Release of microplastics from plastic baby bottles

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1 Abstract

Currently, everything is made of plastic, even baby bottles. Plastic is a material with several advantages: it is cheap, easy to produce, and lightweight [2, 3]. However, plastics also have some disadvantages such as the release of micro- and nanoplastics. Every plastic product loses microplastics, even baby bottles [4]. These microplastics have a (not yet fully known) impact on human health [5].
In this study, we try to quantify the release of microplastics due to high temperatures during the sterilization process in baby bottles. To quantify the release of microplastics, the bottles are exposed to different sterilization times, after that the water from the bottles is filtered and the filter is analyzed. Next, the found particles can be quantified. As a last step, the found microplastics will be characterized using FT-IR, SEM, and EDX.

During all experiments, microplastics were released. The release of microplastics increases slightly with longer sterilization times. What is curious, is that the microplastic release was highest when the baby bottle was not sterilized at all. This can be due to the fact the bottle is not cleaned properly.
Among the characterization experiments, the FT-IR results were inconclusive, and more research is necessary. However, the SEM and EDX results look promising. The images of the particles made with the SEM look a lot like plastic particles and the EDX spectra show a significant presence of a lot of carbon. Suggesting that there are plastic polymers present. This implies that infant feeding bottles do release microplastics which can impact the health of the baby.

2 Introduction

2.1 Background information

Each year around 130 to 140 million babies are born worldwide [6]. About 50 to 60% of these babies are bottle fed by their parents and caretakers [7, 8]. The baby bottles parents use can be made of various materials but most bottles are made of plastic. This is because plastic is easy to make in large quantities, it is cheap, it is lightweight with a low density, and it is easy to produce in different shapes [2, 3].

Various types of plastics are used to make baby bottles, for example, polypropylene (PP), polycarbonate (PC), polyamide (PA), and polyethersulphone (PES) [9]. Although plastics have a lot of advantages, there are also a few disadvantages. Research shows that plastic products can release a lot of micro and nano plastics [4, 9, 10].
The nano- and microplastics (MP's) that are released from plastic packages have a suspected impact on human health. It can cause cancer, inflammatory diseases, pulmonary diseases, cardiovascular problems, and infectious diseases [5]. For children and especially infants exposure to MP's has more risks because they don't have a fully developed immune system yet. The toxins inside the microplastics can lead to an increased likelihood of infections or other diseases [11].
In this report, the possible exposure of babies to MP's coming from PP infant feeding bottles (IFB's) will be researched.

2.2 Research question and hypothesis

The goal of this research is to answer the following research question:
"What is the effect of temperature on the release of microplastics from plastic infant feeding bottles?"

The expectation for the release of MP's is that they will be released during the use and handling of the IFB [4]. Another expectation is that the release of MP's, in the fluid inside the IFB, increases when they are exposed to increasing temperatures (between 70℃ and 100℃) [12]. It is also thought that there is a bigger release of MP's when the bottles have a longer sterilization time.

To answer the research question and test the hypothesis, several experiments will be done. First, it will be verified that the set-up works with clean water. Then the IFB's will be subjected to 3 different sterilization durations and the water will be filtered and the filter will be analyzed. Finally, the filter residue will be characterized.

3 Materials & Methods

In this chapter, the used materials are discussed. Furthermore, the experiments that are conducted and how they are carried out is described here.

3.1 Materials

To limit the scope of research one brand of baby bottle is used. This way, the type or brand of the bottle does not affect the amount of microplastics released. Therefore, the sole focus can be on the effects of heating. The type of baby bottle used in this research is the Difrax natural (transparent) s-bottle 170 ml anti-colic. The choice for the brand Difrax is because it is one of the market leaders in the Netherlands [13].

To sterilize the IFB's, Milli-Q water is used. Milli-Q is purified water made by passing water through a long-life dual-wavelength UV lamp. Next, a Quantum® polishing cartridge removes the remaining ionic and organic contaminants below trace levels. The last step is that the already very pure water recirculates through a loop to the Q-POD® (Quality-Point-Of-Delivery) dispenser, where the last filter removes the last particulates [14, 15]. To sterilize the bottles and heat the water for the experiments a microwave is used. The microwave is set at 900 Watt and the temperature of the water is measured with an infrared laser thermometer.

Figure 1: A schematic overview of the set-up used for all the experiments (from Biorender).

The water used for the verification and quantification experiments is distilled water from a glass round bottom flask, so the water has not been in contact with plastic. To be sure the water used during the verification and quantification experiments is clean, distilled water is used. This water is self-made and stored in a glass bottle to be sure that it has not come in contact with plastic.
The setup used in the filter is shown in figure 1, here is part 1 the glass funnel in which the water from the IFB enters the setup, part 2 is a glass membrane filter holder on this is the filter placed and part 3 is the Erlenmeyer that is used to collect the filtered water.

To stain the MP's on the filter a Nile Red solution will be used. The solution consists of 1 mg of Nile Red in 1 ml methanol. This gives approximately a 120 nM staining solution. Eventually, 15 µl of the 32 µM Nile Red is mixed with 4 ml of distilled water to make a 120 nM solution on the filter.

To filter the MP's out of the water an i3 TrackPor PA (PET/aluminum) filter with 0.8µm pores is used. The filter is made of PET and the surface is coated with aluminium. The coating is to prevent interference with the staining and the filter as much as possible.

To analyze the filter an upright fluorescence microscope is used. The microscope is a Nikon Eclipse E400 microscope with an excitation filter at 480/20 nm and a long pass emission filter of 500 nm. To view the MP's in color, a color camera is used. The camera is connected to the microscope and the computer. To control the microscope from the computer the program Labfiew is used. To analyze the images made with the microscope a script written in Python is used. Lastly to stitch the images and for final image analysis for scale bars Fiji of ImageJ is used.

3.2 Methods

In this section, the different experiments that are done for verification, quantification, and characterization of the set-up and MP's will be discussed.
To test the influence of temperature on the release of MP's the baby bottles will be subjected to three different sterilization variables. First, an attempt will be made to reproduce the data of D. Li, et al [4] with 5 minutes of sterilization. Next, the IFB's will be subjected to 30 minutes of sterilization. Finally, an experiment will be conducted without sterilization and heating. The basic workflow of the experiments is displayed in figure 2, this workflow mimics the use of an IFB of parents that use the bottles. This workflow is in general used for all experiments. In the quantification experiments, the sterilization time varies. The workflow is the same as the researchers D. Li, et al. use [4].

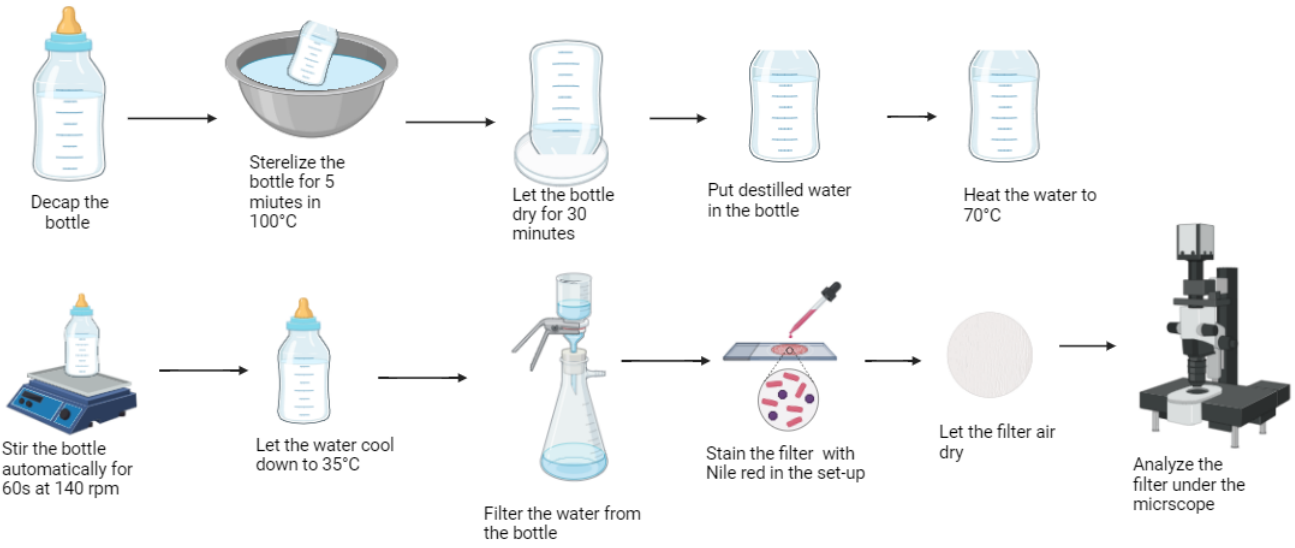


Figure 2: This is a schematic overview of the experiments that will be done with the IFB's to research the release of MP's. (This is made with Biorender).

3.3 Verification

3.3.1 Blank control

To make sure that the distilled water is clean and clear of MP's a blank control is conducted. This is also to set a baseline for all other experiments. This experiment is done at the start of the research. The blank control is conducted by pouring 170ml of distilled water, from the

glass flask, through the filter set-up. Next, the filter is stained with the Nile Red solution and analyzed with the microscope. The protocol is described in Appendix E.1 (Protocol: control experiment).

3.3.2 Verification with beads

To verify and calculate the accuracy of the set-up and experiments 10 µm non-fluorescent PS beads (LOT# A840326) are used. These beads are stained in the Nile Red step of the experiments which makes the counting of the beads possible. The results of this experiment are in section 4.1.2 (Beads). The beads come in a suspension where 1 ml of suspension contains $4.55{\cdot}10^7$ beads. To make the number of beads more manageable a dilution series is used, this series is shown in table 1.

From the stock 10 µl of suspension is taken and diluted with 90 µl of distilled water. So now there are $4.55{\cdot}10^5$ beads in 100 µl of dilution, this is dilution step 1. Now from the diluted suspension (step 1) again 10 µl is taken and diluted in 90 µl of distilled water. Now there should be $4.55{\cdot}10^4$ beads in the (100 µl) diluted suspension, this is step 2. After dilution step 2, the whole 100 µl of the dilution is added to the 170 ml of distilled water inside the IFB (after sterilization), so $4.55{\cdot}10^4$ beads are used in this verification experiment. The protocol used for this experiment is stated in Appendix E.2 (Protocol: Beads).

Table 1: The dilution series of the suspension for the beads.

	Stock	Step 1	Step 2
Polybead® Microspheres suspension	1000 µl	10 µl of the suspension	10 µl from dilution 1
Distilled water	0 µl	90 µl	90 µl
Amount Beads in the (100µl) dilution	$4.55{\cdot}10^7$	$4.55{\cdot}10^5$	$4.55{\cdot}10^4$

3.4 Quantification experiments

To check the effect on the release of MP's under different sterilization conditions, several quantification experiments are done. All quantification experiments are conducted in triplo and with every experiment a new IFB is used. The first experiment is carried out by (trying to) reproduce the data from the article: "Microplastic release from the degradation of polypropylene feeding bottles during infant formula preparation", written by D. Li, et al. [4]. The sample and baby bottle procedure will follow the same method as described in the article. The detailed protocol can be found in Appendix E.3 (Protocol: 5 minutes of sterilization). The second experiment will follow the same procedure but with a longer sterilization time, to determine if the longer exposure to high temperature results in a higher release of MP's. The third and final, experiment will be conducted without any sterilization or heating at all, to check whether high temperatures influence the release of MP's.

3.5 Filter aspects

As mentioned in section 3.1 (Materials) during the experiments an i3 TrackPor PA Filter is used. In this section, several features of the filter are discussed. Like when the water is filtered, the covered area of the filter, how the filter is stained, and how the quantification of the MP's on the filter happens.

3.5.1 Filtering the microplastics

After the baby bottle is sterilized and (air-)dried, the IFB is filled with distilled water and heated to 70℃. This water will be run through the filter (with 0.8 µm pores), the MP's will stay on the filter and the water will go through. To prevent the data from previous experiments from

influencing the results of the next experiment a new filter is used every time. This way potential cross-contamination of data is avoided and accurate and reliable data collection is ensured.

3.5.2 Covered area of the filter

To calculate the contact area of the water and MP's on the filter some diameters are measured with analogue calipers and used for the calculation. The filter has a diameter of 47.00 mm as stated on the packaging and is placed on a glass funnel with a porous membrane holder represented as part 2 in figure 1. On the filter, a glass graduated funnel is placed represented as part 1 in figure 1. This glass funnel has an outer diameter of 58.75mm and an inner diameter of 34.45mm.
Because the glass graduated funnel (part 1 in figure 1) has the smallest inner diameter (34.45 mm), this diameter is used to calculate the (round) contact surface area of the filter with water. The area of the filter that comes in contact with the water is 9.32cm^2.

3.5.3 Staining the filter

To stain the MP's, a 120 nM Nile Red solution is used. As also discussed in section 3.1 (materials), the solution is 4 ml distilled water with 15 µl (of 32 µM) Nile Red solution. To stain the filter a plug is put in the bottom of part 2 in figure1 (in section 3.1 Materials). The solution is poured on the filter and left there for at least 30 minutes so the Nile Red can absorb to the surface. Next, the solution is run through the filter and the filter is air-dried and stored in a Petri dish, or placed under the microscope.

3.5.4 Quantifying the microplastics on the filter

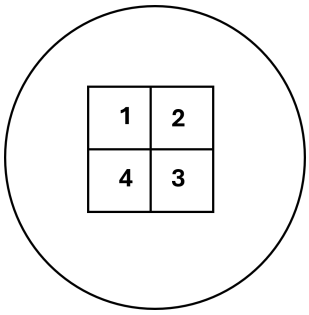


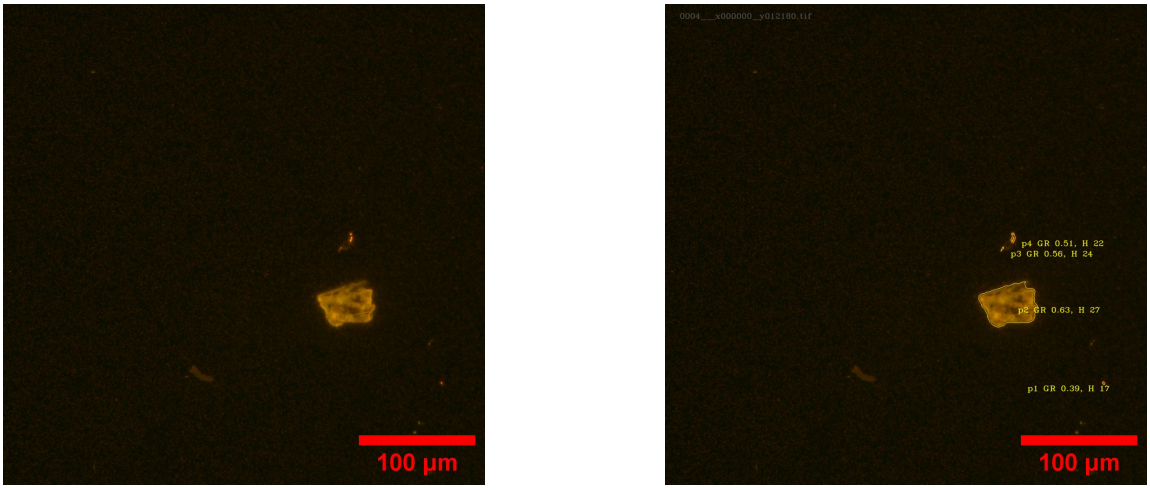
Figure 3: An overview of the area that is analyzed with the microscope.

