

Enzymatic Purification of Microplastics in Environmental Samples

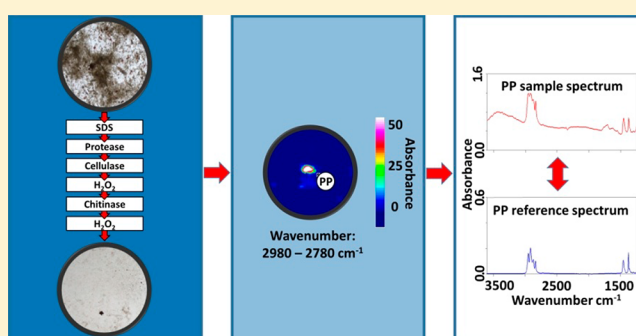
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Supporting Information

ABSTRACT: Micro-Fourier transform infrared (micro-FTIR) spectroscopy and Raman spectroscopy enable the reliable identification and quantification of microplastics (MPs) in the lower micron range. Since concentrations of MPs in the environment are usually low, the large sample volumes required for these techniques lead to an excess of coenriched organic or inorganic materials. While inorganic materials can be separated from MPs using density separation, the organic fraction impedes the ability to conduct reliable analyses. Hence, the purification of MPs from organic materials is crucial prior to conducting an identification via spectroscopic techniques. Strong acidic or alkaline treatments bear the danger of degrading sensitive synthetic polymers. We suggest an alternative method, which uses a series of technical grade enzymes for purifying MPs in environmental samples. A basic enzymatic purification protocol (BEPP) proved to be efficient while reducing $98.3 \pm 0.1\%$ of the sample matrix in surface water samples. After showing a high recovery rate ($84.5 \pm 3.3\%$), the BEPP was successfully applied to environmental samples from the North Sea where numbers of MPs range from 0.05 to 4.42 items m^{-3} . Experiences with different environmental sample matrices were considered in an improved and universally applicable version of the BEPP, which is suitable for focal plane array detector (FPA)-based micro-FTIR analyses of water, wastewater, sediment, biota, and food samples.



INTRODUCTION

To date, plastic debris is almost ubiquitous in aquatic habitats. Plastic particles <5 mm, so-called microplastics (MPs), have been reported to be present in various marine^{1–4} and freshwater ecosystems.^{5,6} As a result of this omnipresence, accompanied by the potential hazards of MPs to the environment, organisms, and human health, the contamination of the ocean with plastic debris has been characterized as one of the top emerging global issues,^{7,8} and a similar risk for freshwater environments is anticipated.⁹

Although the problem has been recognized, to date, a universal standard operating procedure for identifying and quantifying MPs does not exist, which hampers comparing MPs data.¹⁰ Nevertheless, technical advancements using state-of-the-art spectroscopic techniques allow for the reliable identification and quantification of MPs, even in the lower micron range.^{11–13} The analysis of small MPs (<500 μm) with micro-FTIR spectroscopy¹⁴ or Raman microspectroscopy^{10,15} mandatorily requires concentrating the samples on filter membranes. By applying these techniques in combination with chemical imaging and analysis, MPs can be characterized in terms of their composition, quantity, and size.^{13,14,16}

Generally, all spectroscopic approaches are constrained by a mostly unfavorable target (MPs) to nontarget (organic or inorganic particles) ratio in the samples. Hence, analyses are usually hampered by an excess of organic (e.g., algae, plankton, and natural debris) or inorganic particles (e.g., sand and silt) as their signal overlays MPs spectra. Therefore, the purification of MPs from environmental samples is an essential part of sample processing and a crucial step prior to conducting an identification and quantification analysis via chemical imaging.

As a prerequisite, the purification approach should (1) efficiently remove the organic and inorganic material from the samples while not affecting the synthetic polymers, (2) facilitate the concentration of the purified samples on filters with a small pore size (<1 μm) that is suitable for spectroscopic measurements, and (3) be cost and labor effective.

To date, diverse purification approaches have been developed to purify environmental samples for analyses of MPs. Inorganic particles in, e.g., sediment samples, are removed

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using density separation with different solutions of high density, which leads to a significant reduction in the sample volume.^{17–21}

Organic material, often present in high loads in plankton, sediment, or tissue samples, is usually digested by strong acidic^{11,12,22,23} or alkaline solutions,^{23–26} oxidation agents,^{19,27,28} or a combination of these agents.^{24,29}

These digestion approaches, however, are limited by the chemical susceptibility of the MPs, and sensitive synthetic polymers can be lost during digestion,^{22–24,26,29,30} which is furthermore dependent on their size, shape (e.g., fragment, fiber, film, sphere), age, and oxidation.

An alternative, plastic-conserving and expedient approach is to purify environmental samples using enzymes.^{23,30,31} The use of a single enzyme (proteinase-K) to isolate MPs from seawater samples with a high content of planktonic organisms was first suggested by Cole et al.²³ Although a high grade of purification was reached, a significant drawback of the approach is the high cost for the enzyme that is usually used in molecular biology approaches. Meanwhile, inexpensive enzymes have been successfully used for the purification of plastic particles from mussel tissue samples.^{30,31} Unfortunately, none of the studies determined whether their enzymatic approach is suitable for different sample matrices and appropriate for chemical imaging of MPs concentrated on filters.

This underlines the urgent need for a purification protocol that is “plastic-conserving” and applicable for different environmental sample matrices while meeting the above-mentioned prerequisites for spectroscopic analyses.

