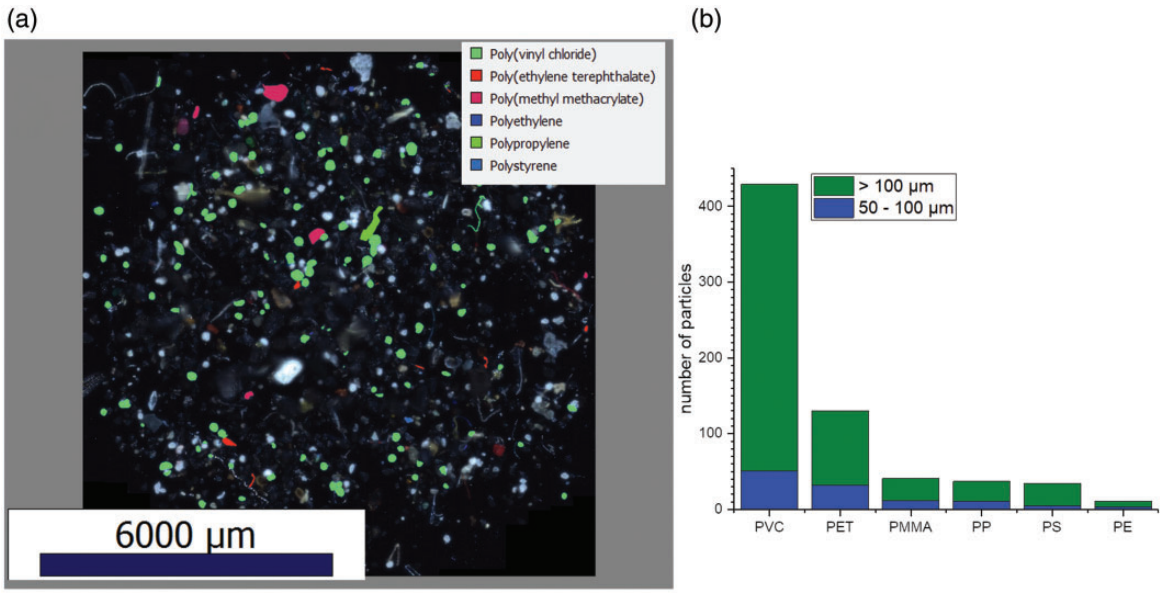

Figure 5. Results of the soil sample. (a) Example photo of one out of a total of nine filters from the complete 500 g (w/w) sample. (b) Summary of MP from a total of 32 211 particles/fibers from all nine filters combined (measurement time: 1.3 d), of which 682 were MP particles.

for the particle recognition, so we very critically review the automated results. In simpler cases, the needed time for that reviewing can be substantially lower. The Investigation of a Soil Sample section (Supplemental Material) gives a complete overview over measurement times of all filters belonging to that sample, together with details of the particle recognition and the obtained spectra.

The example shows that approximately 2% of the measured particles were identified as MP particles, which, according to our experience, is a relatively high value. It