After the filter is prepared, it will be placed under the upright fluorescence microscope. The images of the filter will be made using Labview. To analyze the images made with the microscope a script written in Python is used. The scripts used for the analysis are displayed in Appendix F (Scripts). This script makes a data set that measures the number of particles, colors, and sizes in µm, and the covered area in µm^2 of MP's on the filter. In the next section 3.6 (Image analysis) more information about the image analysis will be given.
The fluorescence microscope can make a tile scan of a 1 by 1 cm square by making 625 images of that area. As shown in figure 3 four 1 by 1 cm squares of the filter in all experiments are imaged. These areas are called areas 1 to 4. In total 4 cm^2 will be analyzed from the filters used during the experiments.

3.6 Image analysis with Python

To analyze the images from the fluorescence microscope a Python-script is used. The Python-scripts are displayed in Appendix F (Scripts). With the analysis, the script finds particles with a certain threshold and puts the data of the particles in a data frame. This data frame eventually becomes an .HTML report reflecting the data acquired from the images. Lastly, a summary of some data is given, this is the total particles counted with a size that is larger than 20 pixels, total particle coverage in µm^2, average Green ratio (GR), the average size of all particles found in pixels, and lastly the average size of all particles in µm^2.
Figure 4 presents images before and after analysis. The image of figure 4a is run through the script next figure 4b emerges as output from the analysis. The Python-script circles the MP's

that have a minimum contour size and brightness. The particle gets a P value which is the number of the particle in the picture. Furthermore, in the image, the P value, Hue value, and GR are written. This information will be visualized with yellow letters inside the image next to the particle where it belongs.
In this research, the total particle coverage in μm^2 and the average size of the particles in μm^2 are used for the calculation of the number of particles per liter.



(a) An image from the fluorescence microscope.

(b) The picture after it is been analyzed with Python-script.

Figure 4: An overview of the Python analysis of the separate pictures made with the microscope.

3.7 Characterization of the microplastics

To prove that the particles found during the quantification experiments are actual plastics from the IFB and not an additive as M.N. Gerhard, et al. [16] states. Several characterization experiments are performed, like FT-IR, SEM, and EDX.

3.7.1 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is used to characterize the particles from the IFB's as plastic. This is a tool for chemical characterization [17]. FT-IR is a rapid and nondestructive spectroscopy technique. It uses a polychromatic beam of IR radiation, which produces an interferogram. The beam will be passed through the sample where the beam is reflected off the surface or transmitted through the sample. The sample-specific frequencies are absorbed and converted into vibrational or rotational energy. Finally, the radiation passes through the detector. Next, a computer transforms the interferogram into an absorption spectrum using the Fourier transformation [18, 19].

To get enough MP's for the sample for the FT-IR measurement, six (already used) baby bottles are reused. The water in the IFB's will be circulated until approximately 33 liters of water have circulated in the bottles. (This is calculated from the average of total coverage in μm^2 in table 8 displayed in Appendix C.2, Results: 5 minutes of sterilization).
To prepare the sample the bottles are sterilized once for 5 minutes next, 4 of the 6 bottles are filled. The bottles are heated in duos (so the circulation within the bottles can be done) to 70℃ and then cooled down to 35℃. After 33L has been through the IFB's, the water will be concentrated above the filter (approximately 10-20 ml) in the filter set-up shown in figure 1 and

the concentrated water is evaporated in a Petri dish. Lastly, the residue is scraped together and the powder is put in the FT-IR spectrometer.

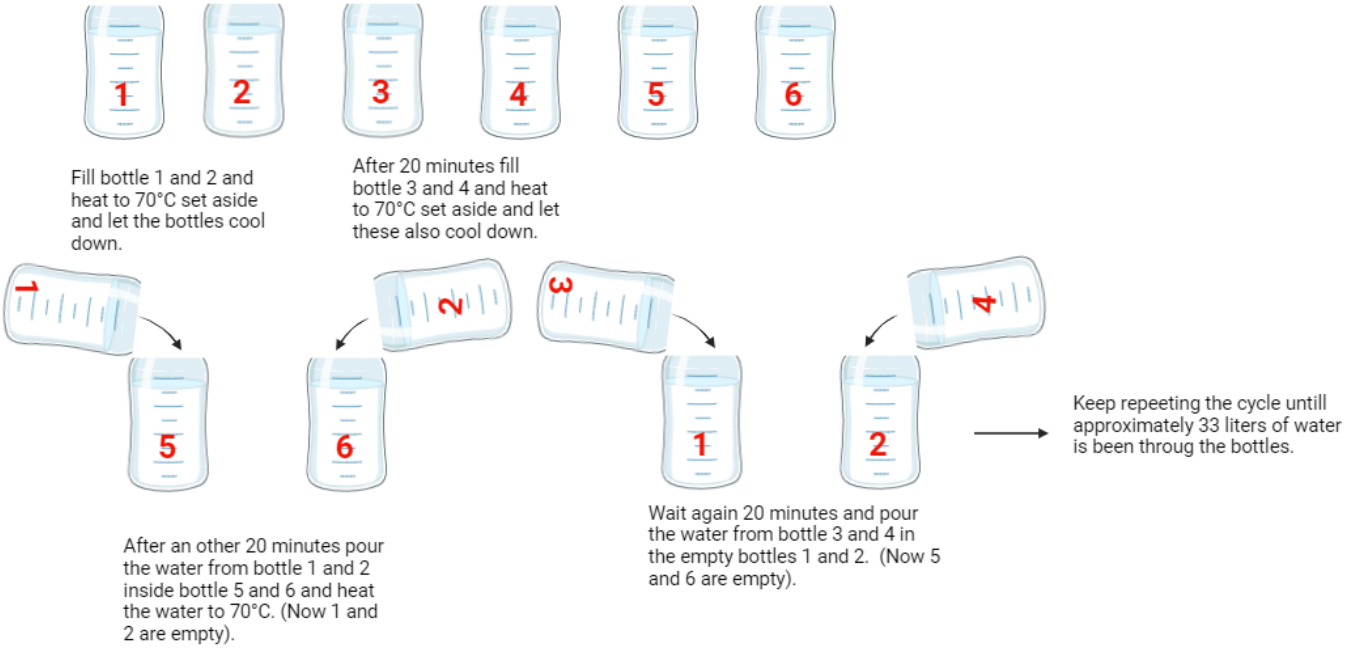


Figure 5: A schematic overview of how the water will be circulated within the 6 IFB's. (Made with Biorender).

3.7.2 Scanning electron microscopy & Energy-dispersive X-ray spectroscopy

In the second characterization experiment, Scanning electron microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDX) are used. SEM is a type of microscopy from which high-resolution images can be obtained. It uses a focused beam of electrons which scans the surface area of a sample. SEM can generate an image at a very high resolution compared to other types of microscopy. The electrons of the SEM interact with atoms in the sample which give different signals that can be used to obtain information about the composition of the sample [20].

A sample for SEM was prepared using a section of the filter used in the FT-IR experiment, which still contained numerous particles. The same piece of filter is also used for EDX. This is a technique used for elemental analysis of the sample after the SEM. EDX is a technique that generates characteristic X-rays in the atoms. Next, elastic and inelastic scattering occurs and the atoms are ionized. As a result, the atoms will emit X-rays and as every element has its own emission band, this X-ray signal can be used to characterize the chemical composition of the elements from the sample. Eventually, an elemental composition graph with intensity data is generated using EDX, this can be used for further analysis [21, 22, 23].

4 Results

In this chapter, the results obtained from the verification, quantification, and characterization experiments are presented. In the next chapter 5 (Discussion) the results are discussed in depth and the results will also be interpreted there.
First, the results of the verification experiments will be presented. Next, the results of the

different quantification experiments (with varying sterilization and heating times) are displayed, and lastly, the results of the MP characterization with FT-IR and SEM & EDX are presented.

4.1 Verification of the set-up

With these verification experiments, the baseline is set for the quantification experiments. First, an experiment is conducted with just distilled water, the blank control. Second, for the yield and margin of error, an experiment with PS beads is done.

4.1.1 Blank control

First, to check whether the distilled water is clean and if the set-up works, a blank control is done with distilled water. Second, this experiment is also done as a baseline for the other experiments, because sometimes some filter pores are also stained during the staining of the MP's. The control experiment is not done in triplo, and it is done by just pouring 170 ml of distilled water on the filter, staining the filter, and analyzing the stained filter. The experiment gave the following results shown in table 2. In this table the data from the analyzed filter is presented, area 3 has a very big area because the measurement with the microscope went wrong and some images were taken beyond the filter. The average of the areas is calculated, and area 3 is excluded from this calculation.

Table 2: The results of the control experiment from the different areas shown in figure 3 analyzed with the microscope.

	Area 1	Area 2	Area 3	Area 4	Average
Total coverage in µm^2	$1.83 \cdot 10^3$	$1.68 \cdot 10^4$	$4.68 \cdot 10^5$	$1.54 \cdot 10^4$	$1.13 \cdot 10^4$
Average particle size µm^2	42.2	19.0	48.7	49.3	36.8

Next, the total coverage of the filter and what that coverage would be for a liter of water are calculated. This data is presented in table 3. With the total coverage per liter, the number of defects per liter will be calculated.

Table 3: Total coverage of the filter is calculated with MP's in micrometers and the total coverage of a micrometer is calculated per liter.

	Measured area	Filter area		IFB volume	volume
cm^2	1	9.32	ml	170	1000
µm^2 coverage	$1.13 \cdot 10^4$	$10.53 \cdot 10^4$	µm^2 coverage	$10.53 \cdot 10^4$	$61.94 \cdot 10^4$

The defects per liter can now be calculated. This calculation will be repeated for the quantification experiments with different exposures to temperature.
The Python script found an average of 542 particles per cm^2, the particles have an average size of 36.8 µm^2, and the total coverage in µm^2 per liter is $61.94 \cdot 10^4$ so the number of defects per liter is:

$$\frac{61.94 \cdot 10^4}{36.8} = 16.83 \cdot 10^3$$

The size distribution of the defects found is displayed in the size distribution histogram in figure 6. There are a lot of defects smaller than 50 µm^2 found with a cumulative percent that starts around 92% in the first bin of the big histogram.

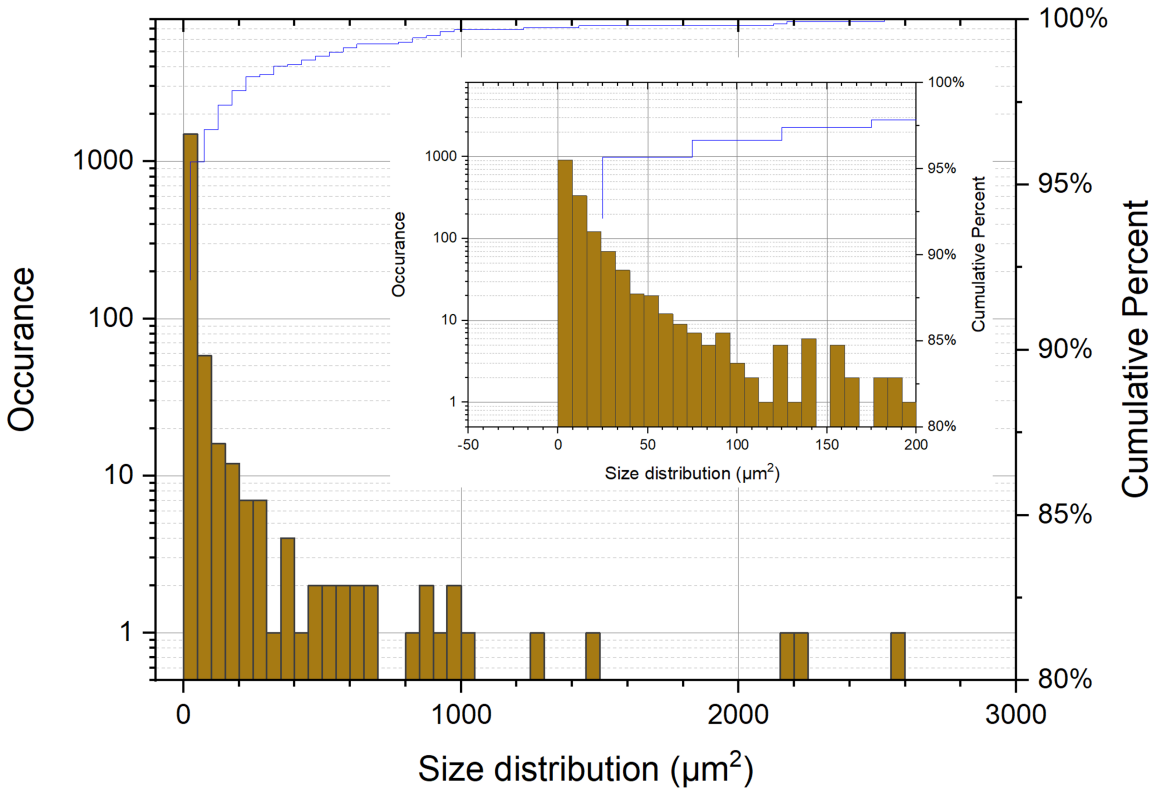


Figure 6: The size distribution histogram of occurrence of the found particles against sizes of the particles in µm² for the blank control experiment. In the top right corner, a zoomed-in histogram is displayed with a bin size of 8 instead of 50.

Of the images made with the microscope also some stitched images are made to get a clear overview of a whole area (all stitched images are of area 1). Figure 27 displayed in Appendix D.2 (stitched: control) shows the stitched inverted image. The red highlighted square in the corner refers to the zoomed-in photo displayed in figure 12a in section 4.2.5 (Results overview). This stitched image was made after the image analysis, all blue dots are stained MP's. In Appendix D.2 (stitched: control) all stitched images are shown. These are of the images made with the microscope before (figure 26a and 26b) and after image analysis (figure 26c, 26d). Both sets of photos are of the normal colors and the inverted colors.

4.1.2 Beads

To calculate the yield and margin of error, an experiment using PS beads was conducted. The calculations for yield, margin of error, and recovery percentage are presented, and the experiment is only done once. Table 4 shows the number of beads found per area, scanned with the microscope. These numbers were obtained using the script from Appendix F.2 (Python-script for beads) and the total yield will be calculated. The script was modified to count only the beads because they have a specific color, Hue value, aspect ratio, shape, and size in µm². After the analysis with the Python-script the number of beads found in each area is given.

Table 4: This table presents the number of beads that are found on the filter per area.