To overcome the existing limitations, the overall aim of our study was to develop a protocol for the reduction of organic and inorganic sample matrices of different environmental origins or types (plankton, sediment, and biota), while leaving all synthetic polymers unaffected. The grade of purification should be suitable for reliably identifying and quantifying MPs using an FPA-based micro-FTIR analysis accompanied by chemical imaging.^{13,14,32}

In the first step, we developed a basic enzymatic purification protocol (BEPP) based on the sequential application of a detergent, technical grade enzymes, an oxidizing agent, and a density separation step, and evaluated the protocol for its purification efficiency and applicability for FPA-based FTIR imaging of MPs in the size range of 20–500 μm . In the second step, the recovery rate of the MPs after the application of the entire purification process was determined. In the final step, important improvements in the BEPP, based on tests conducted with different sample matrices, are described leading to an optimized universal enzymatic purification protocol (UEPP) that is suitable for different environmental matrices, including plankton samples, sediment samples, and biota samples.

■ MATERIALS AND METHODS

General Precautions To Prevent Sample Contamination. All laboratory equipment and materials were thoroughly rinsed with prefiltered (0.2 μm) deionized water (Milli-Q) and 35% ethanol before and during all working steps to prevent sample (cross-) contamination. The enzymes were filtered through a 0.45 μm filter before usage. All materials were covered whenever possible with glass lids or aluminum foil to prevent airborne contamination. Wherever possible, equipment made of plastic was replaced with glass or metal components. Within the laboratory, cotton lab coats and only non-synthetic

clothing were worn. To monitor possible sample contamination, blank samples (Milli-Q water) that underwent the same procedure as the environmental samples were used.

MPs Sampling. Marine plankton samples were obtained from the North Sea by sea surface sampling with a neuston catamaran using a plankton net with a 100 μm mesh size during cruise “HE409” on the “Heincke” research vessel in September 2013 (Table S1). The samples were carefully transferred to PVC Kautex Bottles (Kautex Textron GmbH & Co. KG, Bonn, Germany) and frozen prior to purification with the BEPP and the subsequent FPA-based micro-FTIR analysis.

Basic Enzymatic Purification Protocol (BEPP). The BEPP is based on the results from preliminary purification experiments of plankton samples with commercial enzymatic mixtures produced for organic washing agents (Biozym SE & Biozym F, Spinnrad GmbH, Bad Segeberg, Germany), which contain a detergent, amylase, protease, and lipase. Combined with a preceding detergent step with sodium dodecyl sulfate (SDS) and a subsequent chitinase step, the approach proved to be efficient for the purification of marine surface water samples and mussels.

To increase the efficiency of the purification process, the enzyme mixtures were replaced with technical grade enzymes (protease, cellulase, chitinase; ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) that were applied at their optimum pH and temperature (for detailed information on the used enzymes please refer to the section “Detailed Description of the Sequential Purification Steps” and the Supporting Information). All enzymes were specifically chosen according to the potential corresponding major components of organic material in the environmental samples. Similar to the preliminary experiments, the BEPP included an incubation step with SDS prior to the three enzymatic steps and was additionally combined with a hydrogen peroxide treatment that was performed twice and a final density separation step using a ZnCl_2 solution. As a result, the BEPP involves seven consecutive purification steps (Figure 1), which are described in detail below.

General Procedure. In the first step the samples were defrosted and subdivided into two size fractions using a 500 μm stainless steel sieve. The fraction >500 μm was visually sorted for potential MPs, and they were individually analyzed using attenuated total reflection (ATR) FTIR spectroscopy (Figure 1, data not shown). The sample fraction <500 μm was purified using the BEPP.

The general handling procedure for all samples was as follows for all sequential steps: Each sample was filtered through a stainless steel filter (47 mm diameter, mesh size 10 μm , Wolftechnik Filtersysteme GmbH & Co. KG, Weil der Stadt, Germany) with a bottle top filtration device (Thermo Fisher Scientific Inc., Waltham, MA, USA). One single filter per sample was used consecutively during the whole procedure. All equipment that came into contact with the sample was rinsed thoroughly with Milli-Q water to avoid particle losses. After filtration, the filtrate was discarded, and the filter with the residues was placed in a 100 mL laboratory glass bottle (DURAN Group GmbH, Mainz, Germany). Next, the respective enzyme or chemical solution was added as described in Figure 1. The sequence of the enzymes was chosen according to the lability of the targeted biological substrates – first proteins followed by cellulose and then chitin (for more information refer to the paragraph “Detailed Description of the Sequential Purification Steps”). During each filtration step rinsing with Milli-Q water took place to exclude an interference

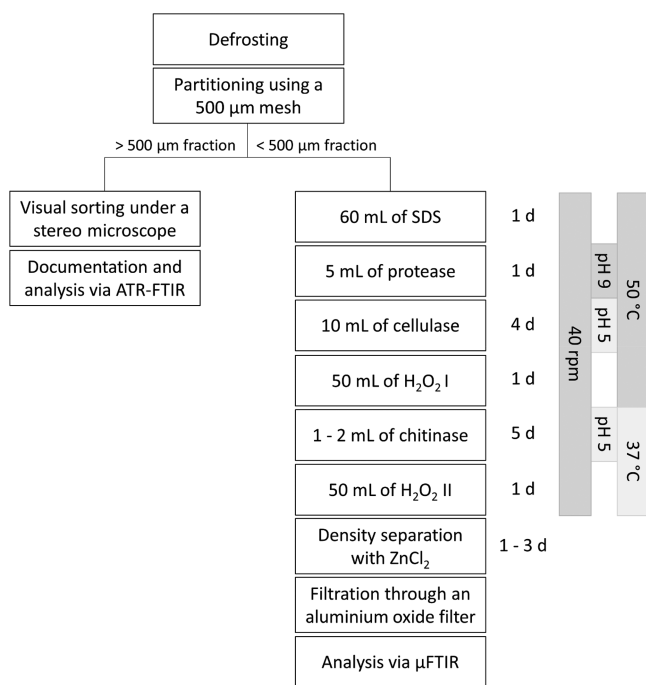


Figure 1. Flowchart of the basic enzymatic purification protocol used for the efficiency validation, determination of the recovery rate determination, and determination of the MPs in the plankton samples.

