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Analysis of Microplastics in Industrial Processes—Systematic Analysis of Digestion Efficiency of Samples from Forestry, Wastewater Treatment Plants and Biogas Industries

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Abstract: Microplastics (MPs) are persistent, globally relevant pollutants that have thus far been rigorously studied in natural waters but have not been as extensively studied in industrial wastewaters. Samples were collected from the forestry industry, wastewater treatment plants and the biogas industry. An enzymatic treatment protocol for MPs' detection was applied to an assortment of industrial samples ranging from wastewaters, effluents and condensates to sludges and digestates. The effects of selected enzymes were studied systematically to develop a basis for digestion protocols on industrial samples. Further, different methods of detection (micro FTIR and Raman) were compared to each other, and the samples were visually examined using SEM. The developed protocols in this study were then compared with blank samples, contamination controls and samples spiked with artificial microplastics. This research aimed to fill some of the gap in the knowledge regarding the analysis methods and especially in the type of samples screened for microplastics thus far and presents a systematic approach to MPs' detection in industrial wastewaters. It highlights the issues with the used analytical methods (such as misidentification) and validates the analysis results with milled, random shape and wide-size-range reference MPs that represent real samples better than standardized, ideal round beads. This study provides the first-ever suggestion for an enzymatic digestion protocol for industrial sample analysis.

Keywords: industrial wastewater; enzymatic digestion; pretreatment protocol; industrial effluent; microplastics

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1. Introduction

Plastic particles of the sizes 1–1000 µm [1] called microplastics (MPs) have been widely studied in environmental samples [2–8] and in municipal wastewater treatment plants [9–12]. For these purposes, several methods have been developed and adapted for MPs' detection, such as Fourier-transform infrared spectroscopy (FTIR) [13–16], Raman spectroscopy [17–20] and pyrolysis gas chromatography [21–23]. Additionally, pretreatment by applying enzymatic digestion protocols [24–26] has been developed for the treatment of environmental samples. However, the study of MPs in industrial processes has much more scarce literature thereon. While they have been analyzed on some occasions [27–31] and on both liquid and sludge samples [32,33], these varied studies do not have unified sample treatment protocols developed and do not make use of enzymatic digestion in the way presented in this study. The information on this topic is fragmented and measured using different techniques. Industrial facilities, as a potential source of microplastics [34–36] require more attention for the proper assessment of the industrial impact on MP pollution, and also to test the existing methods on industrial wastewaters and to conceive future laws and regulations for this area of pollution. A correlation between urban industrialization and MP quantity has been observed, which makes MPs' detection

in industrial process waters particularly important [37,38]. This is additionally relevant due to the upcoming EU regulations that industries will need to follow [39].

The analytical methods for MPs' detection continue to be challenging for various types of samples [40,41]. The existing protocols for environmental samples [24] could hypothetically serve as a base to analyze industrial samples as well. In addition, in regard to specifically sludge samples, the treatment and analysis process are equally difficult [42] and ununified [33,43]. Most previous articles on industrial samples [27,28] did not analyze both sludge and liquid samples alike, thus further highlighting the need for a unified protocol for industrial purposes. A unified protocol would also ensure easily comparable results in future research and consistency in the methodology. Expanding this research to other industrial areas beyond this study would be supported by such a unified protocol.

Furthermore, there is no method comparison nor method validation studies with MP particles that more closely represent a real environment than standard-sized plastic pellets. This research is of great interest to the industrial sector as well. In this study, for the biogas industry, MPs in the digestates after treating sewage sludge are an important quality issue, as this digestate could be used as a fertilizer in the future. For all industries, it is important to know whether certain water treatment processes remove MPs from water or not.

The objective of this study was to analyse the amounts of microplastics in diverse samples of industrial wastewaters, develop a basic protocol for sample preparation and validate the used methods with cryogenically milled MPs that more closely represent a real environment [44]. Further, it aimed to study the uncertainty of MP measurements and provide a blueprint for future research that will be necessary to help the industrial sector satisfy the upcoming EU law on plastic pollution. Different enzymes were tested, and different analytical methods were compared with each other in terms of efficient and accurate MPs' detection in industrial samples.

2. Materials and Methods

2.1. Industrial Sample Collection

Samples from industrial processes in Finland (forestry industry, biogas industry and a municipal wastewater treatment plant) were all obtained in 1 L sealed glass bottles, pre-washed with ultrapure water. The samples included influents and effluents from processes in the forestry industry, filtrates, digestates, influents and effluents taken from a wastewater treatment plant, and influents, digestates, condensates and sludges taken from processes in the biogas industry. Additionally, several control samples from various points in the industrial facilities were collected to account for air contamination and any contamination from handling the bottles during the sampling process. Each sampling location consisted of either one bottle (sludges and digestates with a high amount of sediment) or ten bottles (clear effluent samples, influents, filtrates and condensates). The reason for this difference is that sludge samples can only be processed in small amounts, and one bottle was enough for the experiments. This is due to the much higher solid content, which easily clogs filters and consumes more chemicals during digestion. The selection of samples included opaque and transparent samples with varying degrees of sediment, and further, they represent various matrices and processing steps that are relevant for the industry.

The bottles were filled by either dipping them directly into the process water or filled via a pressurized sampling pipe. The samples were obtained from the three industries with the aim to investigate the efficiency of the enzymatic digestion steps in these different sets of samples. The bottles were sealed immediately after sampling and kept at 4 °C in a refrigerated room until use. Processed samples (after enzymatic digestion) were kept in the same refrigerated room until analysis. All the experiments were conducted in a fume hood with basic laboratory safety equipment (gloves, glasses and lab coat). Field blank samples were collected by opening the bottles at the sampling sites for 30 s, and they were not dipped into the sampling site. Blanks were prepared in the lab to verify the absence of contamination with any plastics in the enzymatic treatment process as well (for example from filters, chemical bottles, lab coat fibres, poorly washed equipment, etc.).

Contamination from the sampling pipes was tested by sampling other liquids (wastewater) that flow through pipes made of the same material to account for the contribution of the piping system to the MPs' content of the samples.