	Area 1	Area 2	Area 3	Area 4	Average
Amount of beads	4001	5258	2072	2597	3482

The average number of beads found on 1 cm^2 is 3482. Now, the variance, standard deviation, and margin of error will be calculated as displayed in equation 1 to 5.

$$variance = \sum \frac{(x_i - \bar{x})^2}{n} \tag{1}$$

$$variance = \frac{(4001 - 3482)^2 + (5258 - 3482)^2 + (2072 - 3482)^2 + (2597 - 3482)^2}{4} = 1546706.25 \tag{2}$$

$$standarddeviation\ \sigma = \sqrt{1546706.25} = 1243.77 \tag{3}$$

$$Margin\ of\ error = \frac{Z \cdot \sigma}{\sqrt{n}} = \frac{1.96 \cdot 1243.77}{\sqrt{4}} = 1218.92 \tag{4}$$

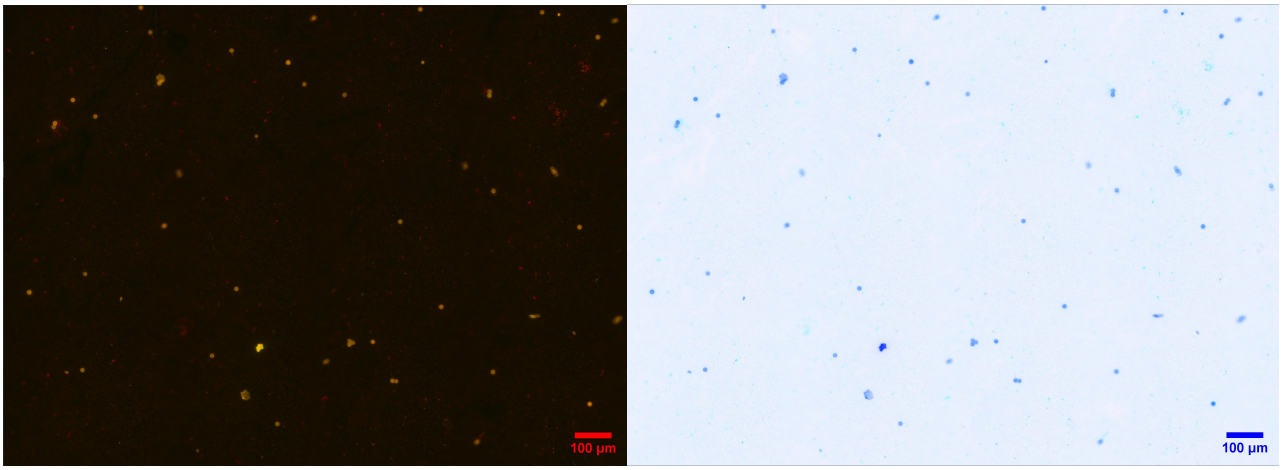
Z is a constant and for a 95% reliability interval, it has a value of 1.96.

$$Margin\ of\ error\ \% = \frac{1218.92}{45500} \cdot 100\% = 2.67\% \tag{5}$$

The margin of error is 2.67% as presented in formula 2 to 5. This margin of error will be used for the error shown within the number of particles per liter found with the quantification experiments.

Lastly, the number of beads found on one cm^2 is 3482 so on the whole filter it is then $3482 \cdot 9.32 = 32452$ beads are found back from the $4.55 \cdot 10^4$ that are put in the IFB, which means that the yield/recovery percentage is 71%, with a loss of 29%. This yield is used for the analysis of the quantification experiments.

In figure 7 two images are displayed from the zoomed-in (highlighted) corner of the stitched images from area 1. The whole stitched picture is displayed in Appendix D.1 (Stitched: beads) in figure 25 (The red square refers to the images shown in figure 7). All small orange dots in figure 7a and blue dots in figure 7b are beads, there are approximately 104 beads (counted by hand) in these two pictures.



(a) The left upper highlighted corner of the stitched images made with the microscope for the beads of area 1.

(b) The left upper highlighted corner of the stitched images made with the microscope for the beads of area 1, but inverted.

Figure 7: Stitched pictures of area 1 for the beads.

4.2 Quantification of the MP's

After the verification experiments, some quantification experiments are performed. First, three IFB's are sterilized for 5 minutes, after that three new IFB's are sterilized for 30 minutes, and lastly, 3 new IFB's are not sterilized or heated at all. With these experiments, the influence of temperature on the release of MP's is tested. All results of the separate experiments are first displayed and will be discussed in the following chapter. A summary of the results is given in section 4.2.4 (Particles per liter overview) and section 4.2.5 (Results overview).
Before calculating the number of particles per liter, the entire filter was scanned to ensure that the particles were evenly distributed. Since the particles were found to be homogeneously distributed, this was taken into account when calculating the number of particles per liter.

4.2.1 5 minutes of sterilization

This first set of experiments is done to try and replicate the data presented in the article: Microplastic released from the degradation of polypropylene feeding bottles during infant formula preparation written by D. Li, et al. [4]. The experiment is done exactly like the article except here a Nile Red staining and fluorescence microscope are used, for the protocol see Appendix E.3 (Protocol: 5 minutes of sterilization).
The results acquired from the experiments are presented in Appendix C.2 (Results: 5 minutes of sterilization) in table 8 and table 9 with these results the total number of particles per liter is calculated for the three separate bottles.

Bottle 1: The Python script found an average of 669 particles per cm^2, the particles have an average size of 77.8 μm^2, and the total coverage in μm^2 per liter is $257.97 \cdot 10^4$ so the amount of particles per liter is:

$$\frac{258.21 \cdot 10^4}{77.8} = 3.31 \cdot 10^4 \pm 8.9 \cdot 10^2$$

Bottle 2: The Python script found an average of 2218 particles per cm^2, the particles have an average size of 66.2 μm^2, and the total coverage in μm^2 per liter is $100.33 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{100.33 \cdot 10^5}{66.2} = 15.16 \cdot 10^4 \pm 4.0 \cdot 10^3$$

Bottle 3: The Python script found an average of 2339 particles per cm^2, the particles have an average size of 121.9 μm^2, and the total coverage in μm^2 per liter is $159.54 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{159.54 \cdot 10^5}{121.9} = 13.09 \cdot 10^4 \pm 3.5 \cdot 10^3$$

The size distribution histogram of the particles found through all three experiments with the 5 minutes of sterilization is shown in figure 8. Again, many particles smaller than 50 μm^2 are found but there are also much larger ones. There is a cumulative percent that starts around 90% in the first bin of the big histogram.

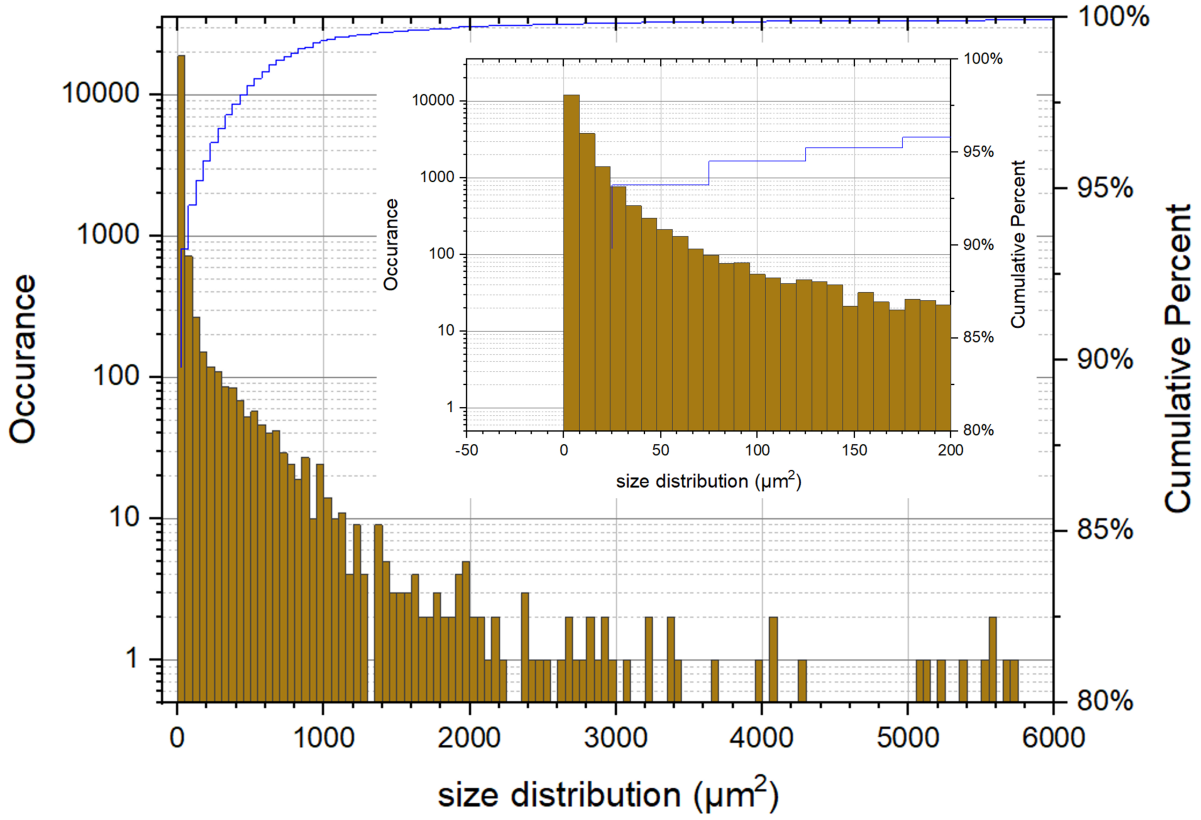


Figure 8: The size distribution histogram of occurrence of the particles against sizes of the particles in μm^2 for the experiment with 5 minutes of sterilization. In the top right corner, a zoomed-in histogram is displayed with a bin size of 8 instead of 50.

Of the images made with the microscope also some stitched pictures are made to get a clear overview of the whole area (all stitched images are of area 1). Figure 29, displayed in Appendix D.3 (Stitched: 5 minutes of sterilization) shows the stitched inverted image. The red highlighted square in the corner refers to the zoomed-in photo depicted in figure 12b in section 4.2.5 (Results overview). This stitched image was made after the image analysis, all blue dots are stained MP's. In Appendix D.3 (Stitched: 5 minutes of sterilization) all stitched images are shown. These are of the images made with the microscope before (figure 28a and 28b) and after image analysis (figure 28c, 28d).

4.2.2 30 minutes of sterilization

This second set of experiments is done to check if the IFB releases more MP's when the bottle is sterilized for a longer time. The experiments are done the same as the previous set of experiments but instead of sterilizing the IFB for 5 minutes, it is sterilized for 30 minutes in this set of experiments, for the protocol see Appendix E.4 (Protocol: 30 minutes of sterilization).
The results acquired from the experiments are presented in Appendix C.3 (Results: 30 minutes of sterilization) in table 10 and table 11, with these results the total number of particles per liter is calculated for the three separate bottles.

Bottle 1: The Python script found an average of 1925 particles per cm^2, the particles have an average size of 72.1 μm^2, and the total coverage in μm^2 per liter is $79.49 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{79.49 \cdot 10^5}{72.1} = 11.02 \cdot 10^4 \pm 2.9 \cdot 10^3$$

Bottle 2: The Python script found an average of 4026 particles per cm^2, the particles have an average size of 30.3 μm^2, and the total coverage in μm^2 per liter is $52.96 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{52.96 \cdot 10^5}{30.3} = 17.47 \cdot 10^4 \pm 4.7 \cdot 10^3$$

Bottle 3: The Python script found an average of 1750 particles per cm^2, the particles have an average size of 83.2 μm^2, and the total coverage in μm^2 per liter is $72.92 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{72.92 \cdot 10^5}{83.2} = 8.76 \cdot 10^4 \pm 2.3 \cdot 10^3$$

The size distribution histogram of the particles found through all three experiments with 30 minutes of sterilization is shown in figure 9. Again, many particles smaller than 50 μm^2 are found but now there are also much larger ones they are also larger than the 5 minutes of sterilization. It has a cumulative percentage that starts around 80% in the first bin of the big histogram.

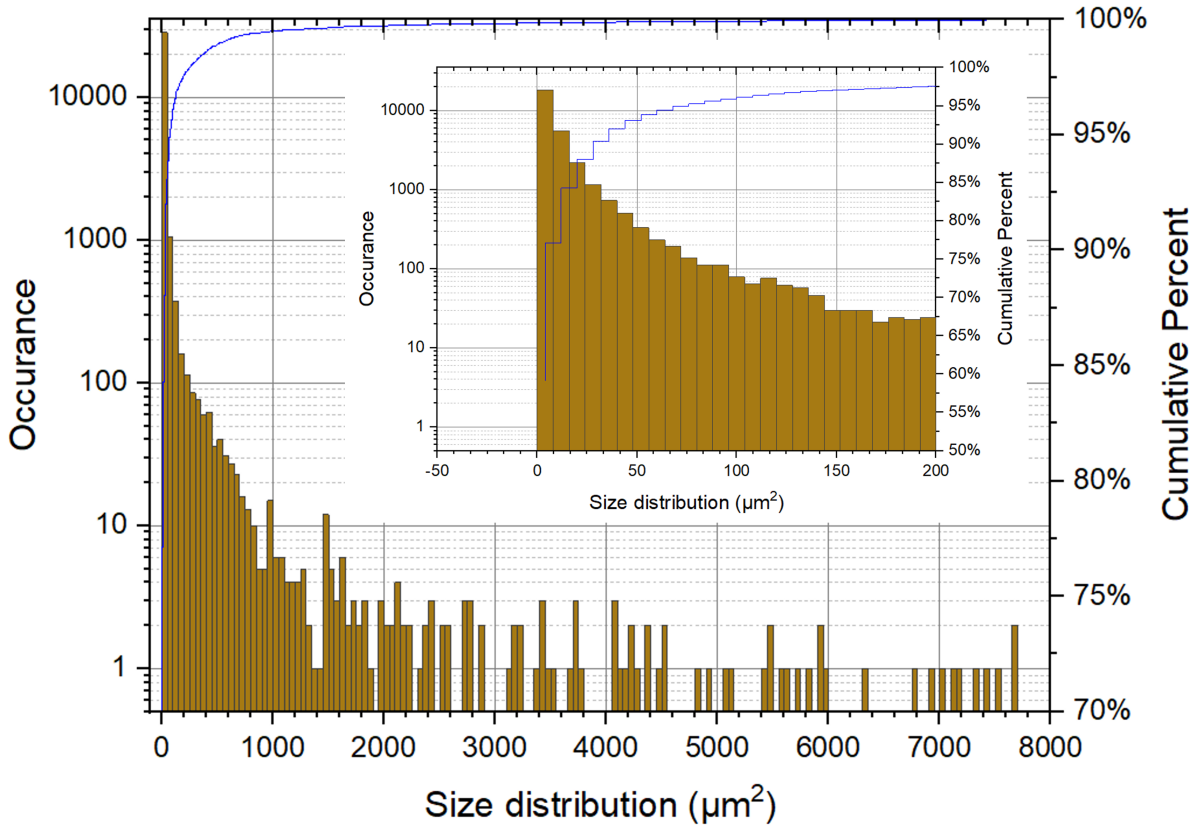


Figure 9: The size distribution histogram of occurrence of the particles against sizes of the particles in µm² for the experiment with 30 minutes of sterilization. In the top right corner, a zoomed-in histogram is displayed with a smaller bin size of 8 instead of 50.

Of the images made with the microscope also some stitched pictures are made to get a clear overview of the whole area (all stitched images are of area 1). Figure 31, displayed in Appendix D.4 (Stitched: 30 minutes of sterilization) shows the stitched inverted image of the images made with the microscope. The red highlighted square in the corner refers to the zoomed-in photo displayed in figure 12c in section 4.2.5 (Results overview). This stitched image was made after image analysis, all blue dots are stained MP's. In Appendix D.4 (Stitched: 30 minutes of sterilization) all stitched images are presented. These are of the images made with the microscope before (figure 30a and 30b) and after the image analysis (figure 30c, 30d).

4.2.3 No sterilization or heating

This third set of experiments is done to check whether the IFB loses fewer MP's when it is not exposed to high temperatures. The experiments are done the same as the previous sets of experiments but now the IFB's are not sterilized or heated at all. They are rinsed 3 times after which they are filled with distilled water and set aside for 45 minutes to mimic the cool-down time (from 70℃ to 35℃). After 45 minutes the water is run through the filter and the filter is then stained with Nile Red and analyzed with the microscope the same as with the other quantification experiments. For the protocol see Appendix E.5 (Protocol: no sterilization or heating). The results acquired from the experiments are presented in Appendix C.4 (Results: no sterilization or heating) in table 12 and table 13, with these results the total number of particles per liter is calculated for the three separate bottles.