of the next enzyme with the enzyme rests of the previous step. The samples were incubated in a Multitron shaking incubator (Infors AG, Bottmingen, Switzerland) at 40 rpm with the respective incubation time and temperature. After the incubation, the stainless steel filter was removed from the bottle, and all remaining residues on the filter were thoroughly rinsed back into the bottle using Milli-Q water. Then, the clean stainless steel filter was remounted on the bottle-top filtration system, and the corresponding sample in the bottle was filtered again to remove the enzyme/chemical solution. The filter with the residue was placed back into the incubation bottle, the filtration equipment was rinsed with the corresponding buffer solution, and the next enzyme or chemical solution was added (Figure 1). After the six purification steps, a final density separation process was included (detailed description below). The residual sample was filtered through aluminum oxide filters (25 mm diameter, 0.2 µm pore size, Anodisc, Whatman, GE Healthcare, Chicago, IL, United States) for the FPA-based FTIR analysis. Here, a specially manufactured filter system, consisting of an acrylic glass filter funnel with a 9 mm diameter mounted on a 25 mm supporting plate and pressed on the fritted stainless steel support base with a laboratory clamp, was used to reduce the final filtration area with respect to the FPA-based micro-FTIR analysis.¹⁴

Detailed Description of the Sequential Purification Steps. Sodium Dodecyl Sulfate (SDS) Treatment. The initial incubation was performed using SDS, which is an anionic surfactant. SDS macerates planktonic organisms and animal and plant residues and increased the contact surface for the following enzymatic treatments. A solution of 5% (w/w) SDS with a volume of 60 mL per incubation bottle was applied. The samples were incubated for approximately 24 h at 50 °C in the incubator.

Protease Treatment. The first enzymatic purification was conducted with protease. Protease catalyzes the decomposition

of protein chains into easily dissolved and dispersed peptides and thus further macerates planktonic organisms and cell residues. Protease A-01 (subtilisin, EC 3.4.21.62, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) was applied, which attains its optimum activity at pH 9.0 and 50 °C. The enzymatic activity is specified as 1,100 U/mL. In total, 5 mL of protease was added to the sample matrix in the laboratory glass bottles, and 25 mL of phosphate-buffered saline (PBS) solution was added. The PBS solution was set to pH 9.0 by adding sodium hydroxide. The samples were incubated at 50 °C for 24 h.

Cellulase Treatment. The cellulase treatment targets the maceration of phytoplankton cell walls and other plant residues. Cellulase TXL (EC 3.2.1.4, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) was applied, which is an endo-1,4-β-glucanase with a high “C1” activity. It cleaves the β-1,4-bonds within cellulose molecules and is used to decompose all kinds of cellulose. Cellulase TXL attains its optimum reaction activity at pH 5.0 and 50 °C and had an activity of >30 U/mL. For this purification step, 10 mL of Cellulase TXL and 50 mL of the PBS solution, set to pH 5.0 by adding hydrochloric acid, were added. The samples were incubated at 50 °C for 4 days.

Hydrogen Peroxide Treatment I. The exoskeletons of crustaceans contain not only chitin but also a protective coating of proteins and calcium carbonate, which makes them very robust and difficult to digest. To facilitate better contact between the chitinase and chitin (in the subsequent step), the samples were initially treated with 50 mL of 35% stabilized hydrogen peroxide (Merck KGaA, Darmstadt, Germany), which is a well-established process for the destruction of organic materials.^{20,33} The samples were incubated for 24 h at 50 °C.

Chitinase Treatment. High amounts of chitin-containing materials were anticipated to be present in both, marine and freshwater environmental samples. The chitinase used (EC 3.2.1.14, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) has a specific activity of >50 U/mL. It consists of chitodextrinase, 1,4-β-poly-N-acetylglucosaminidase, and poly-β-glucosaminidase. These hydrolytic enzymes break down the glycosidic bonds within chitin. The chitinase reaches its maximum enzyme activity at a pH of 5.6 and 37 °C. Depending on the amount of PBS buffer (pH 5) needed to rinse the equipment after filtration (between 15 and 30 mL), a total of 1 to 2 mL of Chitinase were added, and the samples were incubated for 5 days at 37 °C.

Hydrogen Peroxide Treatment II. A second application of hydrogen peroxide (50 mL) was performed to further degrade the partly dissolved organic material. The samples were treated for 24 h at 37 °C.

Density Separation. Frequently, considerable amounts of inorganic material (e.g., sand and diatom frustules) were present in the samples after the enzymatic treatment. Therefore, the samples underwent density separation using a ZnCl₂ solution at a density of 1.7 g/cm³. After the last filtration step, the filter cake was flushed in a small 50 or 100 mL beaker using a prefiltered zinc chloride solution. Then, the samples were transferred into 50 or 100 mL separation funnels where they remained between 1 and 3 days. Every few hours (4–12 h), the settled portion was carefully discarded. When necessary, the separation funnels were carefully swayed to support the separation process and overcome the surface tension. As soon as sedimentation was no longer observed, the separation funnels were carefully emptied until just a few mL of solution,

containing the light fraction with the MPs, were left. This fraction was finally filtered through aluminum oxide filters (0.2 μm , Anodisc, Whatman, GE Healthcare, Chicago, IL, United States) for FPA-based micro-FTIR spectroscopy.

Efficiency Validation of the Enzymatic Purification.