2.2. Digestion Procedure

The obtained liquid (non-sludge) samples were filtered through a 20 µm steel filter (G. BOPP + CO. AG, Zurich, Switzerland). These filters were used for the entire digestion process. For each sample, 1–3 L of the sample was filtered, depending on the amount of sediment to test the enzyme efficiency. Initially, subsampling was performed by splitting the same bottle into three samples of 2 × 350 mL and 1 × 300 mL. Once the method was further optimized, the bottles were filtered in their entirety. These filtered samples and sludges/digestates were subjected to an enzymatic digestion process based on the protocol reported by Löder et al. [24]. The protocol was modified to adapt to the different matrices of industrial samples (see Figure 1). In detail, firstly, 30 mL of 35% H_2O_2 (Merck, Rahway, NJ, USA) was added to the sample and shaken at 50 °C and 100 RPM for 2 days. Then, the hydrogen peroxide was filtered away and 5 mL of protease (Protease A-01, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) was combined with 25 mL of pH 9 phosphate saline buffer (pH 7.4, Sigma-Aldrich, St. Louis, MI, USA), added to the sample and left at 100 RPM and 50 °C for 2 days again. If further enzymes were necessary, after filtering out the protease solution, 10 mL of cellulase (Cellulase TXL, ASA Spezialenzyme, Wolfenbüttel, Germany) in 25 mL of buffer was added and stirred at 100 RPM and 50 °C for 4 days. The cellulase solution was filtered and then 10 mL of Amylase (Amylase FL, ASA Spezialenzyme GmbH) in 25 mL of phosphate buffer was added and left stirring at 100 RPM and 37 °C for 3 days. Cellulase and amylase were tested at pH 4–7 in increments of 0.5 in phosphate buffer. The pH value of the phosphate buffer was regulated using drops of 1 M HCl (prepared from 37% HCl supplied by VWR Chemicals, Radnor, PA, USA) and 1 M NaOH solutions (prepared from NaOH pellets supplied by Millipore Sigma, St. Louis, MI, USA). Multiple enzymes were not used at the same time. Before a new enzyme was added, the previous one was always filtered away using steel filters. If there was still plenty of solid material left after the enzymatic digestion steps, density separation using sodium polytungstate (ρ = 3 g/cm^3) was utilized to remove any heavy inorganics. Density separation was performed by letting the material settle in the sodium polytungstate solution for 2 days, removing the sediment and filtering the remaining solution through 20 µm steel filters.

The sludge samples were treated using the same method that was applied to the liquid samples, but with some adjustments. Firstly, the liquid fraction was filtered (through 20 µm steel filters) and analyzed separately. The solid fraction in the bottle was not filtered. Initially, only 1–5 mL of undried sludge could be digested due to high concentration of solid materials in the samples and the difficulties in the digestion of the sludge samples. However, larger volumes (up to ~150 mL) could be processed after further adapting and modifying the procedure. Initially, 5 mL of 35% H_2O_2 and 50 mL of ultrapure water were added to the sample and stirred at 100 RPM for 24 h at 50 °C. Then, 5 mL of 35% H_2O_2 was added and stirred under the same parameters for another 5 h. A final 5 mL of 35% H_2O_2 was added and left stirring under the same parameters overnight to complete the H_2O_2 treatment step. If the reaction to H_2O_2 was still violent, further H_2O_2 addition steps were added until there was no further reaction. This stepwise addition lowered the intensity of the reaction and prevented excessive foaming. It also eliminated the need for extra process steps, which minimized the possible losses. The rest of the procedure followed the previously described steps for the liquid samples. The entire process for each sample type is shown in Figure 1.

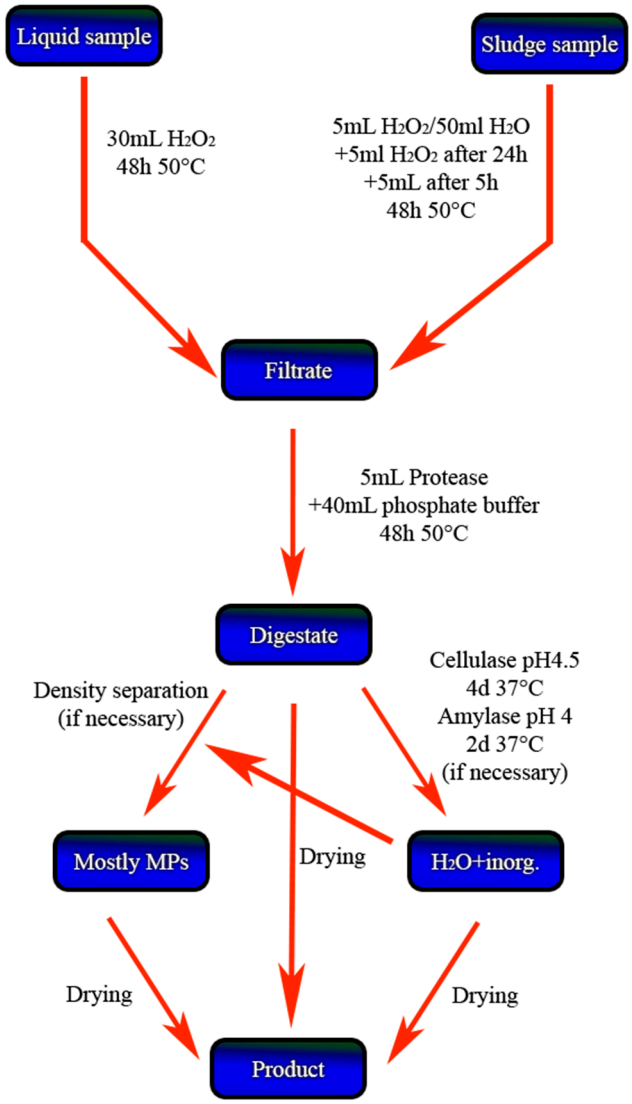


Figure 1. Industrial sample treatment process of the liquid and sludge-like samples.

2.3. Analytical Methods

The samples were analyzed using 2 methods, namely Fourier-transform infrared spectroscopy (micro FTIR) and Raman spectroscopy. For the purposes of micro FTIR analysis, the final samples ("Product" in Figure 1) were filtered through a 15 µm pore size silver filter (Starlitech Co, Auburn, WA, USA) and attached to a glass substrate. Micro FTIR analysis was conducted using an Agilent Cary 670/620 device, equipped with an optical microscope and a 128 × 128 FPA detector with a pixel size of 5.5 µm. A 12 mm diameter area of the filter was scanned in the reflection mode with a 15× objective, in a 3800–800 cm^{-1} spectral range and under an 8 cm^{-1} spectral resolution. The results were obtained using SiMPle (v.1.0.0), analysis software developed by Primpke et al. [45], using reference spectra from common and in-house libraries for the grinded polymers [2]. The software works through Pearson correlation coefficients between a sample and reference spectra. The threshold for particle recognition was adjusted to detect the most particles possible with correct identification. SiMPle provides information regarding the particle number, sizes and types. The particle size was limited by the steel filter pore size (15 µm).

The Raman spectra of the particles were measured using a Raman imaging microscope (Thermo Scientific DXR2xi, Waltham, MA, USA) and applying the particle selection as described in Tsering et al. [46]. The samples were prepared by filtering the final digested sample through a 5 micron gold filter (or anodisc filter for the validation samples) and

placing the filter on a glass substrate. The laser wavelength was 785 nm, the grating had 400 lines/mm, the resolution was 5 cm^{-1}, the spectral range was 50–3300 cm^{-1}, the aperture was a 50 µm confocal pinhole, the objective was 10×/0.25 NA, the exposure time was 0.14 s and the number of scans was 50.