Bottle 1: The Python script found an average of 6637 particles per cm^2, the particles have an average size of 40.6 μm^2, and the total coverage in μm^2 per liter is $112.94 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{112.94 \cdot 10^5}{40.6} = 27.82 \cdot 10^4 \pm 7.4 \cdot 10^3$$

Bottle 2: The Python script found an average of 4391 particles per cm^2, the particles have an average size of 31.4 μm^2, and the total coverage in μm^2 per liter is $77.30 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{77.30 \cdot 10^5}{31.4} = 24.62 \cdot 10^4 \pm 6.6 \cdot 10^3$$

Bottle 3: The Python script found an average of 4476 particles per cm^2, the particles have an average size of 76.3 μm^2, and the total coverage in μm^2 per liter is $182.56 \cdot 10^5$ so the amount of particles per liter is:

$$\frac{182.56 \cdot 10^5}{76.3} = 23.93 \cdot 10^4 \pm 6.4 \cdot 10^3$$

The size distribution histogram of the particles found through all three experiments without sterilization or heating is shown in figure 10. Again many particles smaller than 50 μm^2 were found but now there are also some very large ones found. These are however not much larger or to a greater extent found than with the 30 minutes of sterilization. It has a cumulative percent that starts around 91% in the first bin of the big histogram.

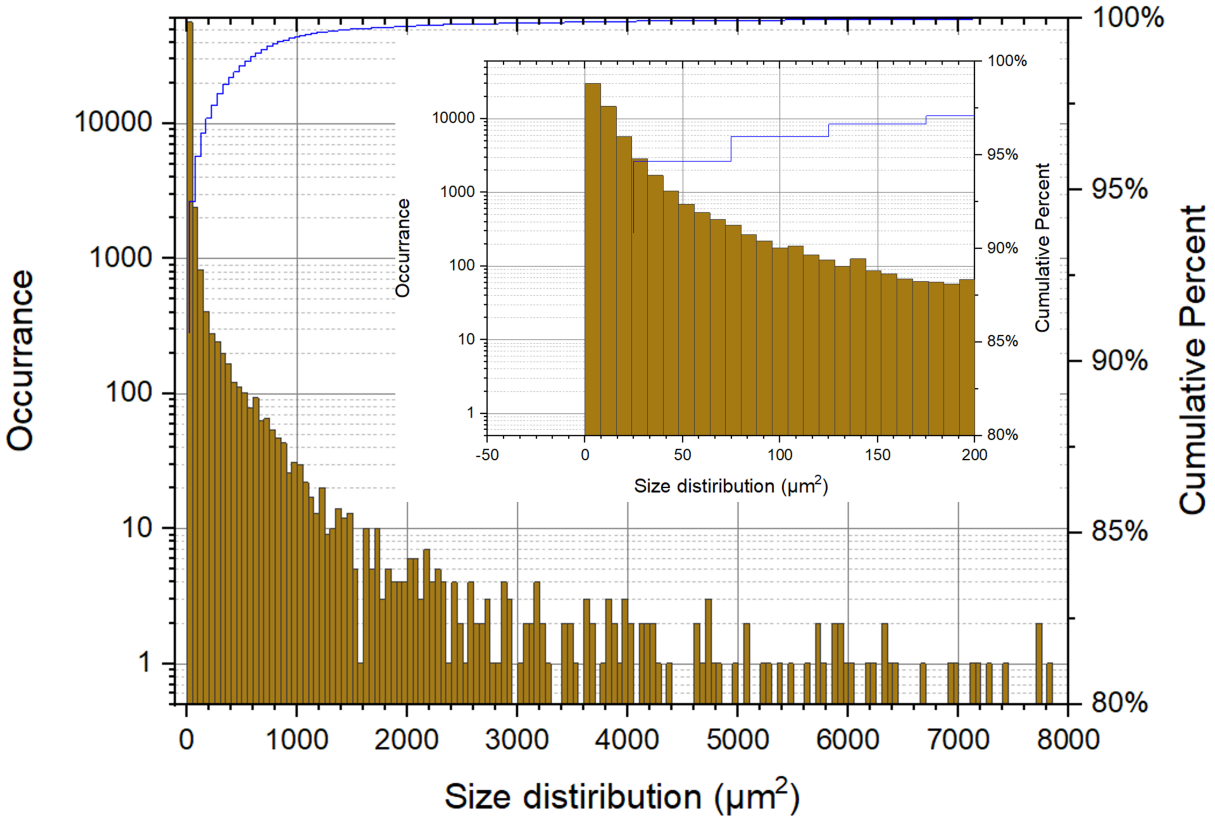


Figure 10: The size distribution histogram of occurrence of the particles against sizes of the particles in μm^2 for the experiment with no heating. In the top right corner, a zoomed-in histogram is displayed with a smaller bin size of 8 instead of 50.

Of the images made with the microscope also some stitched pictures are made to get a clear overview of the whole area (all stitched pictures are of area 1). Figure 33, displayed in Appendix D.5 (Stitched: no sterilization or heating) shows the stitched inverted image of the images made with the microscope. The red highlighted square in the corner refers to the zoomed-in photo displayed in figure 12d in section 4.2.5 (Results overview). This stitched image was made after image analysis, all blue dots are stained MP's. In Appendix D.5 (Stitched: no sterilization or heating) all stitched images are presented. These are of the images made with the microscope before (figure 32a and 32b) and after image analysis (figure 32c, 32d).

4.2.4 Particles per liter overview

In this section, an overview of the number of particles per liter found is presented in tables. Table 5 represents the raw data retrieved from calculations after image analysis. Table 6 represents the data after it is corrected for the blank control and the yield of 71%. This is calculated by subtracting the blank control from the data and then multiplying that number with the yield of 71% which was calculated during the verification with beads.

Figure 11 illustrates the average number of particles including the margin of error of 2.67% for the three different quantification experiments, 5 minutes of sterilization, 30 minutes of sterilization, and no sterilization or heating. These are the raw data and the data corrected for the blank control and the yield.

Table 5: The number of particles per liter acquired from the separate bottles of the different quantification experiments.

	Bottle 1	Bottle 2	Bottle 3	Average
Particles per liter 5 minutes of sterilization	$3.31 \cdot 10^4 \pm 8.9 \cdot 10^2$	$15.16 \cdot 10^4 \pm 4.0 \cdot 10^3$	$13.09 \cdot 10^4 \pm 3.5 \cdot 10^3$	$10.52 \cdot 10^4 \pm 2.8 \cdot 10^3$
Particles per liter 30 minutes of sterilization	$11.02 \cdot 10^4 \pm 2.9 \cdot 10^3$	$17.47 \cdot 10^4 \pm 4.7 \cdot 10^3$	$8.76 \cdot 10^4 \pm 2.3 \cdot 10^3$	$12.42 \cdot 10^4 \pm 3.3 \cdot 10^3$
Particles per liter no sterilization or heating	$27.82 \cdot 10^4 \pm 7.4 \cdot 10^3$	$24.62 \cdot 10^4 \pm 6.6 \cdot 10^3$	$23.93 \cdot 10^4 \pm 6.4 \cdot 10^3$	$25.46 \cdot 10^4 \pm 6.8 \cdot 10^3$

Table 6: The number of particles per liter after the blank control is subtracted and multiplied with the yield.

	Bottle 1	Bottle 2	Bottle 3	Average
Particles per liter 5 minutes of sterilization	$1.16 \cdot 10^4 \pm 3.1 \cdot 10^2$	$9.57 \cdot 10^4 \pm 2.6 \cdot 10^3$	$8.10 \cdot 10^4 \pm 2.2 \cdot 10^3$	$6.27 \cdot 10^4 \pm 1.7 \cdot 10^3$
Particles per liter 30 minutes of sterilization	$6.63 \cdot 10^4 \pm 1.8 \cdot 10^3$	$11.21 \cdot 10^4 \pm 3.0 \cdot 10^3$	$5.02 \cdot 10^4 \pm 1.3 \cdot 10^3$	$7.62 \cdot 10^4 \pm 2.0 \cdot 10^3$
Particles per liter no sterilization or heating	$18.56 \cdot 10^4 \pm 5.0 \cdot 10^3$	$16.19 \cdot 10^4 \pm 4.3 \cdot 10^3$	$15.80 \cdot 10^4 \pm 4.2 \cdot 10^3$	$16.88 \cdot 10^4 \pm 4.5 \cdot 10^3$

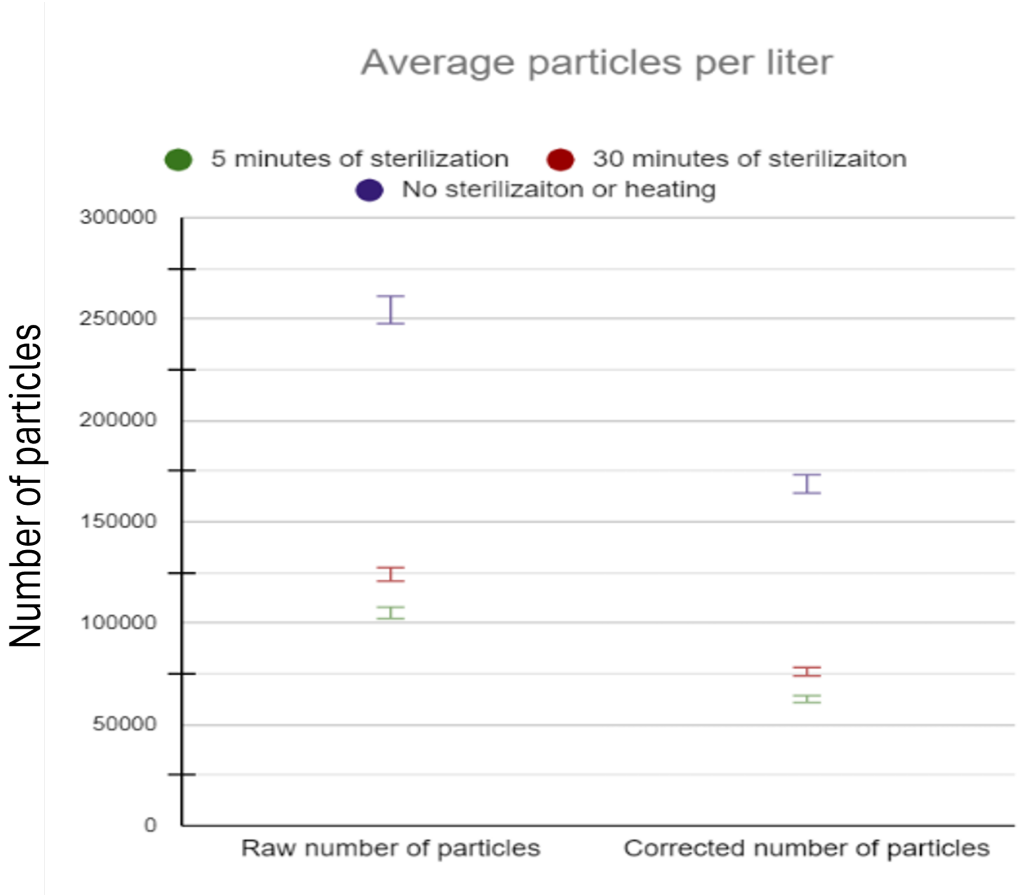


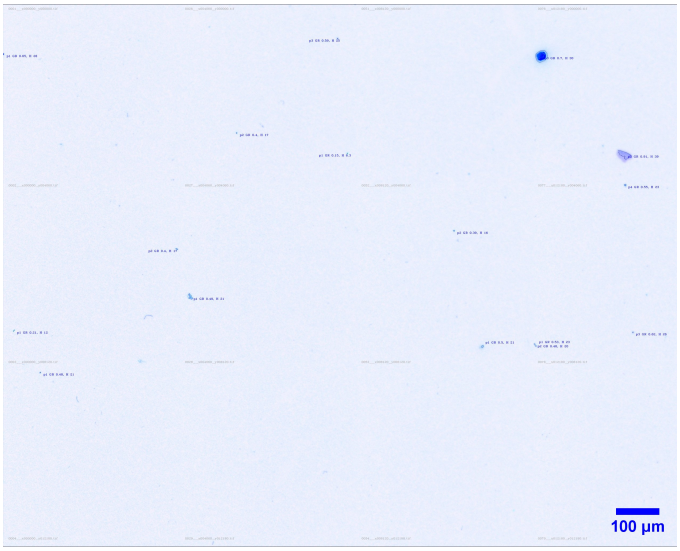
Figure 11: The average particles found for the different quantification experiments before and after correction with the blank control and yield.

4.2.5 Results overview

This section presents the zoomed-in images of the highlighted upper left corners from the stitched images of area 1 (from the microscope analysis 3). The whole stitched images, with the highlighted squares, are shown in Appendix D (Stitched pictures). The grey text lines indicate the names of the individual images that were stitched together. The blue text is from the image analysis with the script using Python. The blue text marks the location where the Python-script found a particle.

The first image is of the stitched picture of area 1 from the blank control experiment. The second image represents the 5 minutes of sterilization experiment. The third image corresponds to the 30 minutes of sterilization experiment and the final image is from the experiment without sterilization or heating. As depicted, the blank control photo contains almost no colored particles only defects. In contrast, the other figures show more particles (and some defects) after closer inspection.

(a) The zoomed-in (highlighted) corner of the blank control filter. (The full picture is in Appendix D.2).



(b) The zoomed-in (highlighted) corner of the 5 minutes of sterilization filter. (The full picture is in Appendix D.3).

(c) The zoomed-in (highlighted) corner of the 30 minutes of sterilization filter. (The full picture is in Appendix D.4).

(d) The zoomed-in (highlighted) corner of the no sterilization of heating filter. (The full picture is in Appendix D.5).

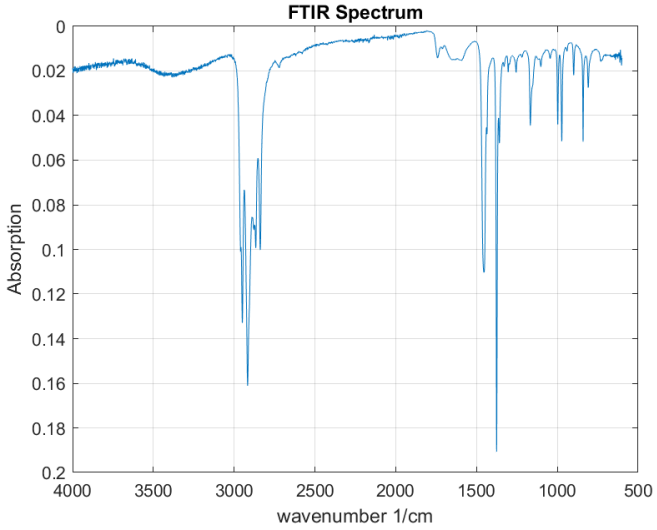
Figure 12: Images of the zoomed-in highlighted corners of the inverted stitched images after image analysis with Python. The blue lines are the text that is put in the picture through the Python-script. The particles are blue and circled and the text next to it is from the Python analysis. The grey text that is visible in some of the sub-figures is the name of the separate images from which the stitched images are made.