The efficiency of the BEPP was determined using representative environmental samples obtained in July 2014 northeast of the coast of Helgoland island, North Sea, German Bight. A plankton net with a mesh size of 100 μm was towed two times at the surface for several minutes resulting in a highly concentrated plankton sample of approximately 10 L that contained a sample matrix with a high content of phytoplankton and zooplankton, such as diatoms, ciliates, flagellates, and copepods as well as organic detritus (marine snow) and sand particles. The sample was mixed and homogeneously divided into 24 aliquots, each with a 250 mL volume. Further, each incubation bottle was doped with a small amount of polypropylene (PP) particles (<150 μm , abrasion from a blue screw cap, Duran group, Wertheim, Germany). The PP doping was conducted to test whether the sample matrix reduction after the purification was sufficiently high to facilitate analyzing the MPs via FPA-based micro-FTIR spectroscopy. As described above, the plankton samples were filtered, and the filter cake of each replicate was transferred into 100 mL laboratory glass incubation bottles for subsequent purification using the BEPP.

The efficiency of the enzymatic purification process was evaluated according to the weight loss of the remaining filter cake after each purification step using a gravimetric analysis. Prior to the experiments, all filter substrates (21 clean 10 μm stainless steel filters and three 0.2 μm aluminum oxide filters) were dried for 48 h at 60 °C and weighed with a laboratory precision scale ($d = 0.01$ mg, Sartorius AG, Göttingen, Germany). All sample aliquots were treated according to the BEPP described above. Prior to the purification and after each purification step, three replicates were filtered through preweighted stainless steel filters, and after the last step (density separation), aluminum oxide filters were used for the subsequent FPA-based micro-FTIR spectroscopy tests. The filters with the residual samples were dried for 48 h at 60 °C and weighed, and the purification efficiency was calculated using the initial mean dry weight per sample aliquot of 94.77 mg. Additionally, the appearance of the residual sample was documented using a stereomicroscope (Olympus SZX16, Olympus Europa Holding GmbH, Hamburg, Germany). Prior to the photos, the filter residue has always been resuspended in the same volume of Milli-Q to facilitate a comparison. No photos have been taken after the density separation step, because due to the 0.2 μm aluminum oxide filters used for the subsequent FPA-based micro-FTIR analysis it was important to keep the filtered volume as small as possible to keep the filtration time at a minimum (compare paragraph “Density Separation” above). Thus, a resuspension in a larger amount of Milli-Q as necessary for the comparison with the previous steps was impossible.

The applicability of the BEPP for FPA-based micro-FTIR chemical imaging was tested on the PP doped samples, as described below.

FPA-Based Micro-FTIR Imaging. The suitability of the BEPP for chemical imaging was tested on a sample from the efficiency validation. The measurements were performed using FPA-based micro-FTIR spectroscopy with a Hyperion 3000 FTIR microscope equipped with a FPA detector coupled to a

Tensor 27 spectrometer (Bruker Optik GmbH, Ettlingen, Germany), similar to the setup in Löder et al.¹⁴ The dried sample on the aluminum oxide filter was placed on a CaF₂ filter holder under the IR microscope and measured in transmission mode at a final magnification of 150 \times . The FTIR measurement was performed in the wavenumber range of 3600–1250 cm^{-1} at a resolution of 8 cm^{-1} and a coaddition of 32 scans. The background was acquired on the blank filter material using the same parameters. Exemplarily, a filter area of approximately 2.5 \times 1.6 mm was measured by combining 126 FPA tiles. The FTIR system was operated, and the data were processed using OPUS 7.5 software (Bruker Optik GmbH). For chemical imaging the wavenumber range between 2980 and 2780 cm^{-1} , corresponding to the C–H stretching vibrations in PP, was integrated. The measured spectra were compared to those from a polymer library generated by the Alfred Wegener Institute, Helgoland, Germany.¹⁴

The measurements of the purified samples from cruise “HE409” were performed using the same system; the measurement settings and the parameters for the subsequent analyses were chosen according to Löder et al.¹⁴ (The measurement time for a filter area of around 10 \times 10 mm with a pixel resolution of around 10.6 μm is 10–12 h; the time needed for the analysis of the spectra depends on the amount of potential particles present and can be several hours).

Determination of the Recovery Rate. Bright red polyethylene (PE) beads (density 1.072 g/cm^{-3} , size 180–212 μm , REDPMS-1.070, Cospheric, Santa Barbara, CA, USA) were counted (109, 84, and 86), picked out under a microscope (Olympus SZX16), and transferred into three laboratory glass bottles filled with 1 L of Milli-Q water. These three samples went through the entire BEPP as described above. Finally, the recovered PE beads were counted under the stereomicroscope, and the recovery rates were calculated.

Development of an Universal Enzymatic Purification Protocol (UEPP). During the daily lab routine, the enzymatic purification approach has been applied during several studies on MPs for samples containing different environmental matrices.^{13,14,32,34} In addition to marine surface water samples, these matrices have included freshwater surface water samples from rivers and lakes, marine and freshwater sediment and beach samples after density separation, wastewater samples, tissue samples of mussels, daphnia, and fish, and commercial fish food samples. During these studies, several modifications in the BEPP were necessary to adjust for the different chemical compositions of each sample matrix, and all samples underwent an enzymatic purification and were concentrated through filters for subsequent analyses of MPs via FPA-based micro-FTIR spectroscopy and chemical imaging.

The experiences of these investigations resulted in an improved universal enzymatic purification protocol (UEPP), which is mainly based on the following main modifications in the BEPP: changing the buffer used, including two optional enzymatic steps (amylase and lipase), and optimizing the incubation conditions and enzyme concentrations, which were developed in cooperation with the enzyme manufacturer (ASA Spezialenzyme GmbH, Wolfenbüttel, Germany). These comprehensive adjustments for the proposed UEPP are not included in the Results section but are suggested in the Discussion, whereas the detailed changes are described in the Supporting Information (SI).

RESULTS

Efficiency Validation of the Enzymatic Purification.