Scanning electron microscope (SEM) images were obtained using a Hitachi S-4800 SEM. The working distance was 8 mm. The voltage and current were altered based on the sample to obtain better-quality pictures. A voltage of 2 kV and current of 2 µA were used for the industrial samples and a voltage of 5 kV and current of 2 µA were used on the grinded MP reference samples. The samples were prepared for SEM by rolling a needle tip over a filter with a sample already analyzed by using micro FTIR and transferring some of the particles from the filter onto a double-sided carbon tape attached to a sample holder.

2.4. Method Validation

The two analytical methods were validated using ultrapure water samples spiked with in-lab artificially prepared microplastics [44] in known quantities (10–20 MPs per sample and for each polymer). The synthetic MPs used (PP, PE, PVC, PS, PA and PET) were in a size range of from 20–200 µm. The recovery rates were defined after subjecting the spiked samples to the same enzymatic digestion protocol as the industrial samples. The reason why the samples were spiked with 10–20 MPs of each polymer type was in order to be consistent with real samples' order of magnitude of the detected number of MPs. The MPs were added and counted under an optical microscope. The average recovered particle from these experiments was 50–60 µm in diameter. Additionally, blank samples were prepared with only ultrapure water and without added MPs to account for lab contamination during the process. The process of MPs' preparation can be found in [44].

A method comparison was conducted by studying the samples from the same bottle subjected to the same enzymatic treatment protocol (H_2O_2+protease) and at the final step filtered either on a silver, gold or anodisc filter. The same sample was then analyzed using different methods (micro FTIR and Raman), if possible. An interlaboratory comparison was included, as the Raman measurements using anodisc filter were performed with the DXR3xi Raman device at Lappeenranta University (LUT) using the same settings [44].

The Hitachi S-4800 SEM microscope was used to compare the MPs in the industrial samples with the reference milled MPs used in the study.

2.5. Contamination Control

Contamination was monitored through the laboratory blank samples and field blank samples collected during the sampling at the industrial facilities. The laboratory blank samples were produced by subjecting 20 mL of ultrapure water to the enzymatic treatment scheme. Both types of blanks were clear of significant contamination (0–3 polypropylene particles per sample). All the equipment was washed with ultrapure water before use, all the experiments were performed inside a fume hood and all the samples, beakers and bottles were covered with aluminium foil when not in use. Before their use, all the reagents were filtered through a separate 20 µm steel filter, which was the same kind of filter as those used in the digestion procedure. All the equipment and samples were handled using laboratory gloves.

2.6. Classification of MPs

The plastics in both the real and control samples were characterized by polymer type, size and shape. The type refers to the specific plastic, such as PP, PS, PET, etc. The size of the microplastics was divided into 5 categories: 18–30 µm, 30–50 µm, 50–80 µm, 80–100 µm and >100 µm. Due to the steel filter size (20 µm) used during the digestion, the smallest particle detected in these categories was 18 µm. The sizes were determined by their major dimension (the largest possible diameter of the particle). The shapes were separated into fragments and fibres. A particle was considered a fibre if its major dimension was at least five times larger than its minor dimension. This method was used in previous research [47].

The minor dimension in the SiMPle software was determined by treating the particle as an ellipse and then calculating the minor dimension based on the surface area and major dimension of the particle. If a particle was not a fibre, it was classified as a fragment.

3. Results

3.1. Liquid Sample Results

Table 1 shows the digestion treatments and the number of MPs per litre in the analyzed industrial water samples. An important finding in the data analysis was that the protease digestion in all the cases across all the sampling locations significantly increased the number of detected MPs. However, no additional particles were observed in the blank samples, and the same effect was not observed on the spiked samples, which indicated that the protease itself was not a source of contamination, but recovered more particles in the real samples. The samples were more clear of non-plastics and the MPs were more efficiently detected by using the micro FTIR and Raman. In this case, the filters contained almost exclusively microplastics. The protease digestion was therefore kept as a constant in the digestion scheme. Cellulase and amylase did not have such significant effects on the samples. The samples treated with only cellulase or amylase were not much clearer, and the same or lower number of MPs were detected by using the micro FTIR.

Table 1. Samples with their pre-treatment methods, detected particles, ranges, polymer types and average particle size. PROT = protease, AMY = amylase and CEL = cellulase. The size range refers to the total sum of detected particles, not split into groups. The analyzed volume of all samples in the table was 1 L. All samples were measured using micro FTIR.

	Sample	Pretreatment Method	No. of Particles/L	Size Range (μm)	Polymer Type	Average Size (μm)
Location 1: effluent						
	1	H_2O_2	4	69–165	PP, PET	119
	2	H_2O_2+PROT	8	25–256	PP	111
Location 2: process wastewater						
	3	H_2O_2	8	28–207	PP	123
	4	H_2O_2+PROT	27	17.8–244	PA, PET, PE, PP	73
	5	H_2O_2+CEL	2	125–288	PE	206
	6	H_2O_2+AMY	1	71	PP	71
	7	H_2O_2+PROT+CEL	10	18–70	PP, PE	43
	8	H_2O_2+PROT+CEL+AMY	8	35–150	PP, PVC	69
Location 3: process wastewater						
	9	H_2O_2	16	37–223	PP	83
	10	H_2O_2	1	34	PET	34
	11	H_2O_2+PROT	28	41–461	PET, PP	148
	12	H_2O_2+PROT	9	30–201	PP, PS, PE	116
	13	H_2O_2+PROT	17	38–307	PE, PP	102
Location 4: condensate						
	14	H_2O_2	13	33–154	PA, PET, PE, PS, PP	81
	15	H_2O_2	9	44–111	PET, PP	73
	16	H_2O_2+PROT	16	30–704	PA, PET, PE, PP	151
	17	H_2O_2+PROT	22	28–360	PET, PE, PS, PP	132

Figure 2 shows the MPs' characterization results for the liquid samples. PP appears to be the vast majority of the detected particles in all the cases. In terms of size, 49% of the particles were larger than 100 μm. However, the smallest particles (<30 μm) are also the most likely to be lost during the digestion process, due to, for example, becoming stuck inside the pores of the filter due to having only a slightly larger size than the pores.