4.3 Characterisation of the material

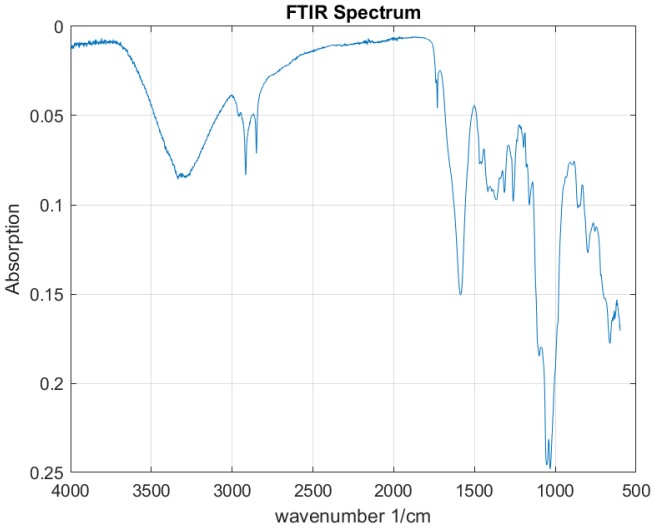
To characterize the particles coming from the IFB's as PP, three different characterization methods are used. First FT-IR is done with a piece of the IFB itself and the powder retrieved after the experiment as explained in section 3.7.1 (Fourier transform infrared spectroscopy). Second SEM and EDX measurements are performed on a piece of the filter that contained some of the particles coming from the IFB's.

4.3.1 FT-IR

To characterize the particles coming from the IFB's as PP microplastics, FT-IR is used. First, an FT-IR spectrum is made of the transparent IFB itself (to get a reference). The bottle is cut up and the piece of the bottle is placed under the FT-IR spectrometer. The spectrum is shown in figure 13a and shows a spectrum of PP when comparing it to literature [24].
Second, an FT-IR spectrum is made of the MP powder retrieved from the IFB after the experiment is done as described in section 3.7.1 (Fourier transform infrared spectroscopy) and Appendix E.6 (Protocol: FT-IR experiment). The spectrum from the FT-IR is depicted in figure 13b. This spectrum shows some overlapping peaks with the pp spectrum but it also shows some different peaks.



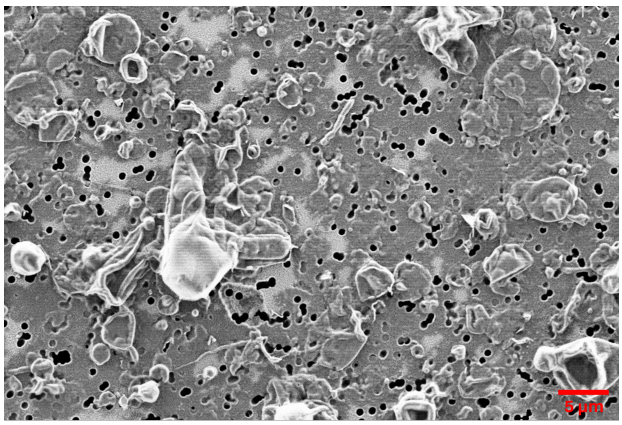
(a) The spectrum of a piece of the IFB.



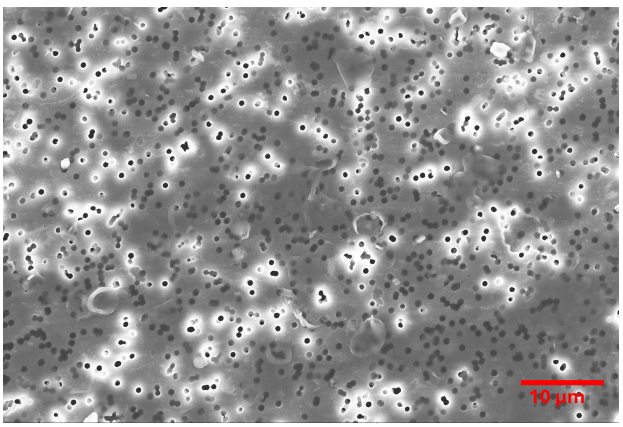
(b) The spectrum of the powder scraped from the dried in MP's.

Figure 13: The FR-IR absorption spectra from the baby bottle and the collected MP's.

4.3.2 SEM



(a) An image of a piece of the filter made with the SEM with a magnification of 5000X and a voltage of 1.40kV

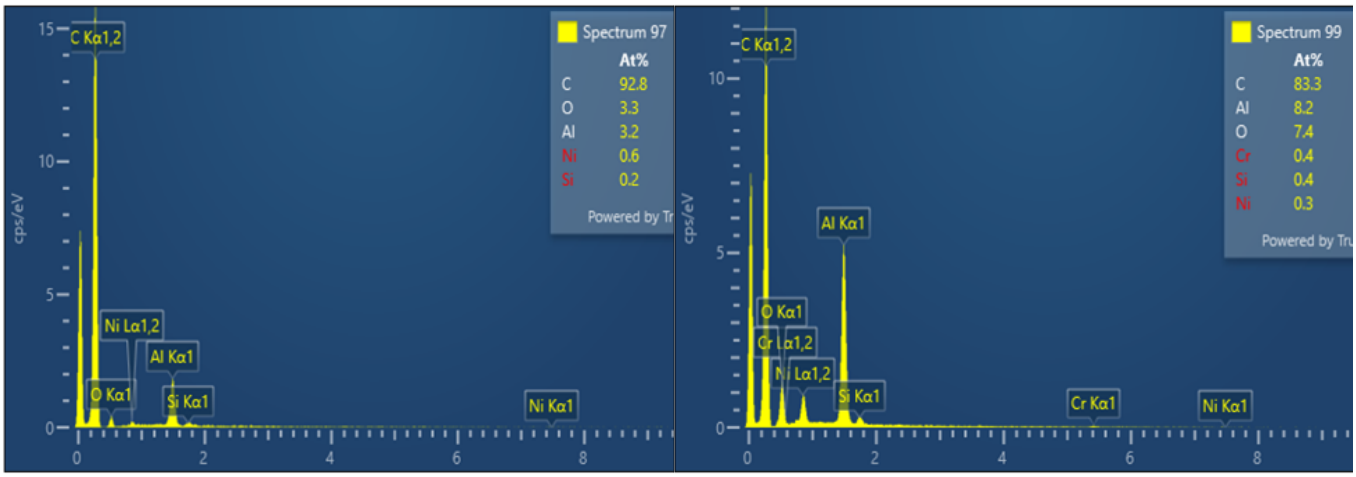


(b) An image of the same piece of the filter made with the SEM with a magnification of 4.03K X and a voltage of 10.00kV

Figure 14: Two images made with the SEM of the filter used in the FT-IR experiment.

To make a sample for the SEM and EDX analysis, the filter used in the FT-IR experiment is cut up and a piece of that filter is placed on the sample holder of the SEM. Two images from the SEM are displayed in figure 14, and the rest of the images are displayed in Appendix C.7 (Results: SEM).
Figure 14a is made with a voltage of 1.4 kV and a magnification of 5000X on this image some big particles are visible. There are also some black spots which means that the pores of the filter are filled. The other figure (figure 14b) is made with a higher voltage (10.00 kV) and magnification of 4.03K X. With this voltage the material (and filter) becomes a bit see-through. The white glow over some pores means that the filter's pores are empty, and a black spot means that the filter's pores are filled with material. The clear grey part in the background is the aluminum filter. Some of the particles on the filter are now also see-through.

4.3.3 EDX



(a) EDX spectrum of spot 97 on figure 24b in Appendix C.8. Spot 97 is a particle found with the SEM.

(b) EDX spectrum of spot 99 on figure 23a in Appendix C.8. Spot 99 is a particle found with the SEM.

Figure 15: Two EDX spectra of different particles and areas on the filter used as a sample for the SEM.

Along with the SEM sample, some EDX measurements were conducted. These measurements were taken at two different areas of the filter. In these areas, different spots/particles were analyzed and EDX spectra were generated for these spots. The spectra show the elemental peaks of the elements present and it shows how much of that element is present. Additionally, these spectra also show the atomic percentage which represents the number of elements of a specific atom present relative to the total number of atoms present.

The images of the two areas that are analyzed with EDX are presented in Appendix C.8 (Results: EDX). These images show the area of figure 24b and 23a. In these images, certain particles/measurement areas are highlighted and numbered from spot 95 to spot 102. The corresponding spectra are also presented in Appendix C.8 (Results: EDX). Two EDX spectra are selected and will be discussed in more detail in section 5.3.3 (Discussion: EDX).

5 Discussion

The results presented in Chapter 4 (Results) are discussed and explained in this chapter.

5.1 Verification

5.1.1 Blank control

The blank control experiment is done as shown in Appendix E.1 (Protocol: Blank control). The results are promising with a low coverage. Except what is obtained from the tile with area number 3 shown in figure 3. Here the microscope had incorrect settings, meaning images were taken outside the filter and gave incorrect results. Some of the images were from the filter but others were of the object the filter placed on. As a result, with analysis, more particles were found than actually present. Since the other tiles gave good results. The average for the number of defects is calculated with the other three areas, where area 3 is excluded.

Furthermore, when the total number of defects per liter is calculated it gives a relatively large number of defects. One explanation for this can be that the aluminum coating of the filter did not coat all PET pores of the filter equally well.
This can mean that the defects found during the blank control are the PET pores colored with Nile Red because the sizes of the defects are very small. This is also shown in the size distribution histogram where a lot of small particles are present in figure 6 in section 4.1.1 (Blank control).

When taking a closer look at the stitched images in Appendix D.2 (Stitched: control) and the zoomed-in image in figure 26a (in section 4.2.5, Results overview). The amount of defects seen by the eye is very low. This means the defects found after analysis are probably of the aluminum-coated PET filter. It is also noticeable that after the analysis with the Python-script, more particles are found than when looking at the stitched image by eye this is displayed in figure 26b and figure 27 in Appendix D.2 (Stitched: Blank control).

5.1.2 Beads

The number of beads found back from the IFB (170 ml) in total is 32452 this gives a recovery of 71% from the starting number of beads, which was $4.55 \cdot 10^4$. This means we have a percentage loss of 29% during the experiment. In the literature, for example, K.B. Olesen, et al. have a recovery percentage of PS beads which is between 97% and 64% [25]. And another research from I. Dimante-Deimantovica, et al. have a recovery percentage between 94% and 37% [26]. With these numbers, the recovery of 71% is quite good.

There are some remarks to be made about the numbers acquired from the beads experiment and why a loss might have taken place. First, in the calculations made in section 3.3.2 (Verification with beads) is assumed that there is a minimal loss in the number of beads. In real life, it may be the case that more beads were lost. This is because the beads come in suspension [27] and they may have sedimented to the bottom of the bottle they are stored in. To make sure to have as many beads as possible, the bottle of the beads was shaken before the dilution series was started. After which 2 drops of suspension were put in an Eppendorf tube. This tube is then put into the sonicator to make sure the beads are not aggregated together.

Next, the dilution series was started. With these dilution steps, some beads may also have been lost. After the bead dilution was put into the IFB, some of the beads may have remained in the Eppendorf tube or the pipette tip, or they might have stuck to the side of the IFB. These are some reasons that there can already be fewer beads in the dilution before it is put in the IFB. These can also be some reasons that there are less beads found than that was put in the IFB.

5.2 Quantification

5.2.1 5 minutes of sterilization

This experiment was done to try and replicate the data of the article from D. Li, et al. [4]. As stated before, the experiment is done the same as in this article except, a staining and a different microscope with analysis methods are used here.

When comparing the data from the article with our results. It appeared that our experiment gave less release of MP's than the article retrieved. The difference between our (raw) results and those of the article is between factors 154 and 12.49 less. In the article, they do not mention the brands of IFB's they used, but between the different brands of IFB's the article also has a difference in the number of particles between their highest number and lowest number with a factor of 12.35 [4].

The difference in the release of MP's can be because of the use of a different brand of IFB than the researchers in the article of D. Li, et al. It can also be because they have a recovery test with a percentage of 94.9% and in this research, the recovery is 71% which is smaller meaning the amount of MP's counted is smaller [4] when the particles per liter are calculated to 94% the number of particles will also be higher.

Furthermore, for bottle two, the average size in μm^2 is different in the results section than stated in the table 8 in Appendix C.2 (Results: 5 minutes of sterilization). In the results section, the size of area 2 is excluded from the calculation for the average size because this size was way larger than the other sizes. When a closer look at that set of pictures was taken a very large fiber was found which made the size of the particles in area 2 much larger. So in the calculation of the number of particles per liter, the average size used was with the sizes from areas 1, 3, and 4.

5.2.2 30 minutes of sterilization

This second experiment was conducted to determine whether the duration of exposure to high temperatures indeed affects the release of microplastics. The bottles for this set of experiments are sterilized for 30 minutes instead of 5 minutes. The influence of temperature is tested differently than in the article of D. Li, et al. [4] they tested different heating temperatures and found a significant change in MP's release. In this research, the sterilization time is longer.

The average of particles per liter is only a bit higher for the 30 minutes of sterilization than the 5 minutes of sterilization. This is different than previously thought because with higher temperatures/longer heating the release of MP's normally goes up [4, 12, 28].

A possible explanation for the lower number of particles than expected could be that the water in which the IFB is sterilized has not been analyzed. The IFB is first sterilized and that water is thrown away after which the IFB is filled with distilled water and heated to 70℃, same as in the first experiment. The longer sterilization may therefore cause a release of more MP's, but the sterilization water (in which maybe the MP's are) is not analyzed/collected. This means that it cannot be determined whether the longer sterilization causes a higher release of MP's.

5.2.3 No sterilization or heating

The last experiment was done to test if no exposure to any heat indeed causes a lower release of MP's. This experiment is done because the article of D. Li, et al. [4] shows that fewer microplastics are released when the IFBs are not subjected to temperatures above 50℃. In

this research, the effect of temperature in combination with the release of microplastics was investigated. According to supplementary notes 2.3 of the article (of D. Li, et al.) [4], the bottles are not sterilized first (when they research different temperatures and MP release) to prevent interference with boiling temperatures. However, they did clean the bottle very well with detergent water, distilled water, and deionized water.

In this research the IFB is also not sterilized before the experiment without heating is done. But what is different relative to the experiments performed by D. Li, et al. is that the IFB's are not thoroughly cleaned. They are only rinsed three times with Milli-Q water before the experiment is started.
When analyzing the results of this set of experiments there are a lot more MP's found than in the experiments where the bottles are sterilized. A cause for this can be because the IFB's were not cleaned properly with different types of water and soap or at least submerged in water for 5 minutes. They were only rinsed 3 times with Milli-Q. It could be that MP's are released during the cleaning and boiling and then disappear in the sink with the cleaning water or boiling water. These MP's (that have not been rinsed away) can now show up on the filter during the experiment. This makes it seem that more MP's are released when the IFB is not boiled or heated compared to when it is heated or cleaned first.

To test this theory, more research is needed, for example by repeating the experiment with the same bottles (because now every time new bottles were used), but without heating. The bottle should first be cleaned thoroughly with cold water and soap and different types of water (normal water, Milli-Q, and distilled water) to rinse all soap residue. Further tests can also be carried out to determine whether fewer microplastics are released if the bottle is sterilized for five minutes. During the second part of the experiment, the water is not heated to 70℃ and simply kept at room temperature.

5.2.4 Size distribution

The blank control experiment and the quantification experiments include size distribution histograms, each paired with a cumulative percent graph. These graphs have a starting percentage between 80 and 93% of the first bin. All experiments give most particles with a size smaller than 50 μm^2 but when the bottles are sterilized also much larger particles were found. Especially when the sterilization is longer some very large particles are released. The experiment without sterilization or heating also resulted in the release of some very large particles.

Using the size distribution data, the amount of plastic ingested by a baby per liter of formula from an IFB is calculated. The average size of all MP's found in the quantification experiments is 64.8 μm^2.
Assuming each particle has a height of 1/4 of its area the volume is 1049.3 μm^3 which equals $1049.3 \cdot 10^{-12}$ cm^3. The average particles released across all experiments is $16.13 \cdot 10^4 \pm 4308$. The density of PP ranges between 0.895 and 0.92 g/cm^3 [29]. For this calculation, the density of 0.908g/cm^3 is assumed.
Therefore, one MP weighs $0.908 \cdot 1049.3 \cdot 10^{-12} = 9.53 \cdot 10^{-10}$ grams. The total mass of MP's released from one liter of water is $9.53 \cdot 10^{-10} \cdot 16.13 \cdot 10^4 = 1.54 \cdot 10^{-4}$ gram which equals 0.15 mg.