During the whole purification process, the initial sample mass (dry weight) was reduced by $98.3 \pm 0.1\%$ (standard deviation = SD), which was equal to a mean reduction of $93.18 \pm 0.1 \text{ mg}$ for an initial mean dry weight of $94.77 \pm 3.8 \text{ mg}$ (Figure 2).

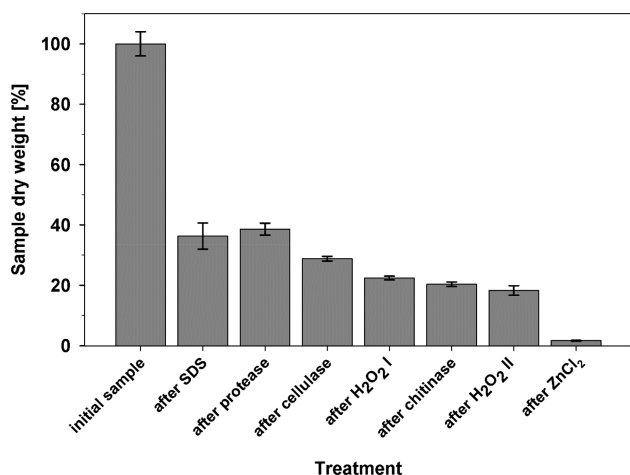


Figure 2. Changes in the sample dry weight in percent after each purification step (SD: $n = 3$ for all results; after ZnCl₂, SD: $n = 2$).

Within the single treatment steps, the largest reduction in the natural matter content was observed after the first treatment with SDS. During this step, the sample mass was reduced by $63.7 \pm 4.3\%$. With the exception of the protease treatment, the

sample mass was continuously reduced by the subsequent enzymatic and hydrogen peroxide purification steps (Figure 2), resulting in a further halving of the sample mass. This was also reflected in the reduced amount of visible residual material in the microscopic picture (Figure 3). The second highest mean reduction value ($16.6 \pm 0.1\%$) was attained during the final zinc chloride density separation, which removed high-density particles, mainly sand grains. The result of this last treatment was based on two instead of three replicates, because one sample was lost due to a leakage in the filtration device.

Suitability Test of the BEPP for FPA-Based Micro-FTIR Imaging. The above-mentioned prerequisites for a successful analysis of MPs via FPA-based micro-FTIR chemical imaging were clearly fulfilled. The chemical imaging based on the integration of the wavenumber range between 2980 and 2780 cm^{-1} , corresponding to the C–H stretching vibrations, allowed for the clear discrimination of the doped PP particles (Figure 4). Only minimal amounts of organic residues were present, which are also highlighted by the integration within this wavenumber range. The efficiency of the BEPP was very high and resulted in a good contrast between the background and PP particles due to the reduced intrinsic interference background signals from the residuals of the organic matrix (Figure 4). The residual sample matrix showed only a very weak signal in the wavenumber ranges between 1500 and 1800 cm^{-1} and between 3000 and 3600 cm^{-1} (Figure 4). The high grade of purification furthermore facilitated the visualization of PP particles smaller than 20 μm .

Determination of the Recovery Rate. The determined recovery rate for the red fluorescent MPs in the three water samples processed with the BEPP yielded a mean value of 84.5

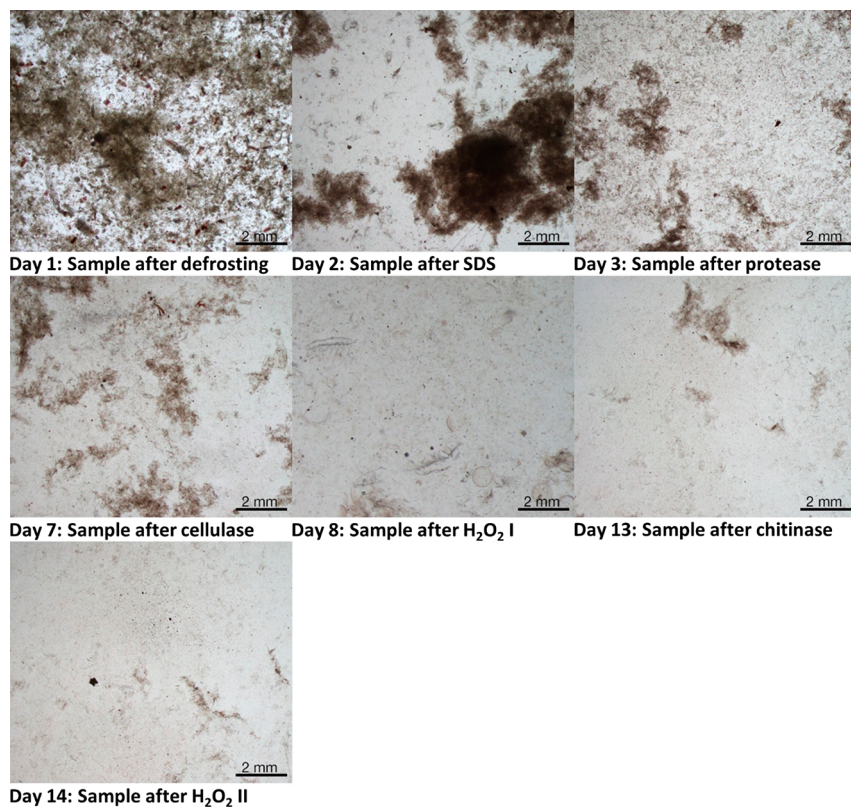


Figure 3. Digestion efficiency evaluation after each consecutive purification step. Due to differences in sample treatment, the final density separation step was not documented.