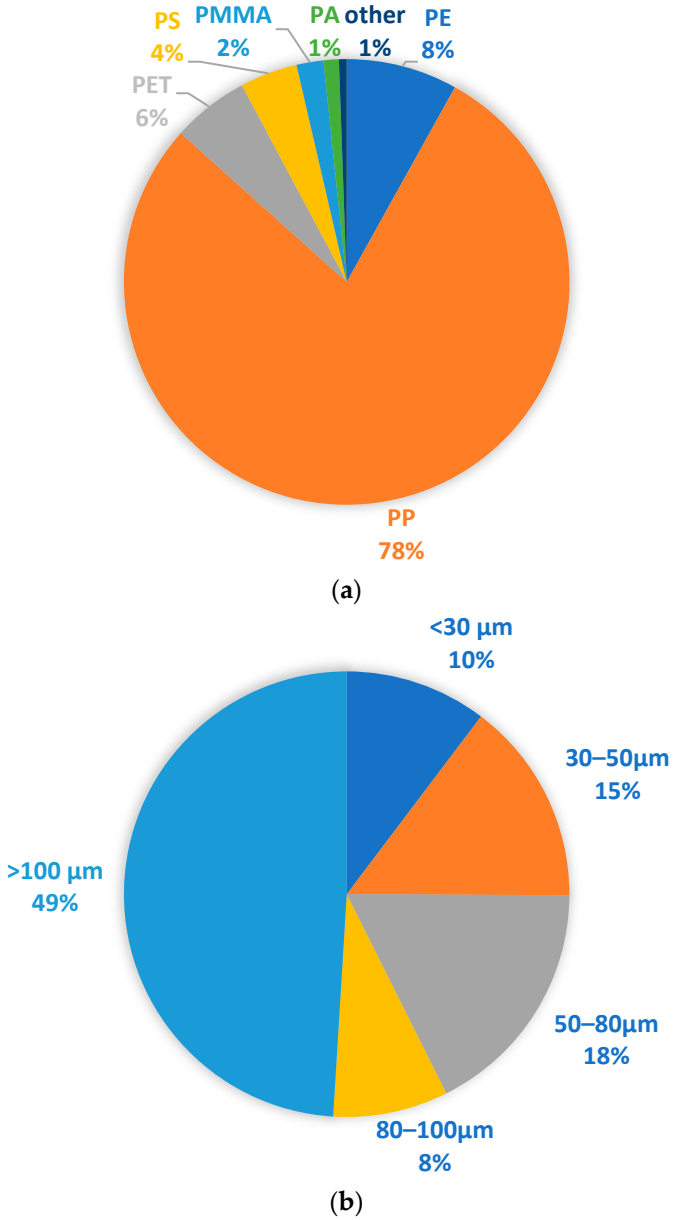


Figure 2. (**a**) Plastic types and (**b**) sizes of the detected plastic particles. The figure presents the size range section in Table 1 split by size category as outlined in Section 2.6.

When treating the samples with cellulase and amylase in the enzymatic treatment process, in both the liquid and some sludge samples, white solid precipitations were observed that could not be analyzed. In the case of amylase, the digestion at pH 4–4.5 and the digestion for 3 days at 37 °C was successful in preventing white precipitate formations. Similarly, cellulase worked at pH 4.5–5 and 4-day digestion at 37 °C. Under these parameters, cellulase was effective in the treatment of some of the sludge samples. Outside of these parameters, precipitations would form that would make the samples impossible to analyze. The above-mentioned parameters should be used if a sample requires digestion with those specific enzymes. In this study, amylase was not necessary for the collected samples. Cellulase was required for some of the sludge samples, but not for the liquid samples.

3.2. Sludge Samples

The results for the sludge sample digestions are given in Table 2. As can be observed, the sludges and samples with a higher solid content required more processing than the

clearer, liquid samples. Similarly to the liquid samples, Figure 3 displays the results regarding the plastic types and sizes observed from the sludge samples. In the sludges, the plastic types (Figure 3a) show a more even spread of particles between PE, PP and PS. These polymer types for both the liquid and sludge samples could be influenced by the materials used in the industrial pipes or reactors. To test this theory, a pipe test sample (water running through pipes made from the same material) was taken from the wastewater treatment plant. The results of the contamination tests are shown in Table 3.

Table 2. Digestion protocols and MPs' content analysis results for the sludge samples. N/A denotes samples that had too much solid material on the filter to be analyzed, i.e., the employed pre-treatment method was not sufficient to clear up the sample enough for analysis. Sample volumes varied depending on how quickly the filter became full. Generally, liquid samples were analyzed in ~1 L amounts, and solid samples were in the range of 2.5–100 mL. Liquid refers to the liquid in sludge samples above the sediment while solid refers to the wet sediment only. All data were obtained by using micro FTIR.

	Sample	Pretreatment Method	No. of Particles/L	Size Range (µm)	Polymer Type	Average Size (µm)
Location 1 (Digestate)						
Solid	1	H_2O_2	4400 (11 *)	23–225	PET, PS, PP, PE	71
Solid	2	H_2O_2+PROT	5333 (16 *)	30–291	PET, PS, PP, PE	99
Solid	3	H_2O_2+PROT+CEL	6333 (19 *)	35–281	PET, PS, PP, PE	117
Solid	4	H_2O_2+PROT+CEL	3194 (23 *)	18–209	PET, PS, PP, PE	93
Location 2: (Reject water)						
Solid	5	H_2O_2	N/A	/	/	/
Solid	6	H_2O_2+PROT	N/A	/	/	/
Solid	7	H_2O_2+PROT+CEL	730 (40 *)	30–235	PET, PS, PP, PE	81
Location 3: (Wastewater sludge)						
Solid	8	H_2O_2	N/A	/	/	/
Solid	9	H_2O_2+PROT	N/A	/	/	/
Solid	10	H_2O_2+PROT+CEL	500 (5 *)	48–241	PE, PP, PMMA	128
Solid	11	H_2O_2+PROT+CEL	700 (7 *)	18–83	PET, PE, PP	69
Solid	12	H_2O_2+PROT+CEL	400 (4 *)	30–135	PP	79
Liquid	13	H_2O_2	N/A	/	/	/
Liquid	14	H_2O_2+PROT	7	54–314	PET, PE, PP	152
Liquid	15	H_2O_2+PROT+CEL	10	33–445	PE, PS, PP	180
Location 4: (Process influent water)						
Solid	16	H_2O_2+PROT	230 (23 *)	40–280	PET, PP	145
Liquid	17	H_2O_2+PROT	1	175	PP	175

*. . . the number in brackets refers to the actual number of particles detected in a low-volume sample. The larger number next to the brackets refers to the particles per L of starting sludge sample, calculated by dividing the detected number of particles by the analyzed volume in L.

Table 3. Results of the contamination tests and inside-process tests.