Assuming a baby drinks 1.2 liters a day from the IFB's until they are four years old, they ingest a total of 0.26 grams of plastics over those four years. This is lower than what researchers like K. Senathirajah, et al. and the WWF have calculated [25, 30], according to them we eat

approximately 0.1 to 5 grams of plastic per week. This seems not realistic just as researchers like M. Pletz, et al. [31] also argue and they prove that the claims made by K. Senathirajah, et al. and the WWF are wrong.

5.3 Characterisation of the material

5.3.1 FT-IR

Figure 16: The FT-IR spectra of the IFB itself and the particle powder retrieved from the FT-IR experiment in figure 5.

Figure 16 shows the spectra of the IFB and the powder retrieved from the FT-IR experiment. When comparing the spectra with an FT-IR spectrum of PP from the article written by I. Prabowo, et al. [24] it can be stated that the spectrum of figure 13a (in section 4.3.1, FT-IR) and the red line in figure 16 has peaks at the same spot of the graph in figure 19 (displayed in Appendix C.1, FT-IR from literature) and thus that the IFB indeed is made of PP.

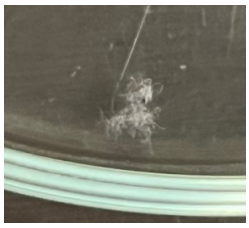


Figure 17: The powder that is harvested with the experiment shown in figure 5.

The graph in figure 13b (in section 4.3.1, FT-IR) and the green line in figure 16 are of the powder shown in figure 17 which is retrieved after the FT-IR experiment as explained in section 3.7.1 (Methods: FT-IR) and figure 5. This graph has some peaks that overlap with the places on the reference graph of PP and the IFB but it also has some peaks that do not overlap with the spectrum of PP.
The peaks that overlap with the reference graph are between the wave numbers of 3100-2500 cm^{-1}. These peaks are at the place of the spectrum causing a C-H bond of an alkane or an alkene. At 1450, 1370, and 840 cm^{-1} there are also some overlapping peaks. These peaks mean sequentially that there are more C-H bending alkane methyl groups, C-H bending alkane gem diol groups, and C=C bending alkene bonds [32, 33]. All these bonds with the presence of carbon are indications for a (plastic) polymer this can mean that the powder is PP.

However, some peaks are not in the spectrum of the IFB but in that of the powder. Such as the peak between 3300 and 3200 cm^{-1} which means that an O-H stretching is present. This can be of an alcohol, a carboxylic acid, or just some remaining water. There is also a broad peak around 1050 cm^{-1} which can mean that there is a CO-O-CO stretching of an anhydride present [32, 33].

The differences in spectra may arise from the appearance of the powder in figure 17 which looks a bit like fibers. This could lead to peaks in the spectrum reflecting fiber properties. The concentrated water containing the MP's was evaporated in a laminar flow cabinet, which theoretically prevents fibers entering the petri dish, during this process. It is possible that the fibers were present in the water before drying or that the contamination occurred during the experiment. Further research using FT-IR spectroscopy is necessary with a larger sample size of water through the IFB required. This ensures a sufficient amount of powder to enable a better verification of whether the particles released from the IFB are indeed PP.

5.3.2 SEM

The images made with the SEM are displayed in section 4.3.2 (SEM) and Appendix C.7 (Results: SEM). In these images, some particles are found and depicted. The black spots visible in the images are pores of the filter that are clogged with particles. The visible particles are angular and somewhat squared and round. The particles lie on top of the filter and stick a bit together. When compared to the literature these particles look like plastic particles with PP in particular [34, 35, 36, 37]. In these papers, the particles depicted in the SEM images also look somewhat squared and round.
Image 14b (and figure 22e in Appendix C.7, Results: SEM) is made with a higher voltage which makes the material a bit see-through. The white glow means that a pore is empty. The particles (and possible MP's) found on the filter are now also see-through. The higher voltages used in the SEM gives also some advantages for example the signal-to-noise ratio is improved, and it can also give higher contrast [38].

5.3.3 EDX

From the SEM sample, also some EDX measurements were conducted. The areas that were measured are displayed in figure 24b and 23a in Appendix C.8 (Results: EDX). Of these areas, some spots are highlighted from which EDX spectra are produced. In section 4.3.3 (EDX) two of the eight spectra are selected. The EDX spectra show the presence of carbon, oxygen, aluminum, and a bit of nickel, silicon, and chrome.

According to the manufacturer nickel and chrome are used as an adhesion layer between the PET and aluminum coating of the filter. The aluminum that is measured is from the filter coating. The remaining compounds are carbon, oxygen, and sometimes a bit of silicon. The carbon is present to the greatest extent and has the greatest peaks. It also has the greatest atomic percentage. This indicates that the measured particles consist of (plastic) polymers. The silicon may be indicated as an additive or it is also part of the adhesion layer of the filter coating. Lastly, the low amount of oxygen can be measured due to surface oxidation [39] or due to contamination of the sample or during the measurement [40].
When comparing the acquired EDX spectra with EDX spectra of plastic and especially PP, carbon has a big peak in the spectrum and there is also some oxygen and silicon present in the spectra [35, 41].

5.4 Recommendations for further research

5.4.1 Shaking the bottle

The article of D. Li, et al. [4] shakes the IFB on a shake table for 60 seconds at 180 rpm. During our test run it became clear that 180 rpm was too fast for the IFB's. In the experiments during this research, the IFB's were shaken at a shake table at 140 rpm. In further research, it would be a good idea to test it with 180 rpm.

5.4.2 Other bottle

With the order of transparent IFB's, Also two pink/blossom-colored IFB's were accidentally delivered. The thought was that these bottles may be fluorescent so some experiments were done to check this hypothesis. The experiments conducted were UV-VIS and an excitation measurement with Fluoromax-4. Some explanations about the experiments and the results from these experiments are displayed in Appendix B (Pink baby bottle). From these experiments can be concluded that the pink IFB is fluorescent for 470 nm and will emit green/yellowish light. In further research, it is possible to use this kind of bottle and then the Nile Red staining is probably not necessary. It is also easier to conclude that the particles found are coming from the IFB because they will be fluorescent same as the IFB.

It is also a possibility that a different brand of IFB's has another fabrication process or another mixture of polymers in their plastic. These things can cause different releases of MP's, so another recommendation is that the same experiments be conducted with a different brand of IFB.

5.4.3 Analyze sterilization water

Now the water the sterilization water of the IFB is not analyzed but it is just poured in the sink. The MP's that are released during the sterilization process is poured also in the sink. With the different sterilization times more MP's can be released and these were not analyzed in this research. To get a better view of the MP release with longer sterilization times it is recommended to also analyze the sterilization water.

6 Conclusion

In this chapter, conclusions will be drawn from the conducted experiments, whose results have already been presented and discussed. Finally, our research question will be answered.

6.1 Verification

The distilled water that is used during these experiments is clean of MP's. The data obtained from the blank control verification experiment can be used as a baseline for the results of the quantification experiments.
Furthermore, the recovery percentage/yield of the beads is 71%. The loss of beads (29%) can be due to the suspension in which the beads are delivered. It can also be that the beads have sedimented at the bottom of the bottle (in which they are stored) and did not leave the bottle with the suspension. It can also be that the beads stuck to the side of the Eppendorf tube, the pipette tip used during the dilution series, or it can also be that the beads stuck to the IFB itself.

6.2 Quantification of the MP's

To answer the question *"What is the effect of temperature on the release of microplastics from plastic infant feeding bottles?"*
Several experiments with different sterilization times or no sterilization or heating are done to quantify the release of MP's during these conditions.

With the current results, we cannot draw convincing conclusions on the influence of temperature and the release of MP's.

When the results were analyzed most released MP's were found in the experiment where no sterilization or heating was used. As discussed this can be due to the lack of cleaning the bottle before starting the experiments. Furthermore when the sterilization time is increased the release of MP's is a bit higher than with the sterilization of 5 minutes. To draw more definitive conclusions and better answer the research question, further research is necessary, such as analyzing the water used for sterilization.

To reduce the release of MP's coming from the IFB's, they should be rinsed and cleaned thoroughly before use. After cleaning the bottles should be sterilized again for 5 minutes after which it is recommended to rinse the IFB again.

6.3 Characterisation of the material

To characterize the residue collected after concentrating the MP's out of the 33 liters of water. Coming from the IFB's concentrated above the filter and letting the water evaporate leaving the MP's behind. FT-IR, SEM, and EDX were used.

The FT-IR graph of the residue has a lot of overlapping peaks with PP but also a view of different peaks that do not overlap with the spectrum of PP. The peaks that do overlap (for example between 3100 and 2500 cm^{-1}) suggest the presence of a lot of carbon which can mean that the residue is plastic. To draw better conclusions about the residue/powder more research is necessary.

Furthermore, as a second a third characterization method, SEM and EDX are used. when looking at the SEM images it can be that the particles found are MP's. The particles look somewhat squared and round. When the voltage is higher the particles and material become a bit see-through. The EDX spectra show that carbon and aluminum are present in large quantities in various particles on the filter this can mean that the particles are plastics. The large quantity of aluminum is due to the aluminum coating of the filter.

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A Abbreviations

Table 7: In this table the abbreviations used throughout the paper are shown.

Abbreviations	Meaning
PP	Polypropylene
PC	Polycarbonate
PS	Polystyrene
PA	Polyamide
PES	Polyethersulphone
IFB	Infant feeding bottle
MP's	Microplastics
PET	Polyethylene terephthalate
rpm	Rounds per minute
ml	Milliliter
µl	Microliter
FT-IR	Fourier transform infrared
SEM	Scanning electron microscopy
EDX	Energy dispersive X-ray
nm	Nanometer
mg	milligram

B Pink baby bottle

With the transparent bottles also two pink (blossom) bottles were delivered instead of the transparent ones. These pink IFB's are not used in the experiments discussed in Appendix E.3 (Protocol: 5 minutes of sterilization) through E.6 (Protocol: FT-IR experiment). However, a question about the fluorescence of the pink color was raised. So, a fluorescence and a UV-VIS measurement with the pink IFB were conducted.

B.1 Experiments with the pink baby bottle

B.1.1 UV-VIS

UV-VIS is a technique that measures the wavelength of visible or UV light that is either transmitted or absorbed by the sample with a blank sample as a comparison. This is used to analyze the pink IFB and see whether it has different properties than the transparent IFB and if it may be fluorescent [42].

B.1.2 Fluorescence

A few fluorescence measurements were done with Fluoromax-4 to determine if the pink IFB is fluorescent. The IFB was also put under the fluorescence microscope to determine if any light came from it. The Fluoromax-4 is a spectrofluorometer that can measure the fluorescence of a sample for certain wavelengths. It is an instrument that can do a broad range of measurements for fluorescence with high resolution and it is flexible with the kind of samples that can be used [43].

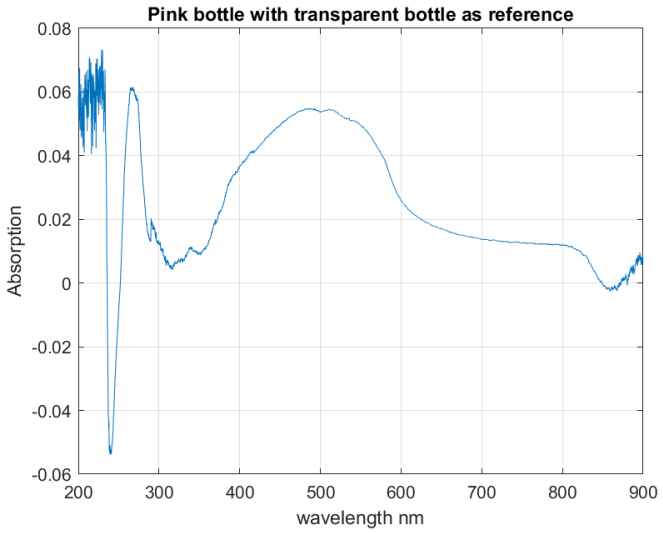
B.2 Results of the pink baby bottle

B.2.1 UV-VIS and fluorescence

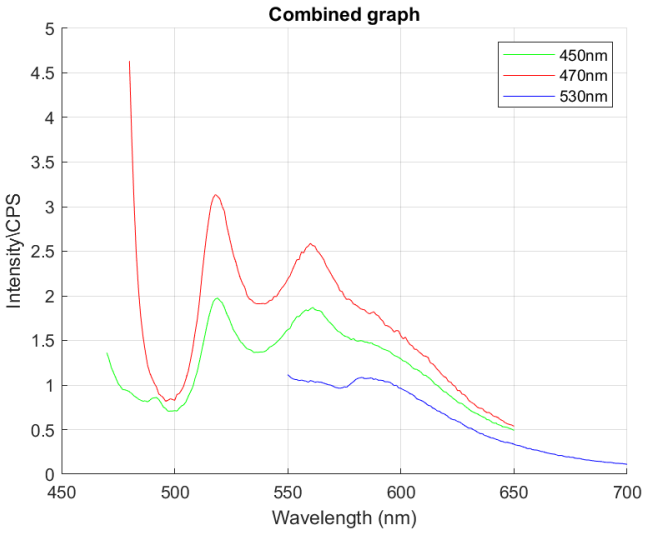
UV-VIS is conducted on the transparent and pink IFB. The results of the UV-VIS are displayed in figure 18a and the separate and all graphs in one are shown in Appendix C.5 (Results: UV-

VIS). The graph in figure 18a is made with the pink IFB as a sample and the transparent IFB as a reference.

In figure 18b the three emission spectra made with the Fluoromax-4 are displayed (the separate graphs are shown in Appendix C.6, Results: Fluoromax-4). The Fluoromax-4 was set first at 530 nm because of the broad peak between 450 and 550 nm on the UV-VIS absorption spectrum and 530 nm is the wavelength for the green color which will be the excitation wavelength for pink samples. After that, it was put at 450 nm and 470 nm for blue light because pink is a mix of red and blue light, and these numbers still fit the broad peak at the UV-VIS absorption spectrum.



(a) The Absorption spectrum obtained with the UV-VIS for the pink bottle with the transparent bottle as reference.



(b) The combined emission spectrum of the three measures done with the Fluoromax at 450, 470, and 530 nm.

Figure 18: The absorption and emission spectra of the pink IFB.

B.3 Discussion of the experiment with the pink baby bottle

B.3.1 UV-VIS and fluorescence

The UV-VIS results show absorption peaks where the pink IFB absorbs the light strongly. There are two noticeable peaks around 250 nm. This indicates a strong absorption in the UV region. There is also a big peak around 530 nm this is in the visible region indicating the pink color, because the green color is absorbed and the pink/red wavelength can be reflected or transmitted [44, 45]. The absorption values are between 0.07 and -0.06 the negative absorption values are probably caused by instrumental noise [46]. Instrumental noise can also be the explanation for the minimal fluctuation in the baseline [47].

The emission spectra for the different wavelengths are shown in figure 18b. The graphs at wavelength 450 and 470 nm have two peaks around 518 and 560 nm. The graph of excitation at 530 nm also has a small peak at 560 nm. The big peaks around 518 and 560 nm suggest that the pink sample can best emit light at these two wavelengths. These wavelengths have the color green and yellow [48, 49]. The spectrum suggests that the 470 nm excitation has the strongest fluorescence for the pink sample. It has the highest intensity and the highest peaks at 518 and 560 nm. This data can be used to identify the properties of the sample for

fluorescence and use it for optimal excitation.
The pink sample was also placed under the fluorescence microscope with blue light. When looked through the microscope at the time, green yellowish light came from it.