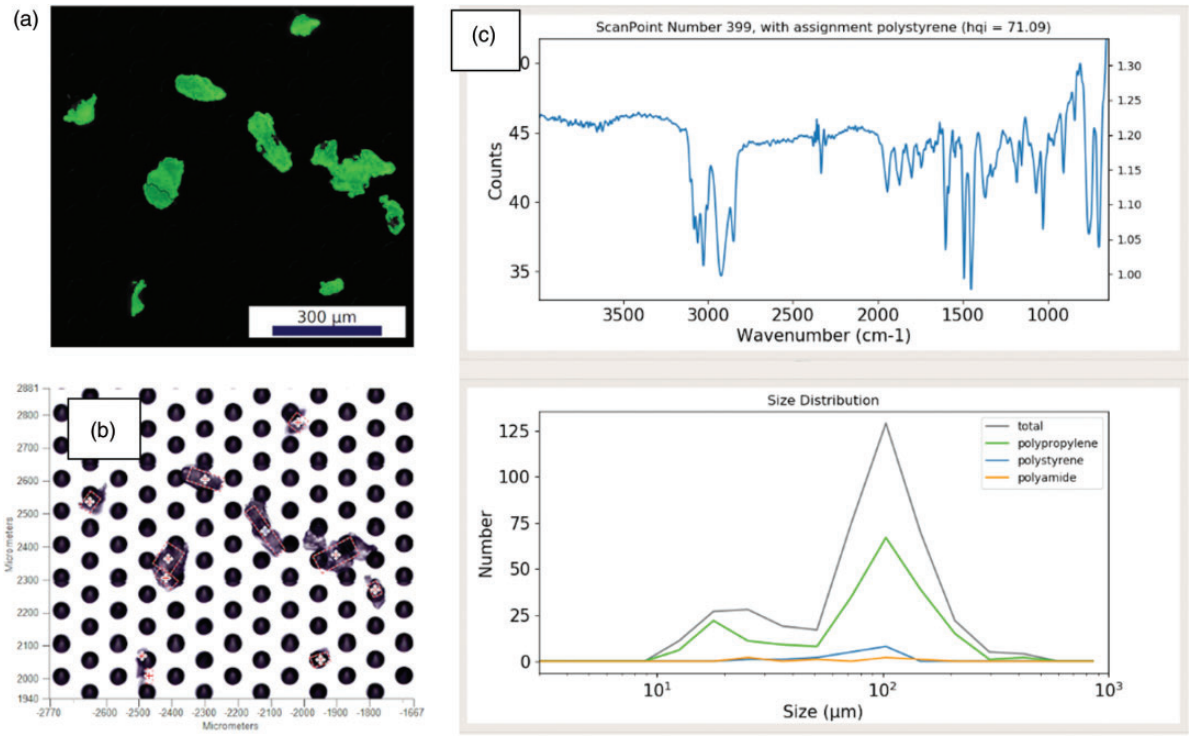

Figure 6. FT-IR measurement of a model sample containing polypropylene and polystyrene on a 50 µm silicon filter. (a) Detail of particle recognition in GEPARD based on dark field image recorded with Zeiss Imager. (b) Preview image in FT-IR microscope of the same position showing imported aperture shapes. (c) Analysis of the FT-IR results in GEPARD.

is interesting to observe that the distribution of the particles on the filter is neither homogenous nor does it follow any distinct pattern. This type of unpredictable particle distribution makes reliable subsampling, i.e., selection of small but representative regions, difficult. We currently measure each detected particle in order not to omit any MP particle, which results in relatively short measurement times per particle. Typical Raman setups use a laser power of 5 mW and the acquisition of five accumulations with 0.5 s integration time per particle. Consequently, it is necessary to handle many spectra with a low signal-to-noise ratio. The current database approach does not always seem to yield ideal results. Chemometric models based on spectral descriptors could probably improve the overall quality,[18] but are not yet implemented in the program.

Several methods for subsampling are described in literature to reduce the number of particles to measure without significantly increasing the uncertainty of the measurement result.[39,60] Their validity, however, was not yet assessed practically on the example of environmental samples. We now start evaluating the suitability of different subsampling methods retrospectively on the base of real-world samples. The results to be published soon.

An example of the analysis of a water sample is shown in the Investigation of a River Water Sample section (Supplemental Material). The water sample was prepared by fractionated filtration on both 50 µm and 10 µm silicon filters. Due to the large hole-to-hole distance of 55 µm on the 10 µm filters, also smaller particles can be found. Their number, however, cannot be considered quantitative. The example shows that the vast majority of particles is found on the 10 µm filters, due to the exponential increase in particle number with decreasing particle size. Eighty-five percent of a total of almost 68 000 particles were found on the 10 µm filters, which shows the importance of including smaller particle sizes, if possible. As a result, 407 MP particles were found, which corresponds to approx. 0.6%. Again, approx. 85% of these MP particles were found on the 10 µm filters.

Figure 6 shows the result of a model sample measured by FT-IR in transmission (resolution 4 cm^{-1}, eight scans per spectrum). For the model sample, cryo-milled particles of polypropylene, polystyrene and polyamide were dispersed in water and filtered onto our silicon filters, as described in the Filtration onto Silicon Filters section of the Supplemental Material. The capability to work with FT-IR and Raman seamlessly is a key advantage of GEPARD and helps in optimizing the general analysis strategy. For instance, large particles can be measured by FT-IR and smaller particles can be measured by Raman. Previous works by Käppler et al. showed that there is no clearly preferable method between FT-IR and Raman to identify MP particles.[37] Thus, being able to measure with one method, identify particles with unclear result and measure them

with the other technique, is a promising approach that becomes accessible with GEPARD.