Sample	No. of Particles	Size Range (µm)	Polymer Type	Shapes
Pipe test	19	41–335	4 PS, 11PP, 4PE	1 fibre, 18 fragments
Filtration 1	40	16–1137	3PET, 1PS, 28PP, 8PE	5 fibres, 35 fragments
Filtration 2	20	17–2113	1PA, 4 PE, 1 PS, 14PP	4 fibres, 15 fragments
Blanks	0	/	/	/

As can be observed from Table 3, the pipes themselves could carry some contamination, although they are made from PVC and as explored in our previous work, PVC is difficult to detect by using these methods [44]. The results of the tests inside the filtration processes can be used for a comparison of the MP content between the influents and effluents, but it is also important to notice any unusual new particle patterns that may appear as contamination from the process itself. There was no discernible pattern that would indicate significant contamination from these processes. This would mean that the results of the

sample measurements were not significantly affected by the process equipment. Different polymers were observed in the sludge compared to the liquid samples, which might be related to the polymer density. In terms of size, interestingly, the sludge samples contained, on average, smaller MP particles than the liquid samples, which is different from what is reported in the literature; for example, comparing the results from [27,32], generally larger particles would be expected in sludge. However, the sludge samples contained more undigested solid material than the liquid samples, and particles could have still been covered with non-plastic matter in some cases. Through manual corrections, these errors were mitigated as much as possible. In future studies, a simulation of sludge, spiked with artificial microplastics could be used as a process test to further analyze these results.

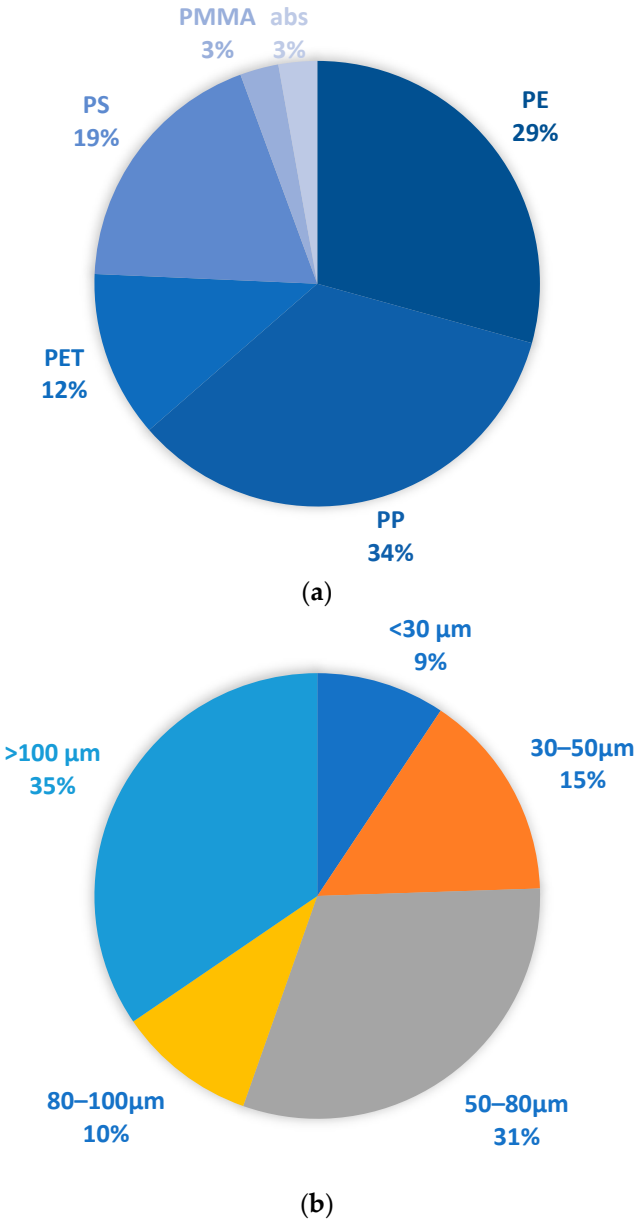


Figure 3. (**a**) MP types and (**b**) size distribution in sludge samples.

The spiked sample controls for the enzymatic digestion procedure had a 61% recovery rate, on average, by using the imaging FTIR, without manual corrections, and 76% with manual corrections, which is good, considering the amount of process steps and possible losses. In previous work [2], a 75% recovery rate was observed in a process with a similar number of process steps with standardized beads. Manual corrections entailed performing

SiMPle analysis with adjusted parameters (such as a decreased correlation coefficient for PVC) or correcting the issues where one particle was identified as several or vice versa. In the spiked samples, the agglomeration of particles was observed, as well as later in the industrial samples, so additional human corrections were needed after the SiMPle software returned its calculations. The particularly poor detection of thin fibres and PVC was observed, as is also stated in our previous work [43]. Particles of all shapes, except thin fibres, were recovered successfully. The smallest particles recovered were in the size range of 20 µm, as expected from the steel pore filter used. The misidentification of particles in the spiked samples was somewhat common, meaning that a polymer particle was detected as the wrong polymer. This also required manual corrections and checks.

The shape of the MPs in the sludge samples differed from that in the liquid samples only slightly; however, while the MP fibre content in the sludge samples was low, several other fibres of a non-plastic origin could be observed. A comparison of the shape distribution of MPs across the different samples is shown in Figure 4. It seems that MP fibres were present in 5–10% of the detected particles. Currently, determining the fibre content depends highly on manual corrections and revisions of the SiMPle results to ensure their accuracy, as outlined in our previous work [44]. If a particle is partially covered by non-plastic material, or a long fibre is bent upwards out of focus, the software detects it as two or more particles, and manual corrections such as overlaying the microscope image with the MP map from SiMPle (shown in Appendix C, Figure A2) are needed to account for such errors.