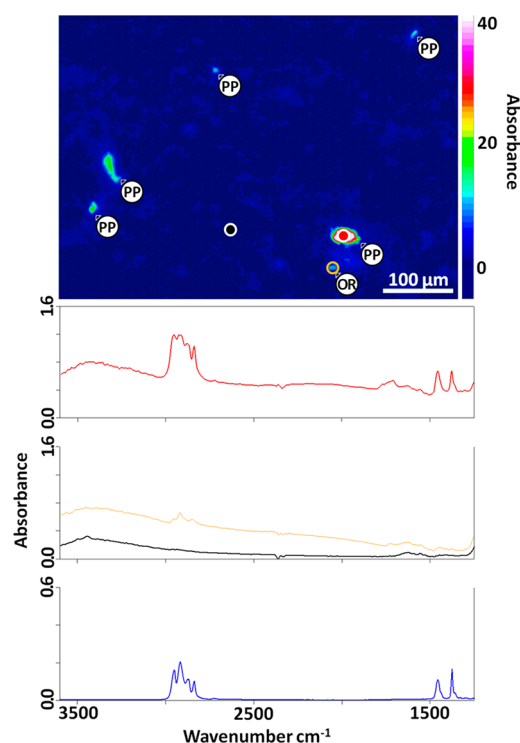


Figure 4. FPA-based micro-FTIR chemical imaging of a sample after the purification process. Upper figure: Chemical image of the wavenumber range from 2980–2780 cm^{-1} , corresponding to the C–H stretching vibrations. The particles marked with PP are the polypropylene particles from the sample doping; OR is an organic residue. The color bar represents the intensity of the integrated band region. Particles with peaks in the integrated region are highlighted according to their peak height. The scale bar corresponds to 100 μm . Lower figure: The red spectrum was measured at the location marked by the red point in the upper figure and identified as PP; the black spectrum was measured at the location of the black point in the upper figure and represents the background signal; the orange spectrum was measured at the location of the organic residue which is encircled in orange, and the blue spectrum is a PP reference spectrum from the reference database.

$\pm 3.3\%$. Approximately 15% of the beads were lost during the purification process (see Table 1).

Table 1. Recovery Rate

replicate	no. of beads added	no. of beads recovered	recovery rate [%]
1	109	89	81.65
2	84	74	88.10
3	86	72	83.72
average \pm SD			84.5 \pm 3.3

Determination of MPs in the Plankton Samples. Sea surface plankton samples from the northern German Bight sampled during cruise “HE409” were purified using the BEPP. The geographic positions and further additional data are provided in the Supporting Information (Table S1).

In all samples, MPs < 500 μm were found, and the concentrations of the total polymer content and assigned polymer types are summarized in Table 2. MPs were found in concentrations between 0.05 and 4.42 particles m^{-3} , the particles were only fragments. The most abundant polymers found were PE and polystyrene (PS). The samples were

corrected for a possible MP sample contamination during the purification by the values of three procedural blanks. Only one polyamide particle and one polyethylene terephthalate fiber were found in the procedural blanks. However, we found a relatively high number of PP particles in the procedural blanks that could be later traced back to the screw caps of the glass bottles the enzymes were stored in. Thus, PP was excluded from the analysis, and the screw caps were replaced by aluminum foil in subsequent studies.

DISCUSSION

Basic Enzymatic Purification Protocol (BEPP). The main aim of the enzymatic purification approach was (1) to reduce the sample matrix to allow for a reliable analysis during FPA-based micro-FTIR imaging and, simultaneously, (2) to conserve the natural composition of the MPs.

The samples that were purified with the BEPP contained a great variety of microalgae (e.g., diatoms and dinoflagellates) and crustaceans (e.g., copepods and decapod larvae), as well as small fragments of insects, macroalgae, and higher plants, that were not removed by the 500 μm sieving step. Despite the high load of organic material, the described BEPP efficiently reduced the natural matter during the enzymatic and oxidative purification steps, whereas inorganic material (mainly sand) was removed during the final density separation step. This resulted in a minimum amount of inorganic and organic particles on the aluminum oxide filters and facilitated the successful and time efficient identification and quantification of MPs down to a size of 20 μm using FPA-based micro-FTIR spectroscopy according to Löder et al.¹⁴

Although the BEPP reached in total a general high grade of purification in our efficiency validation, the efficiency of the single purification steps was of different magnitude (compare Figure 2). This is certainly dependent on the amount of the targeted substrata which is present in the respective sample. For example, no weight loss was observed for the protease step during our purification of the plankton samples. In this case, probably most of the proteins were already degraded by the previous SDS step. However, during the purification of other samples the protease step led to a significant reduction of organic material.

FPA-based micro-FTIR spectroscopy according to Löder et al.¹⁴ facilitates the reliable analysis of particles down to a size of at least 20 μm , thus we used 10 μm stainless steel filters during the purification process. Nevertheless, only aluminum oxide filters allow for good quality FPA-based micro-FTIR measurements in transmission mode, as this filter material is IR transparent in the wavenumber range important for microplastic analyses.¹⁴ Due to the fact that during the purification process we excluded small particles that are not in the measurable size range, 10 μm filters would be theoretically appropriate for FPA-based micro-FTIR measurements; however, unfortunately no aluminum oxide filters with a higher porosity than 0.2 μm are standardly available.

A great advantage of an enzymatic approach as proposed herein is the ability to conserve the sensitive synthetic polymers, as already described by Cole et al.,²³ Catarino et al.,³⁰ and Courtene-Jones et al.³¹ In contrast, in several studies in which strong acid, alkaline, or oxidative treatments were applied, detrimental effects on sensitive synthetic polymers were observed.^{22–24,26,29,30} Furthermore, the usage of aggressive chemicals increases the risk of further fragmenting the

Table 2. Abundances and Polymer Types of MPs < 500 μm Found at the Stations of the Cruise “HE 409” in Units of Number of Items $\text{m}^{-3\text{a}}$

station	total	PS	PE	PA	ABS	PVA	PET	PUR	EVA
1	0.266		0.061	0.051	0.061		0.061	0.031	
2	0.096		0.072		0.024				
3	0.437	0.309	0.077	0.051					
4	0.049				0.049				
5	1.815	1.210	0.519		0.043				0.043
6	0.551	0.551							
7	4.415	1.766	1.876		0.221	0.552			
8	0.209	0.070	0.139						
9	0.137	0.023	0.092				0.023		
10	1.762	0.576	0.105	0.977	0.052		0.052		

^aPS = polystyrene, PE = polyethylene, PA = polyamide, ABS = acrylonitrile butadiene styrene, PVA = poly(vinyl alcohol), PET = polyethylene terephthalate, PUR = polyurethane, and EVA = ethylene vinyl acetate.