Conclusion

Spectroscopic methods are still key for a deeper understanding of the occurrence of MP particles in the environment. We portrayed our contribution to improve both the analysis speed and reliability of these methods. We developed a software to cover an analysis pipeline, which is compatible with not just Raman, but also with FT-IR-microspectroscopy. The GEPARD package follows the concept of particle analyses in four main steps:

(i) Optical image acquisition
(ii) Particle recognition
(iii) Spectroscopic measurement
(iv) Data evaluation and reporting.

Size, shape, color, and chemical classification are registered for each detected particle. GEPARD allows for the reviewing and correcting of all results at any time via a user-friendly interface, thereby increasing confidence in the final result. All results can be visualized graphically in the form of false-color overlays on the optical image as well as various other diagrams, exported as Excel tables or uploaded into an SQL database. Integrating all required steps into one software package makes consistent data handling significantly easier and reduces the required time to process a sample.

GEPARD not only gives great flexibility in setting up and performing MP measurements but also allows for the use of different instruments, making GEPARD compatible with a greater number of analyses laboratories.

We successfully applied our approach on a variety of environmental samples from various different compartments, ranging from air to soil samples. As such, we assert that all included steps are not only designed to work on prepared model samples, but can also be (and have successfully been) applied to real-world samples. The continuous and critical evaluation of all analysis steps assists in the ascertaining of areas where further improvements can be made. Main aspects to improve are: (i) a more powerful particle recognition, especially in the case of overlapping particles, and (ii) a fully implemented spectral evaluation that can work, both by correlating spectra with databases and by applying chemometric tools. Random decision forests proved to be efficient in classifying noisy spectra of environmental MP samples in relatively short time[18] and thus, there is the potential to increase both confidence and speed of the spectra evaluation. Additionally, we have further improved the analysis tool by enabling it to process multiple measurements simultaneously. This facilitates the consolidation of data in cases where multiple filters belong to a single sample. Moreover, comprehensive statistics on particle occurrence across multiple samples are useful to identify point contaminations.

The GEPARD package is open source and free to use and edit. Thus, it can be extended and modified by any researcher whom requires particular adjustments for specific objectives.

Acknowledgments

The authors thank Dr. Martin Armbruster at LUFA Speyer for providing soil samples from their experimental fields.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This research was funded by the Federal Ministry of Education and Research (BmBF) project MicroCatch_Balt grant number 03F0788A and the BONUS MICROPOLL project supported by BONUS (Art 185), funded jointly by the European Union and BmBF (03F0775A).

Supplemental Material

The supplemental material mentioned in the text, consisting of figures and tables, is available in the online version of the journal.

ORCID iDs

Josef Brandt https://orcid.org/0000-0002-3434-4771
Elisavet Kanaki https://orcid.org/0000-0001-7714-0536
Robin Lenz https://orcid.org/0000-0003-4156-7380

References

1. R. Lenz, M. Labrenz. "Small Microplastic Sampling in Water: Development of an Encapsulated Filtration Device". Water. 2018. 10(8): 1055.
2. A. Käppler, F. Windrich, M.G.J. Löder, 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.
3. S. Ghosal, M. Chen, J. Wagner, et al. "Molecular Identification of Polymers and Anthropogenic Particles Extracted from Oceanic Water and Fish Stomach: A Raman Micro-Spectroscopy Study". Environ. Pollut. 2017. 233: 1113–1124.
4. A.L. Lusher, N.A. Welden, P. Sobral, et al. "Sampling, Isolating and Identifying Microplastics Ingested by Fish and Invertebrates". Anal. Methods. 2017. 9(9): 1346–1360.
5. M. Simon, N. van Alst, J. Vollertsen. "Quantification of Microplastic Mass and Removal Rates at Wastewater Treatment Plants Applying Focal Plane Array (FPA)-Based Fourier Transform Infrared (FT-IR) Imaging". Water Res. 2018. 142: 1–9.
6. S. Primpke, P.A. Dias, G. Gerdts. "Automated Identification and Quantification of Microfibres and Microplastics". Anal. Methods. 2019. 11(16): 2138–2147.
7. D. Schymanski, C. Goldbeck, H.U. Humpf, et al. "Analysis of Microplastics in Water by Micro-Raman Spectroscopy: Release of