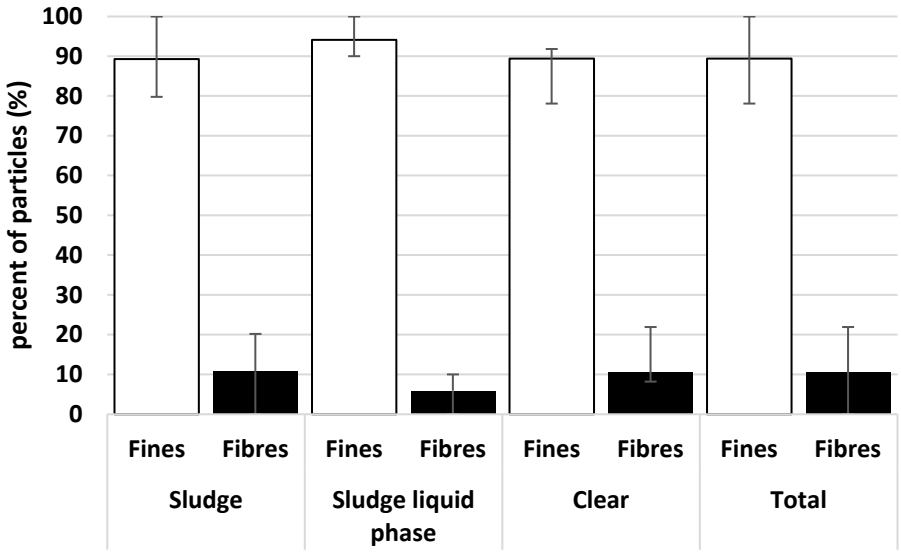
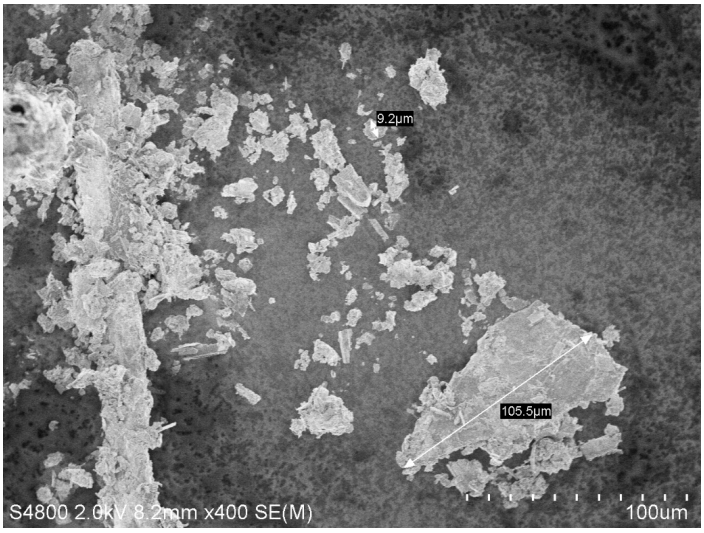
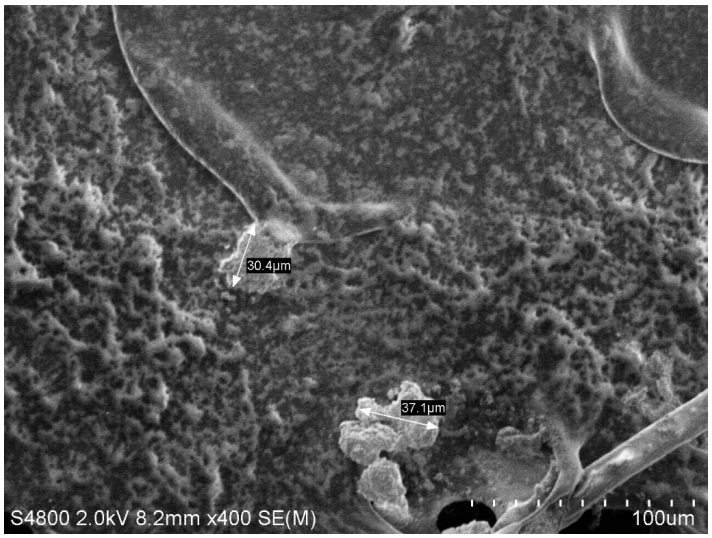


Figure 4. Shape distribution across all the samples.

The MP particles found in the industrial samples were examined using SEM. A sample was first scanned using micro FTIR; then, a needle was used to transfer some particles from the filter to a piece of double-sided carbon tape for SEM measurements. The surface characteristics and morphology of the particles were studied in order to reveal any changes in the particles caused by the industrial processes or the enzymatic treatments. The results were compared to the milled reference MPs. Figure 5 shows a comparison of these SEM images between the industrial samples and reference particles. The MP particles, as well as non-MP content of the industrial samples is within the same size range as the milled reference MPs (20–250 µm). The shapes are also very irregular in both the industrial and reference samples and are similar to each other.



(**a**)



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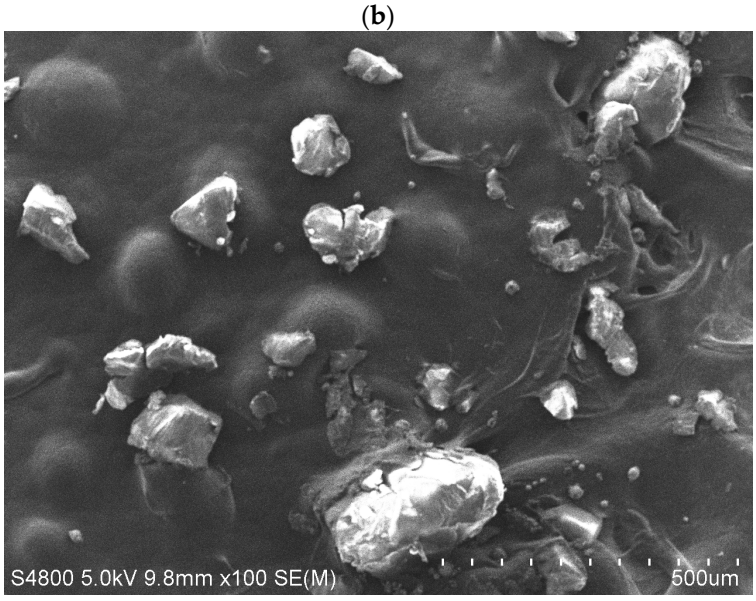

(**c**)

Figure 5. SEM images of industrial samples, where (**a**) represents sludge samples, (**b**) represents liquid samples and (**c**) represents reference grinded PET particles.

3.3. Method Comparison

A final test was conducted for the purpose of analytical method comparison. A liquid industrial sample and the most effective enzymatic treatment was applied in the test to reveal the differences between the analytical methods. Two 350 mL samples and one 300 mL liquid sample from the same bottle were subjected to the enzymatic treatment protocol (H_2O_2+protease) and at the final step were filtered on a silver, gold or anodisc filter. The same sample was then analyzed using different methods (micro FTIR and Raman). The results are given in Table 4. Only the samples on the gold filters could be analyzed using both methods, due to the silver filter being unsuitable for Raman (due to the scattering effects with the silver filter) and the anodisc filter being unsuitable for the micro FTIR.

Table 4. Method comparison tests between three filters (silver, gold, anodisc) and two methods (micro FTIR, Raman). The top three samples are industrial samples, and bottom three examples are sets of recovery rate samples (explored in more detail in [44]). Results of several tests are compiled together in the table for conciseness.