MPs, thus potentially falsifying the analysis results regarding the quantity of MPs.^{22,23}

In addition to using SDS and enzymes, hydrogen peroxide was used in our protocol to degrade the organic matrices. Nuelle et al.¹⁹ reported visible changes in MPs, which became more transparent and thinner, after exposure to 30% hydrogen peroxide over 7 days. In contrast to that, our samples were never treated longer than 24 h with hydrogen peroxide; however, we investigated the influence of the enzymatic purification protocol – including the two hydrogen peroxide steps – on films of eight different plastic polymers (polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR), polyamide (PA), polyethylene terephthalat (PET), polystyrene (PS), polycarbonate (PC)) in terms of IR spectra and weight loss. The effects of the enzymatic purification on all polymers, even on the sensitive polymers like PA, PET, PC, PUR, and PS, were negligible, while these polymers were strongly affected by other treatments with acids or bases (own unpublished data). We are aware that synthetic fibers with their higher surface area to volume ratio, very small aged, and oxidized particles or paint particles are probably more sensitive to the enzymatic purification approach with the two hydrogen peroxide steps than the virgin polymer films tested. However, the fact that we regularly find such MPs in the environmental samples that were processed with the enzymatic purification protocol suggests that the influence on these types of MP is also negligible.

Concerning the efficiency of the enzymatic purification approaches, it was previously reported in Courteney-Jones et al.³¹ that the digestion of mussel tissues with trypsin reached a purification efficiency of 88%. In contrast, Catarino et al.³⁰ reported that mussel tissues could obviously be 100% digested using protease, which is similar to the digestion achieved using acidic or alkaline treatments. Cole et al.²³ reported that an enzymatic treatment with proteinase-K reached a higher purification efficiency for biota-rich plankton samples than using acid or alkaline treatments. The efficiency of our BEPP (98.3%) is similar to the values reported by Cole et al.²³ for proteinase-K (>97%) used for the purification of plankton samples. However, the BEPP requires an incubation time of up to 16 days instead of hours. While the BEPP requires a long duration, the actual handling time to filtrate and add new solutions is of course much shorter (in total around 3–4 h per sample), and many samples can be processed in parallel, which relativizes the time requirement. In contrast, our protocol does not require – besides filtration – any pretreatment steps, such

as drying and grinding the sample as reported in Cole et al.,²³ which bears the risk of fragmenting the larger and brittle MPs into smaller pieces and thus potentially biasing the results.

A further advantage of our approach is the use of technical grade enzymes that are comparably inexpensive. Concerning the costs, a direct comparison with the other studies that used an enzymatic purification is difficult, as we used a series of enzymes and the other studies only one. However, the technical grade protease we used, for example, is by a factor of around 20,000 cheaper than e.g. proteinase-K. Furthermore, the use of a modular approach with different specialized enzymes allows for the digestion of different matrices regardless of the sample type. Our approach was additionally combined with a final density separation step with ZnCl_2 , which proved to be important for the efficient elimination of the remaining inorganic residues. To reduce costs, the ZnCl_2 can be recycled by filtration. The total costs for the purification of one sample with the BEPP including all chemicals and enzymes lie in the single-digit euro range.

Although we were able to show that the BEPP is a very effective approach for the purification of MPs in environmental samples, the multiple filtration and rinsing steps involved in the purification protocol pose the risk of losses of MPs during processing. We thus estimated the potential equipment-specific loss of MPs during the purification process without the additional effect of an environmental sample matrix. We therefore purposely chose Milli-Q water as matrix and PE beads as reference MPs. We are aware that every different sample matrix will potentially have its own intrinsic recovery rate, which is potentially different for different polymers and size classes of MPs. Nevertheless, the determination of the equipment-specific recovery rate with Milli-Q as sample matrix revealed that only minor particle losses can occur during the entire purification process, thus allowing realistic quantification results. Careful rinsing during the sample processing can further mitigate the potential losses of MPs.

A series of environmental samples from the German Bight, in which a high amount of water was filtered (12.7–61.2 m^3) – resulting in a high load of organic matrix – were processed successfully with the BEPP and showed different numbers of MPs ranging from 0.05 to 4.42 items m^{-3} . To compare these values to numbers reported in other studies, it has to be mentioned that a mesh size of 100 μm was used. However, the determined numbers were comparable to the results from studies performed in the Northern Atlantic (mesh size 250 μm , 0–22.5 items m^{-3}),³⁵ the English Channel (mesh size 200 and