B.4 Conclusion about the pink baby bottle

The pink bottle that accidentally came with the transparent bottles, was checked for fluorescence. The pink bottle is fluorescent for excitation at 470 nm. It also lit up under the fluorescence microscope. This means that with further research the pink baby bottle can be used and there is no need for Nile Red staining anymore.

C Results

C.1 FT-IR from literature

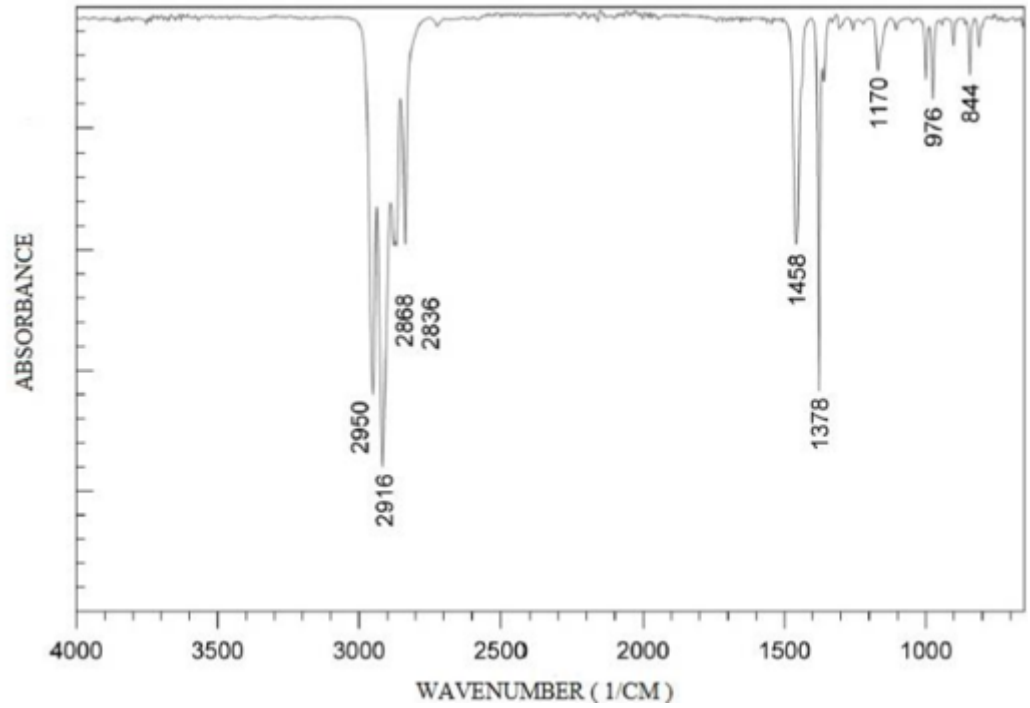


Figure 19: The FT-IR spectrum of PP retrieved from the article of I. Prabowo, et al. [24] used as reference.

C.2 Results: 5 minutes of sterilization

Table 8: The results obtained from the bottle after the experiment was done as described in Appendix E.3.

	Area 1	Area 2	Area 3	Area 4	Average
Bottle 1					
Total covarage in μm^2	$3.43 \cdot 10^4$	$4.79 \cdot 10^4$	$6.91 \cdot 10^4$	$3.69^* 10^4$	$4.705 \cdot 10^4$
Average particle size in μm^2	105.7	59.3	62.6	83.5	77.8
Bottle 2					
Total covarage in μm^2	$8.55 \cdot 10^4$	$3.75 \cdot 10^4$	$1.69 \cdot 10^5$	$1.03 \cdot 10^5$	$1.83 \cdot 10^5$
Average particle size in μm^2	54.5	367.4	31.5	112.2	141.5 we exclude Area 2 due to excessive difference in value the size is 66.2
Bottle 3					
Total covarage in μm^2	$2.68 \cdot 10^5$	$1.25 \cdot 10^5$	$6.48 \cdot 10^5$	$1.23 \cdot 10^5$	$2.91 \cdot 10^5$
Average particle size in μm [5]	120.1	42.2	207.7	117.1	121.9

Table 9: The total coverage per liter in μm^2 for the bottles during the experiments.

Bottle 1		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$4.71 \cdot 10^4$	$43.9 \cdot 10^4$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$43.9 \cdot 10^4$	$258.21 \cdot 10^4$
Bottle 2		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$1.83 \cdot 10^5$	$17.06 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$17.07 \cdot 10^5$	$100.33 \cdot 10^5$
Bottle 3		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$2.91 \cdot 10^5$	$27.12 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$27.12 \cdot 10^5$	$159.54 \cdot 10^5$

C.3 Results: 30 minutes of sterilization

Table 10: The results obtained from the bottle after the experiment done as described in Appendix E.4.

	Area 1	Area 2	Area 3	Area 4	Average
Bottle 1					
Total particle coverage μm^2	$2.50 \cdot 10^5$	$1.38 \cdot 10^5$	$7.69 \cdot 10^4$	$1.13 \cdot 10^5$	$1.45 \cdot 10^5$
Average size in μm^2	87.9	74.3	53.0	73.1	72.1
Bottle 2					
Total particle coverage μm^2	$1.33 \cdot 10^5$	$3.19 \cdot 10^4$	$8.85 \cdot 10^4$	$1.33 \cdot 10^5$	$9.66 \cdot 10^4$
Average size in μm^2	24.5	24.9	19.9	51.8	30.3
Bottle 3					
Total particle coverage μm^2	$4.33 \cdot 10^4$	$1.03 \cdot 10^5$	$2.34 \cdot 10^5$	$1.53 \cdot 10^5$	$1.33 \cdot 10^5$
Average size in μm^2	27.5	40.6	149.3	115.4	83.2

Table 11: The total coverage per liter in μm^2 for the bottles during the experiments.

Bottle 1		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$1.45 \cdot 10^5$	$13.51 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$13.51 \cdot 10^5$	$79.49 \cdot 10^5$
Bottle 2		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$9.66 \cdot 10^4$	$9.03 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$9.03 \cdot 10^5$	$52.96 \cdot 10^5$
Bottle 3		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$1.33 \cdot 10^5$	$12.40 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$12.40 \cdot 10^5$	$72.92 \cdot 10^5$

C.4 Results: No sterilization or heating

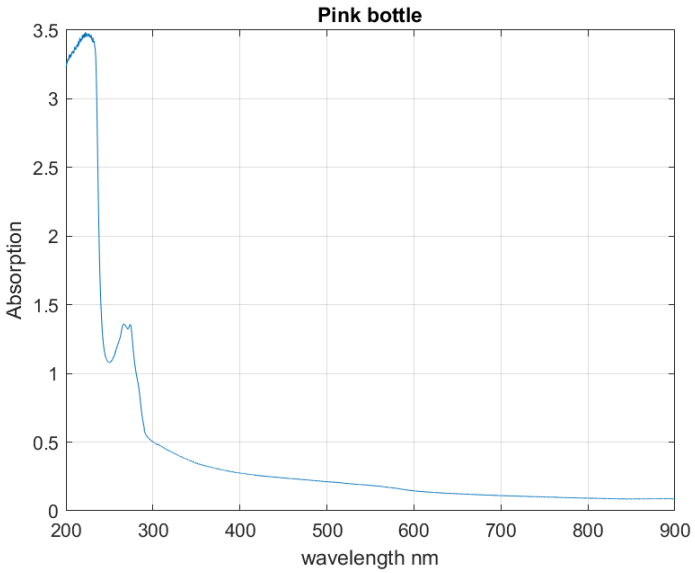
Table 12: The results obtained from the bottle after the experiment were done as described in Appendix E.5.

	Area 1	Area 2	Area 3	Area 4	Average
Bottle 1					
Total particle coverage μm^2	$2.70 \cdot 10^5$	$2.34 \cdot 10^5$	$2.39 \cdot 10^5$	$3.18 \cdot 10^5$	$2.06 \cdot 10^5$
Average size in μm^2	40.6	33.5	48.6	39.8	40.6
Bottle 2					
Total particle coverage μm^2	$1.20 \cdot 10^5$	$3.88 \cdot 10^4$	$1.49 \cdot 10^5$	$2.55 \cdot 10^5$	$1.41 \cdot 10^5$
Average size in μm^2	28.1	29.9	24.2	43.4	31.4
Bottle 3					
Total particle coverage μm^2	$3.02 \cdot 10^5$	$3.31 \cdot 10^5$	$3.49 \cdot 10^5$	$3.48 \cdot 10^5$	$3.33 \cdot 10^5$
Average size in μm^2	95.1	73.2	66.0	70.7	76.3

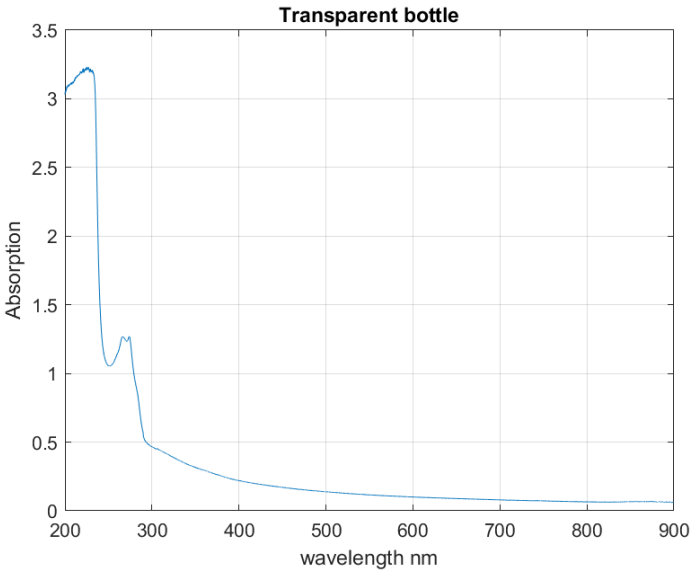
Table 13: The total coverage per liter in μm^2 for the bottles during the experiments without heating.

Bottle 1		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$2.06 \cdot 10^5$	$19.20 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$19.20 \cdot 10^5$	$112.94 \cdot 10^5$
Bottle 2		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$1.41 \cdot 10^5$	$13.14 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$13.14 \cdot 10^5$	$77.30 \cdot 10^5$
Bottle 3		
	Measured area	**Filter area**
cm^2	1	9.32
μm^2 coverage	$3.33 \cdot 10^5$	$31.04 \cdot 10^5$
	Volume IFB	**Volume**
ml	170	1000
μm^2 coverage	$31.04 \cdot 10^5$	$182.56 \cdot 10^5$

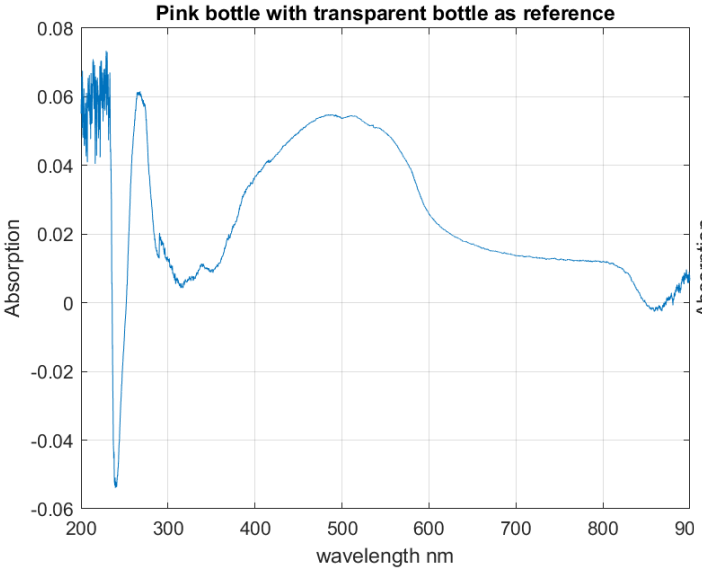
C.5 Results: UV-VIS



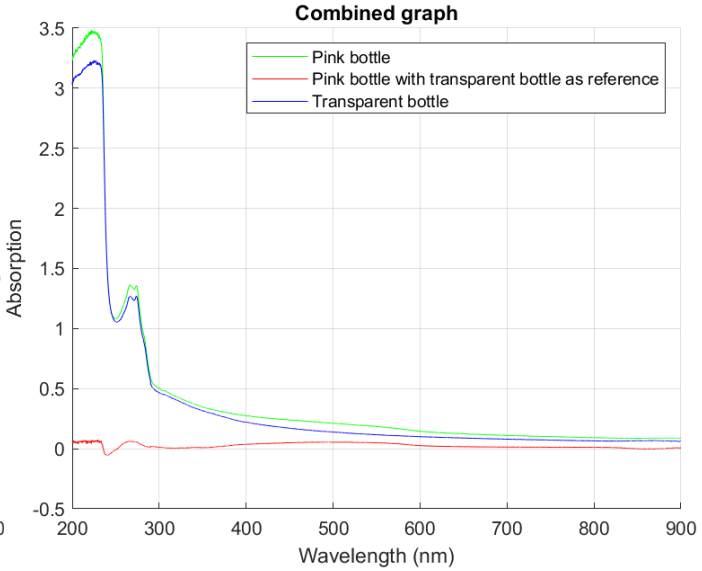
(a) The absorption spectrum of the pink baby bottle.



(b) The absorption spectrum of the transparent baby bottle.



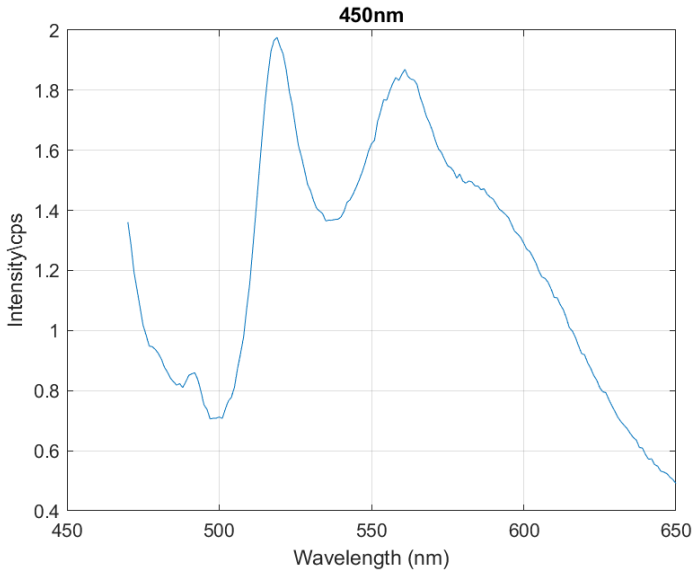
(c) The absorption spectrum of the Pink baby bottle as a sample and the transparent bottle as a reference.



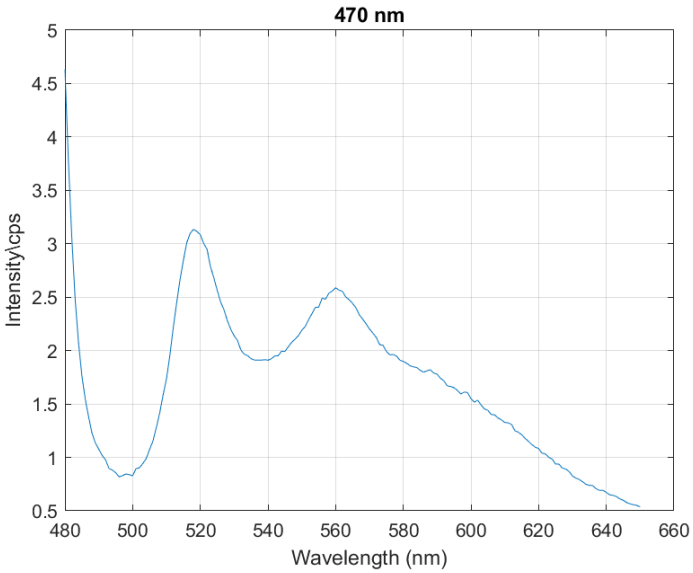
(d) The Absorption spectra obtained with the UV-VIS for all bottles.