Sample (Reject Water)	Filter	No. of MPs/L (Micro FTIR)	No. of MPs/L (Raman)	Polymer Type	Average Size (µm)	Size Range (µm)
1	Silver	40	/	PET, PS, PE, PP	81	30–235
2	Gold	33	25	PET, PE, PS, PP	78	18–335
3	Anodisc	/	15	PE, PP	/	/
Sample sets (Spiked MPs samples	Filter	% of MPs recovered (micro FTIR)	% of MPs recovered (Raman)	Polymer type	Average size	Size Range
1	Silver	76	/	PP, PE, PET, PS, PA, rec. PP, rec. PE, PVC	101	17–342
2	Gold	70	49	PP, PE, PET, PS, PA, rec. PP, rec. PE, PVC	92	17–220
3	Anodisc	/	32	PP, PE, PET, PS, PA, rec. PP, rec. PE, PVC	/	/

From the obtained results in Table 4, it would seem that for the industrial samples, the micro FTIR analysis on silver filters is the most suitable, since it detects more particles than Raman analysis (notable particularly when using the gold filters). Micro FTIR on silver filters also proved to be the most efficient method in the recovery rate tests. The validation of the used methods using artificial MPs is explored in more detail in [43]. In the wider context, these results would have to be repeated on more than two samples to reach more solid conclusions. This is because in previous similar studies on environmental samples, Raman was a more preferred option, also uncovering smaller particles than the micro FTIR [48]. However, a comparison with different filters has not been conducted. This perhaps highlights a need to study and compare the methods with the filter type variable in mind in the future.

4. Discussion

An enzymatic treatment protocol was developed over the course of this study. In this process, treating samples with protease in all the cases uncovered a larger number of MPs and a larger number of polymer types than in the samples without it. This could be due to protease dissolving the protein films that might envelop MPs in the industrial samples. The analysis methods used in this study are surface-sensitive, so if a film forms on the surface of the MPs, it could prevent the detection of the MP particles. One can assume that other films of biological origin could be formed in industrial processes to hinder MPs' analysis. A difference in particle size was not found, i.e., protease does not seem to degrade/break MPs into smaller particles. This further supports this study's finding that protease is a necessary and non-destructive step in the digestion process for samples possibly containing proteins, such as the industrial samples. While other studies [49–52] have used protease

for the treatment of samples and also noticed a positive effect of protease or enzymatic digestion [51,52], the effects of protease have not been assessed in this way or tested on industrially relevant sample sets before. A full comparison of previous studies can be found in Appendix A (Table A1).

In terms of enzymatic treatment efficiency, in most cases, protease treatment was enough for the liquid samples; only the sludge samples required an extra step of cellulase digestion. Thus, the enzymatic protocol developed in this study significantly reduces the process steps needed for enzymatic digestion compared to the previously employed procedures [24]. The sample processing is thereby faster and less loss of microplastics is expected, which increases the recovery rates and accuracy of the obtained results. Chitinase was not used in these experiments, despite being used on environmental samples [24], and the reason for this is that the sample matrices in this study did not contain chitin. However, it should be noted that various enzymes may be needed for samples with different matrices, which highlights the importance of studying samples from an even wider variety of industries in the future.

During the sample processing, the sludge samples presented many practical challenges compared to the clear liquid samples. The low sample amount (1–5 mL) was sufficient for analysis and method development, but the representativity of such samples is questionable. Even though multiple repeats of each sample were tested, and eventually larger sample volumes (>100 mL) could be tested, the combined amount of studied sludge might still not be representative of an industrial scale plant. In this study, sludge subsampling was conducted by vigorously stirring the sample bottle to homogenize the solid material and quickly pouring it into a beaker. The results from the analysis of the sludge samples in this research were very consistent when measuring small volumes, but when extrapolated to MPs per 1 L, these small measurement margins can become significant. Thus, larger, more representative volumes should be studied in the future. Measuring the volume of analyzed sludge was also problematic in the case of thicker sludges. They could not be pipetted, and the sludges stick to the surface of glass beakers, which made traditional pouring very inaccurate as well. The density of the various sludge samples was measured by weighing the sludge in a 100 mL beaker, and subsequently the volume of sludge was determined from the mass of added sludge instead. The subsampling methods, such as representative subsampling from the actual sample material, are not thoroughly described in the current literature, and there is currently no standardized procedure for the sampling and subsampling of sludges [53]; however, subsampling, for example, randomly selected circles in the filter area after the digestion process, has been employed [29]. Subsampling guidelines and methods should be given more attention in the future.

The prepared samples were analyzed using micro FTIR, Raman and SEM. The results of the size and shape analyses offer an insight into the contents of the industrial process waters. The most represented polymer type was PP, which was expected due to how commonly used it is. The lack of PVC detection seems like an inaccurate result, since PVC pipes are commonly in use, but this was an expected result due to the previously known difficulties with PVC detection. The PVC detection issue continues to be an important topic that needs to be addressed. The sizes of MPs were mostly in the higher size categories set in this study; however, this could be due to several reasons, such as the smallest particles becoming stuck in the filters during processing, the increased difficulty of detecting the smallest particles and smallest particles still being covered by non-plastic matter. From these results, the most common size and thus the biggest target size of particle in industrial process waters for future laws and regulations would be 50–100 µm. The SEM analysis of the various samples showed that the milled MPs accurately represent real samples from the industry, as well as shows the shapes and sizes of MP and non-MP particles found in the industrial samples. Those sizes and shapes are comparable, which is further supported by the findings in Tables 1 and 2. This further supports the notion that milled MPs should be used as references in future studies. In the SEM images, it seems that there may still be

a biofilm enveloping the particles, based on the surface morphology. This would further support the necessity of the protease digestion step used in this study.

An important issue detected during the analysis was misidentification. Misidentification was relatively common in the blank samples (containing artificial MPs) and was more common for some polymer types (PVC) than others (PP). It was also noticed that plastics' ease of identification under different measurement parameters varies, and thus, choosing parameters suitable for the detection of all MPs is challenging. Samples have to be tested under several different parameters to truly account for all the possible MP content in the various matrices. Raman spectroscopy was more efficient in identifying the correct polymer type than micro FTIR, but selecting the particles on the filters is more difficult, and agglomerates are harder to discern using Raman, which can lead to a loss of information. In the case of micro FTIR, the misidentifications related to the polymer type, which can be manually corrected in some cases. In those cases where the correct polymer type is unknown, the particle is still correctly identified as a plastic. Thus, FTIR would still be the more accurate method of detection overall, based on the results observed in this study. The blank samples ruled out laboratory contamination and did not indicate errors from the process. With the spiked samples, the correct polymers in both methods could be identified with high accuracy, especially after manual corrections to the analysis parameters used. The issue with this approach is that the contents of real samples are unknown, so it is not always possible to adjust the analysis parameters like that. Additionally, manual correction greatly extends the already long measurement times, which may sacrifice too much speed for accuracy especially in industrial monitoring or quality assurance.

The samples used in this study varied greatly between each other in regard to solid content, different matrices, different organic content, different colours and different levels of transparency. This diversity is exemplified also in Appendix B, Figure A1. While the samples may have come from only three industries, they presented a very diverse array of samples from within these industries. The results were consistent and provided information on the MPs' abundance in the industrial processes. Thus, it is hypothesized that these tested protocols and guidelines could be applicable to other industrial sectors as well with minimal or no changes. In the future, this hypothesis should be further explored.