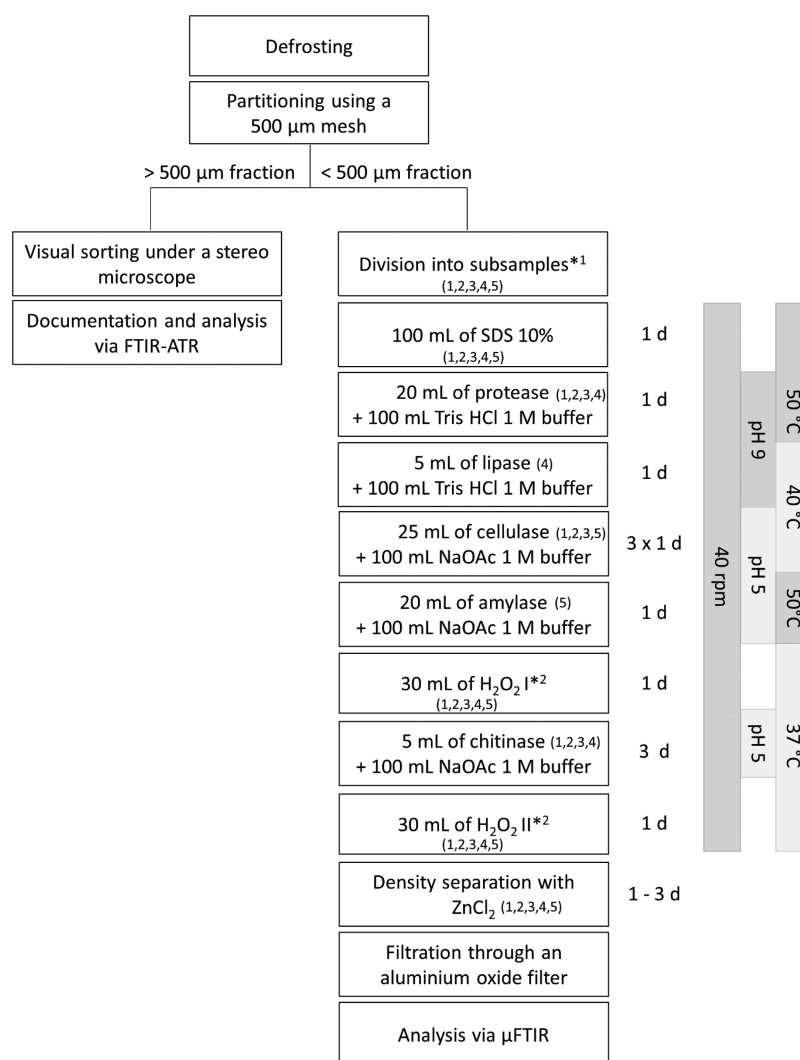


Figure 5. Universal enzymatic purification protocol. The optimized protocol (detailed description of the modifications in the SI) is suitable for purifying MPs from a wide range of different environmental matrices including plankton, extracted sediment, and biota. The incubation times represent the minimum values. The numbers represent the types of samples that are suggested to be purified with the respective purification step: 1 – plankton samples, 2 – extracted sediment samples, 3 – wastewater samples, 4 – lipid-rich biota samples (e.g., mussels, fish gut content, etc.) and other lipid-rich samples, 5 – samples with a high polysaccharide content, e.g. food samples, samples with high loads of plant material or algae. ^{*1} Depending on the amount of matrix present, the samples can be divided before purification; depending on the amount of residue present, they can be reunified for analysis after purification. ^{*2} The hydrogen peroxide steps can be replaced with wet peroxide oxidation, as described below.

500 μm , 0.26–0.31 items m^{-3}),²³ the Atlantic Ocean (mesh size 250 μm , 0–8.5 items m^{-3}),³⁶ and the Portuguese coast (mesh size 180–335 μm , 0–0.04 items m^{-3}),³⁷ in which the particles were also reliably identified using FTIR or Raman microspectroscopy.

Universal Enzymatic Purification Protocol (UEPP).

Although the BEPP was originally developed for seawater surface samples,¹⁴ in modified versions, it can be used to purify other environmental sample matrices, including freshwater plankton samples,³⁴ extracted sediment samples, wastewater samples,³² tissue samples of mussels, daphnia, and fish organs,³⁸ and commercial fish food (partly unpublished data). The general procedure of filtration and incubation as described above remains the same; however, slight adjustments in the BEPP were necessary while considering the chemical composition of each sample matrix.

The final changes in the BEPP that resulted in the UEPP (Figure 5) are described here briefly, whereas a detailed description of the methods and results from the experiments

that led to these adjustments is available in the SI (Optimization procedures). Experiments regarding the application of the enzymes were conducted in cooperation with the manufacturer of the enzymes (ASA Spezialenzyme GmbH).

Important improvements that led to the UEPP are suggested here as follows: (1) larger incubation bottles were used, (2) an optional subdivision of the samples was conducted prior to the purification for cases with high loads of the sample matrix, (3) the SDS concentration was increased to 10% (w/w), (4) the used buffers were replaced with tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 9) and sodium acetate buffer (pH 5), (5) two optional steps (lipase and amylase) for samples with a high content of lipids or polysaccharides were added (e.g., food, biota samples, and water samples with a high organic plant or algae content; lipase is applied after the protease step, to account for lipids released during the digestion of e.g. tissue; amylase is applied after the cellulase step to further digest degradation products of the previous step), (6) the incubation conditions were changed to improve the

efficiency of the enzymatic purification (higher turnover/less time required), and (7) an option to replace both hydrogen peroxide steps with a wet peroxide oxidation protocol was added.

Generally, the modified version – the UEPP – incorporates all the above-mentioned advantages of the BEPP. However, improvements made by experience facilitate now the purification of a broader range of environmental sample matrices and their final concentration through filters for a subsequent reliable analyses via FPA-based micro-FTIR spectroscopy. The UEPP has thus a great potential to be implemented as a standard operation protocol for purifying samples during routine monitoring studies on MPs. Nevertheless, the samples that are purified with the UEPP should be examined carefully for their matrix composition. The necessary steps strongly depend on this composition, and the relevant steps for a sample type given in Figure 5 are just a suggestion, e.g. if a sample does not contain heavy material like sand a density separation with zinc chloride is not necessary.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.est.7b03055](https://doi.org/10.1021/acs.est.7b03055)

Sample stations of RV Heincke cruise “HE409” and optimization procedures for the universal enzymatic purification protocol (UEPP) (PDF)

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Notes

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