Plastic Particles from Different Packaging into Mineral Water". Water Res. 2018. 129: 154–162.
8. S.M. Mintenig, I. Int-Veen, M.G.J. Löder, et al. "Identification of Microplastic in Effluents of Waste Water Treatment Plants Using Focal Plane Array-Based Micro-Fourier-Transform Infrared Imaging". Water Res. 2017. 108: 365–372.
9. M. Bergmann, V. Wirzberger, T. Krumpen, et al. "High Quantities of Microplastic in Arctic Deep-Sea Sediments from the HAUSGARTEN Observatory". Environ. Sci. Technol. 2017. 51(19): 11000–11010.
10. J. Bertling, L. Hamann, M. Hiebel. "Mikroplastik und Synthetische Polymere in Kosmetikprodukten Sowie Wasch-, Putz-, und Reinigungsmitteln". Fraunhofer Umsicht. 2018. http://publica.fraunhofer.de/eprints/urn_nbn_de_0011-n-4907737.pdf [accessed May 19 2020].
11. J. Bertling, R. Bertling, L. Hamann. "Kunststoffe in der Umwelt: Mikro- und Makroplastik. Ursachen, Mengen, Umweltschicksale, Wirkungen, Lösungsansätze, Empfehlungen. Kurzfassung der Konsortialstudie". Fraunhofer Umsicht. 2018. https://www.umsicht.fraunhofer.de/content/dam/umsicht/de/dokumente/publikationen/2018/kunststoffe-id-umwelt-konsortialstudie-mikroplastik.pdf [accessed May 19 2020].
12. R. Geyer, J.R. Jambeck, K.L. Law. "Production, Use, and Fate of all Plastics Ever Made". Sci. Adv. 2017. 3(7): e1700782.
13. Ellen MacArthur Foundation. The New Plastics Economy: A Circular Economy for Plastic in Which it Never Becomes Waste. 2016. https://www.ellenmacarthurfoundation.org/our-work/activities/new-plastics-economy [accessed May 19 2020].
14. N.B. Hartmann, T. Hüffer, R.C. Thompson, et al. "Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris". Environ. Sci. Technol. 2019. 53(3): 1039–1047.
15. E.R. Zettler, T.J. Mincer, L.A. Amaral-Zettler. "Life in the 'Plastisphere': Microbial Communities on Plastic Marine Debris". Environ. Sci. Technol. 2013. 47(13): 7137–7146.
16. M. Masó, E. Garcés, F. Pagès, et al. "Drifting Plastic Debris as a Potential Vector for Dispersing Harmful Algal Bloom (HAB) Species". Sci. Mar. 2003. 67(1): 107–111.
17. L. Cabernard, L. Roscher, C. Lorenz, et al. "Comparison of Raman and Fourier Transform Infrared Spectroscopy for the Quantification of Microplastics in the Aquatic Environment". Environ. Sci. Technol. 2018. 52(22): 13279–13288.
18. B. Hufnagl, D. Steiner, E. Renner, et al. "A Methodology for the Fast Identification and Monitoring of Microplastics in Environmental Samples Using Random Decision Forest Classifiers". Anal. Methods. 2019. 11(17): 2277–2285.
19. A.M. Elert, R. Becker, E. Duemichen, et al. "Comparison of Different Methods for MP Detection: What Can We Learn from Them, and Why Asking the Right Question Before Measurements Matters?". Environ. Pollut. 2017. 231: 1256–1264.
20. O. Setälä, K. Magnusson, M. Lehtiniemi, et al. "Distribution and Abundance of Surface Water Microlitter in the Baltic Sea: A Comparison of Two Sampling Methods". Mar. Pollut. Bull. 2016. 110(1): 177–183.
21. H.R. Kricheldorf, G. Schwarz. "Cyclic Polymers by Kinetically Controlled Step-Growth Polymerization". Macromol. Rapid Commun. 2003. 24(56): 359–381.
22. D. Brown, L. Cheng. "New Net for Sampling the Ocean Surface". Mar. Ecol. Prog. Ser. 1981. 5: 225–227.
23. H.K. Imhof, J. Schmid, R. Niessner, et al. "A Novel, Highly Efficient Method for the Separation and Quantification of Plastic Particles in Sediments of Aquatic Environments". Limnol. Oceanogr. Methods. 2012. 10: 524–537.
24. B. Quinn, F. Murphy, C. Ewins. "Validation of Density Separation for the Rapid Recovery of Microplastics from Sediment". Anal. Methods. 2017. 9(9): 1491–1498.
25. A.S. Tagg, J.P. Harrison, Y. Ju-Nam, et al. "Fenton's Reagent for the Rapid and Efficient Isolation of Microplastics from Wastewater". Chem. Commun. 2017. 53(2): 372–375.