5. Conclusions

An enzymatic digestion protocol for industrial processes, wastewaters and sludge samples was successfully tested for a wide array of different industrial samples. In this study, the role of protease seems to be the dissolving of protein films that form around the MPs, which made it a necessary step in the digestion process, while cellulase served to digest the cellulose fibres that may have covered the MPs and prevented detection (particularly in sludge samples), but amylase did not have a significant effect on the studied samples. A higher number of MPs was detected in the sludges than in the liquid samples, and the sludges contained, in general, smaller MP particles than the liquid samples did. Fibres represented only a small fraction of the detected particles in both cases. The differing matrices of the industrial samples remain a challenge and require adaptation to the specific sample origins and compositions. However, this protocol was applied to a wide assortment of different samples from various industries to give consistent, universal results, and it is hypothesized that it can be used as a base for future research. The authors encourage further exploration of this method in even more sample matrices. During the method validation studies, misidentification was observed, which highlights the potential gaps in knowledge in regard to the methods commonly applied today, as well as subsequently prompts further research into improving and perfecting these analysis methods used in the future. The tests on the different sample filters with micro FTIR and Raman uncovered the strengths and weaknesses of both methods, and based on the results obtained in this study, micro FTIR detection on silver filters was found to be the optimal detection method for this type of sample. However, this result should be further explored in the future. The impact of this study is that it provides important starting steps that will help industries adapt to

the upcoming regulations regarding MP pollution and give greater control over the release of MPs into the environment from the industrial sector.

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Appendix A

Table A1. Table of previous studies with highlighted novelty in this study compared to past studies. WWTP = wastewater treatment plant.

Study	Industries Studied and Type of Sample Studied (Liquid/Sludge)	Methods Used	Methods Validated	Enzymes Used	Enzyme Effects Studied Individually	Reference
Long et al.	WWTP, liquid	Raman	Yes, with a flowmeter and camera	none	no	[29]
Hidayaturrahman et al.	WWTP, liquid	Microscope	no	none	no	[30]
Xu et al.	WWTP, liquid	FTIR	no	none	no	[11]
Bitter et al.	Polymer processing, WWTP, liquid	DSC	no	none	no	[27]
Franco et al.	Food, furniture, marine construction, liquid	FTIR, microscope	no	none	no	[28]
Magalhaes et al.	Paint, resin, textile, pharmaceutical, PVC, liquid	FTIR, fluo-rescence microscopy	no	none	no	[31]
Catarino et al.	None, natural samples	FTIR	Yes, with standard sized MPs	Only protease	yes	[49]
Li et al.	Sewage treatment, sludge	FTIR	no	none	no	[53]
Cole et al.	None, biological samples	Microscope	no	Only protease	yes	[52]
Hrovat et al.	Forest, biogas, WWTP, liquid and sludge	FTIR and Raman	YES, with cryogenically milled MPs	Protease, cellulase, amylase	YES	This study

Appendix B

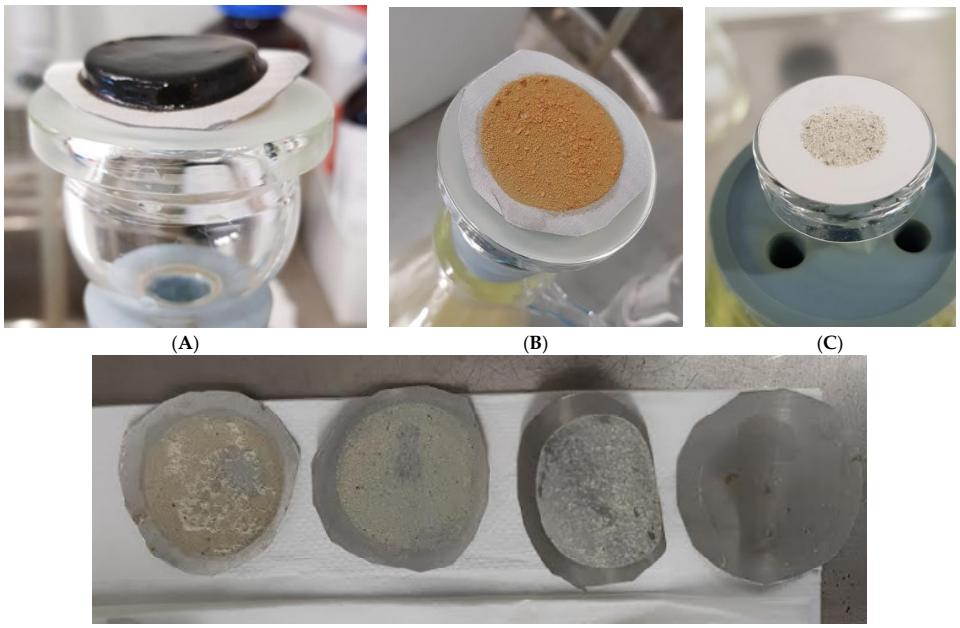

Figure A1. Pictures of various samples after filtration to show sample diversity. (**A**) is a sludge sample after initial filtration of 20 mL. (**B**) is a filtered influent sample of 1 L. (**C**) is a filtered influent sample of 1 L from a different process. (**D**) presents subsamples from one effluent sample that was treated with varying pH values for amylase in descending order from pH 6 (far left), 5.5, 5 and to 4.5 (far right).

Appendix C

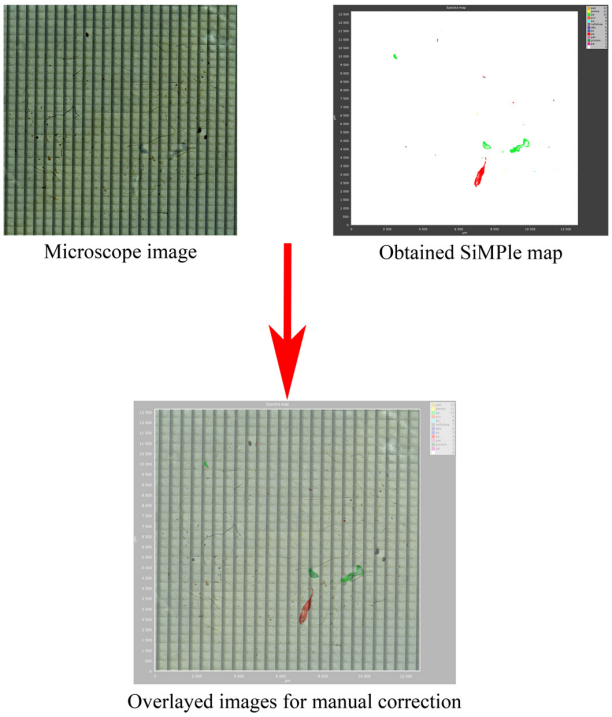

Figure A2. The process of overlaying the microscope image and the MP map obtained from SiMPle before performing manual corrections of the results.

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