26. M.G.J. Löder, H.K. Imhof, M. Ladehoff, et al. "Enzymatic Purification of Microplastics in Environmental Samples". Environ. Sci. Technol. 2017. 51(24): 14283–14292.
27. C. Schwaferts, R. Niessner, M. Elsner, et al. "Methods for the Analysis of Submicrometer- and Nanoplastic Particles in the Environment". TrAC, Trends Anal. Chem. 2019. 112: 52–65.
28. A. Käppler, M. Fischer, B.M. Scholz-Böttcher, et al. "Comparison of μ-ATR-FTIR Spectroscopy and py-GCMS as Identification Tools for Microplastic Particles and Fibers Isolated from River Sediments". Anal. Bioanal. Chem. 2018. 410(21): 5313–5327.
29. J. Wagner, Z.M. Wang, S. Ghosal, et al. "Novel Method for the Extraction and Identification of Microplastics in Ocean Trawl and Fish Gut Matrices". Anal. Methods. 2017. 9(9): 1479–1490.
30. I.R. Comnea-Stancu, K. Wieland, G. Ramer, et al. "On the Identification of Rayon/Viscose as a Major Fraction of Microplastics in the Marine Environment: Discrimination between Natural and Manmade Cellulosic Fibers Using Fourier Transform Infrared Spectroscopy". Appl. Spectrosc. 2017. 71(5): 939–950.
31. M.G.J. Löder, M. Kuczera, S. Mintenig, et al. "Focal Plane Array Detector-Based Micro-Fourier-Transform Infrared Imaging for the Analysis of Microplastics in Environmental Samples". Environ. Chem. 2015. 12(5): 563–581.
32. M.A. Browne, T.S. Galloway, R.C. Thompson. "Spatial Patterns of Plastic Debris along Estuarine Shorelines". Environ. Sci. Technol. 2010. 44(9): 3404–3409.
33. M. Compa, A. Ventero, M. Iglesias, et al. "Ingestion of Microplastics and Natural Fibres in *Sardina pilchardus* (Walbaum, 1792) and *Engraulis encrasicolus* (Linnaeus, 1758) Along the Spanish Mediterranean Coast". Mar. Pollut. Bull. 2018. 128: 89–96.
34. A. Vianello, A. Boldrin, P. Guerriero, et al. "Microplastic Particles in Sediments of Lagoon of Venice, Italy: First Observations on Occurrence, Spatial Patterns, and Identification". Estuar. Coast. Shelf Sci. 2013. 130: 54–61.
35. A.S. Tagg, M. Sapp, J.P. Harrison, et al. "Identification and Quantification of Microplastics in Wastewater Using Focal Plane Array-Based Reflectance Micro-FT-IR Imaging". Anal. Chem. 2015. 87(12): 6032–6040.
36. M. Poulain, M.J. Mercier, L. Brach, et al. "Small Microplastics as a Main Contributor to Plastic Mass Balance in the North Atlantic Subtropical Gyre". Environ. Sci. Technol. 2019. 53(3): 1157–1164.
37. A. Käppler, D. Fischer, S. Oberbeckmann, et al. "Analysis of Environmental Microplastics by Vibrational Microspectroscopy: FTIR, Raman, or both?". Anal. Bioanal. Chem. 2016. 408(29): 8377–8391.
38. S. Primpke, C. Lorenz, R. Rascher-Friesenhausen, et al. "An Automated Approach for Microplastics Analysis Using Focal Plane Array (FPA) FTIR Microscopy and Image Analysis". Anal. Methods. 2017. 9(9): 1499–1511.
39. P.M. Anger, E. von der Esch, T. Baumann, et al. "Raman Microspectroscopy as a Tool for Microplastic Particle Analysis". TrAC, Trends Anal. Chem. 2018. 109: 214–226.
40. L. Hermabessiere, C. Himber, G. Duflos, et al. "Pyrolysis–GC/MS: A Powerful Tool for Microplastics Identification". Paper presented at: ECsafeSEAFOOD Conference: Industrial Stakeholder Meeting and Final Conference: 'Seafood Safety: New Findings and Innovation Challenges'. Brussels, Belgium; January 25–26, 2017.
41. M. Fischer, B.M. Scholz-Böttcher. "Microplastics Analysis in Environmental Samples-Recent Pyrolysis–Gas Chromatography–Mass Spectrometry Method Improvements to Increase the Reliability of Mass-Related Data". Anal. Methods. 2019. 11(18): 2489–2497.
42. G. Dierkes, T. Lauschke, S. Becher, et al. "Quantification of Microplastics in Environmental Samples via Pressurized Liquid

Extraction and Pyrolysis-Gas Chromatography". Anal. Bioanal. Chem. 2019. 411(26): 6959–6968.
43. E. Dümichen, A.-K. Barthel, U. Braun, et al. "Analysis of Polyethylene Microplastics in Environmental Samples, Using a Thermal Decomposition Method". Water Res. 2015. 85: 451–457.
44. E. Duemichen, U. Braun, R. Senz, et al. "Assessment of a New Method for the Analysis of Decomposition Gases of Polymers by a Combining Thermogravimetric Solid-Phase Extraction and Thermal Desorption Gas Chromatography Mass Spectrometry". J. Chromatogr. A. 2014. 1354: 117–128.
45. E. Dümichen, P. Eisentraut, C.G. Bannick, et al. "Fast Identification of Microplastics in Complex Environmental Samples by a Thermal Degradation Method". Chemosphere. 2017. 174: 572–584.
46. E. Besseling, J.T.K. Quik, M. Sun, et al. "Fate of Nano- and Microplastic in Freshwater Systems: A Modeling Study". Environ. Pollut. 2017. 220: 540–548.
47. M. Siegfried, A.A. Koelmans, E. Besseling, et al. "Export of Microplastics from Land to Sea. A Modelling Approach". Water Res. 2017. 127: 249–257.
48. H. Zhang. "Transport of Microplastics in Coastal Seas". Estuar. Coast. Shelf Sci. 2017. 199: 74–86.
49. K.M. Dorling, M.J. Baker. "Rapid FTIR Chemical Imaging: Highlighting FPA Detectors". Trends Biotechnol. 2013. 31(8): 437–438.
50. F. Liu, K.B. Olesen, A.R. Borregaard, et al. "Microplastics in Urban and Highway Stormwater Retention Ponds". Sci. Total Environ. 2019. 671: 992–1000.
51. L. Bittrich, J. Brandt. GEPARD: Gepard-Enabled PARticle Detection for Raman Microscopes. 2018. gitlab.ipfdd.de/gepard/gepard [accessed May 19 2020].
52. G. Papandreou, L.-C. Chen, K.P. Murphy, et al. "Weakly and Semi-Supervised Learning of a Deep Convolutional Network for Semantic Image Segmentation". In: 2015 IEEE International Conference on Computer Vision (ICCV). Santiago, Chile: December 7–13, 2015. Pp. 1742–1750.
53. L.-C. Chen, G. Papandreou, I. Kokkinos, et al. "DeepLab: Semantic Image Segmentation with Deep Convolutional Nets, Atrous Convolution, and Fully Connected CRFs". IEEE Trans. Pattern Anal. Mach. Intell. 2018. 40(4): 834–848.
54. V. Badrinarayanan, A. Kendall, R. Cipolla. "SegNet: A Deep Convolutional Encoder-Decoder Architecture for Image Segmentation". IEEE Trans. Pattern Anal. Mach. Intell. 2017. 39(12): 2481–2495.
55. J. Wang, B. Liu, K. Xu. "Semantic Segmentation of High-Resolution Images". Sci. China Inf. Sci. 2017. 60(12): 123101.
56. S. Beucher. "The Watershed Transformation Applied to Image Segmentation". Paper presented at: 10th Pfefferkorn Conference, Signal and Image Processing in Microscopy and Microanalysis. Scanning Microsc. Int. 1991. Supplement 6: 1–26.
57. P.M. Anger, L. Prechtl, M. Elsner, et al. "Implementation of an Open Source Algorithm for Particle Recognition and Morphological Characterisation for Microplastic Analysis by Means of Raman Microspectroscopy". Anal. Methods. 2019. 11(27): 3483–3489.
58. H.P. Ng, S.H. Ong, K.W.C. Foong, et al. "Medical Image Segmentation Using K-Means Clustering and Improved Watershed Algorithm". In: Proceedings of the 2006 IEEE Southwest Symposium on Image Analysis and Interpretation. 2006. Pp. 61–65..
59. V. Cerny. "Thermodynamical Approach to the Traveling Salesman Problem: An Efficient Simulation Algorithm I". J. Optim. Theory Appl. 1985. 45(l): 41–51.
60. S. Huppertsberg, T.P. Knepper. "Instrumental Analysis of Microplastics: Benefits and Challenges". Anal. Bioanal. Chem. 2018. 410(25): 6343–6352.