Figure 20: The separate and combined absorption spectra obtained with the UV-VIS.

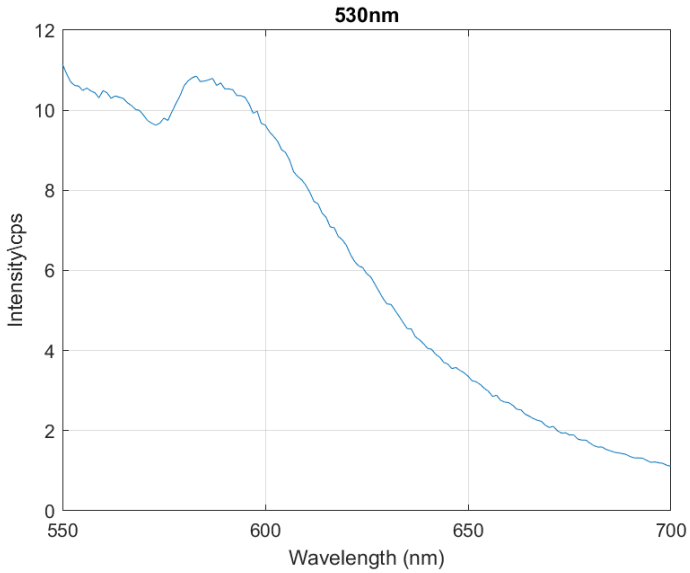
C.6 Reulsts: Fluoromax-4



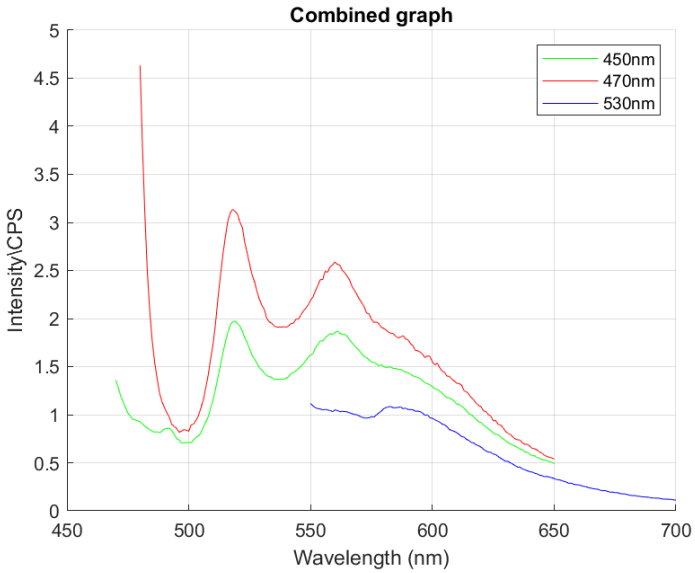
(a) The emission spectrum of the Fluoromax at 450 nm.



(b) The emission spectrum of the Fluoromax at 470 nm.



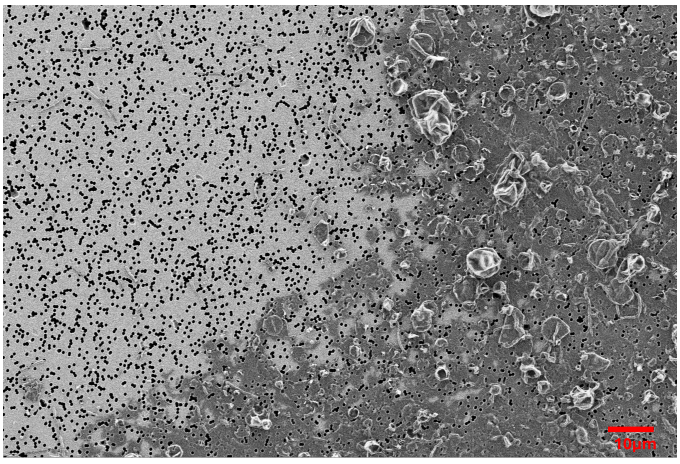
(c) The emission spectrum of the Fluoromax at 530 nm.



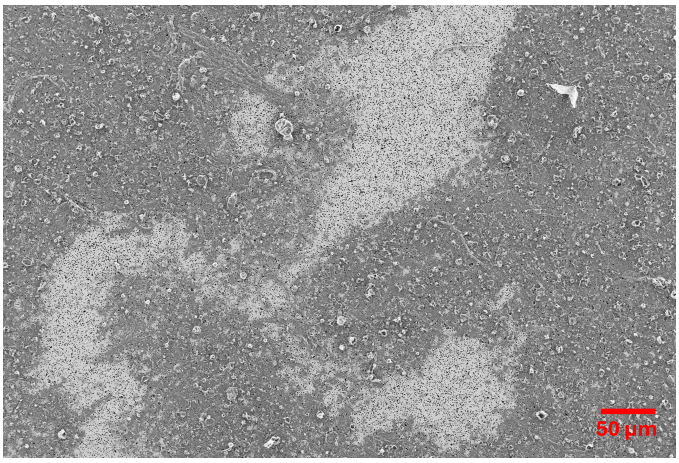
(d) The combined graph of the three measures done with the Fluoromax at 450, 470, and 530 nm

Figure 21: The separate and combined emission spectra of the pink baby bottle.

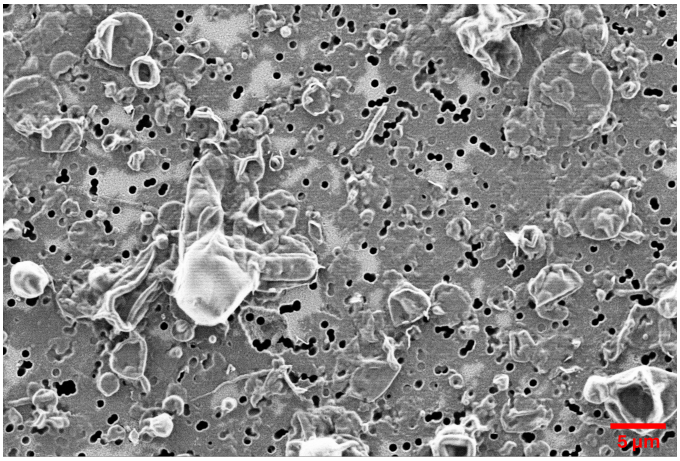
C.7 Results: SEM



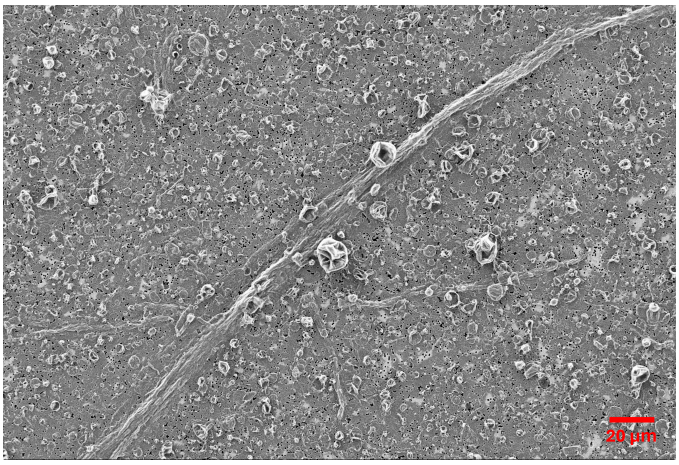
(a) Image of particles on the filter at 1.40kV and a 2.00K X magnification.



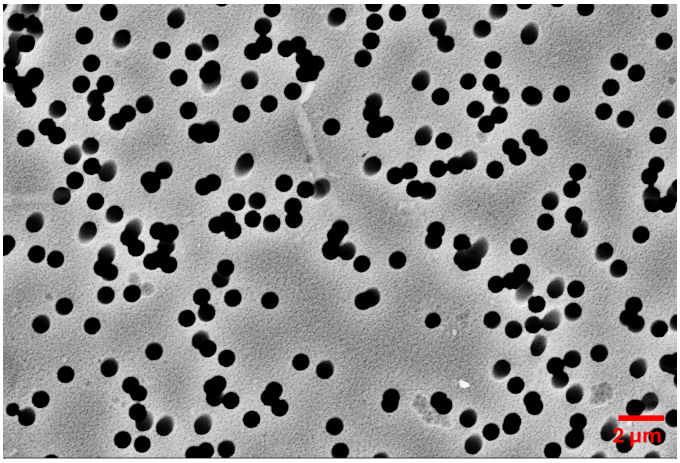
(b) Image of particles on the filter at 1.40kV and a 500X magnification.



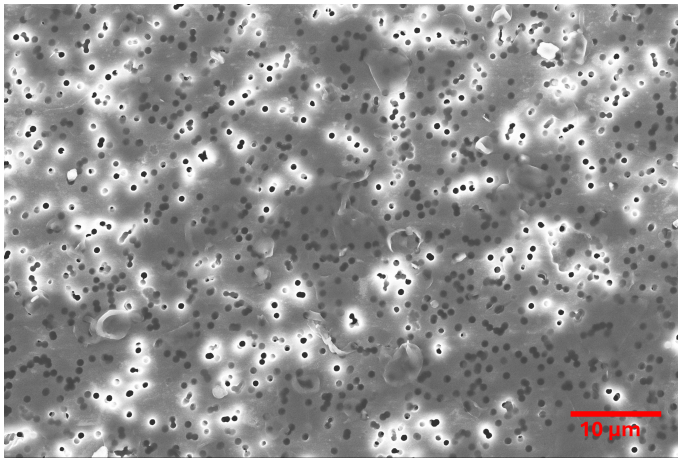
(c) Image of particles on the filter at 1.40kV and a 5.00 K X magnification.



(d) Image of particles on the filter at 1.40kV and a 1.01 K X magnification.



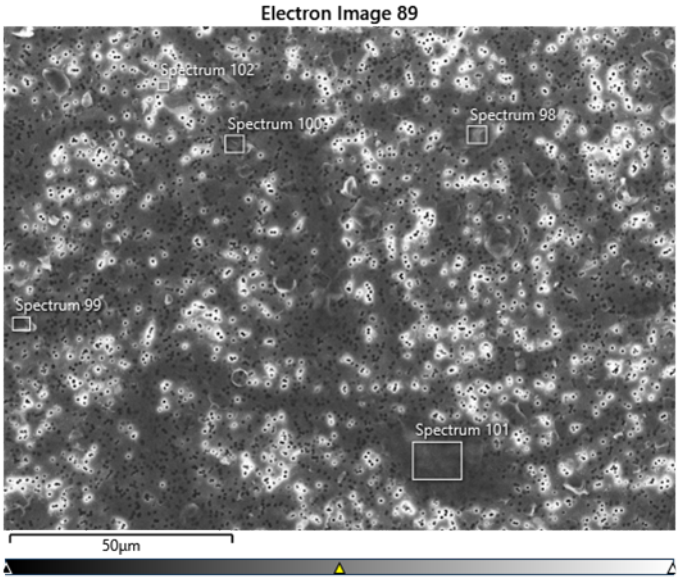
(e) Image of particles on the filter at 10.00kV and a 10.00 K X magnification.



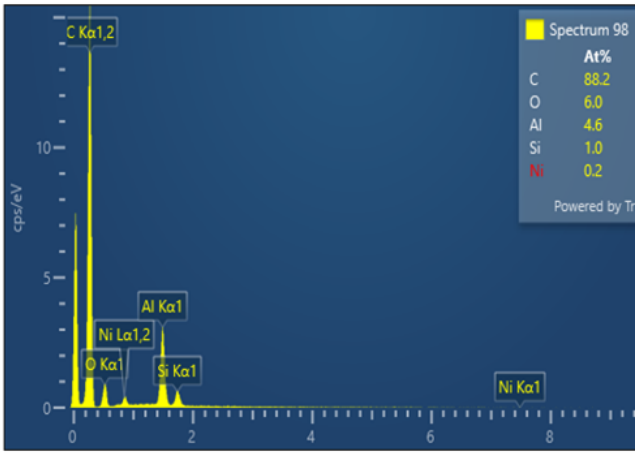
(f) Image of particles on the filter at 10.00kV and a 4.03 K X magnification.

Figure 22: Images of the particles on the filter from the SEM with different voltages and magnifications.

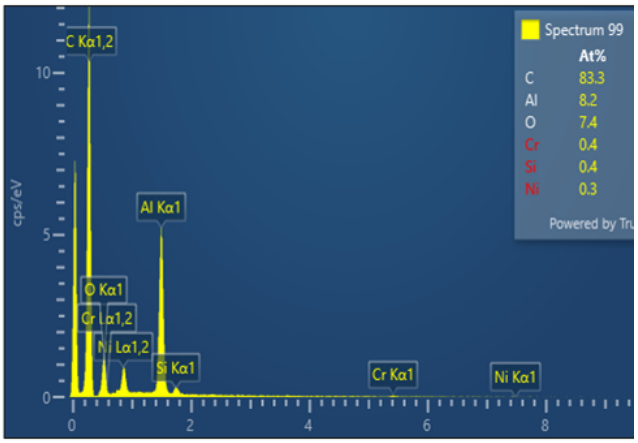
C.8 Result: EDX



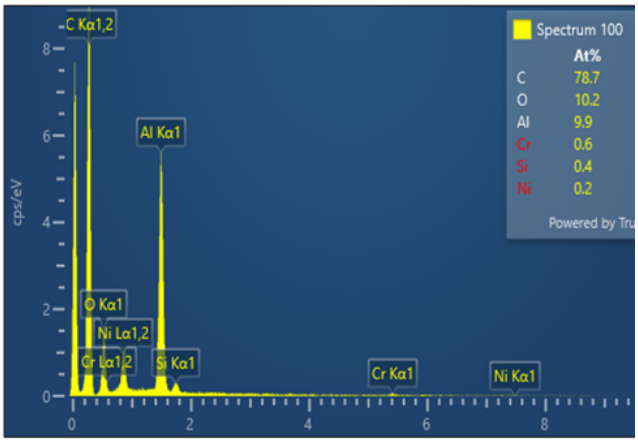
(a) Electron image overview of the different spots where an EDX spectrum is made of. All spots are possible MP's.



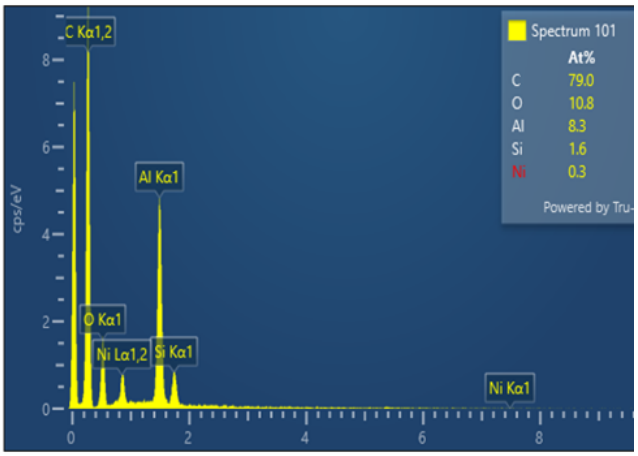
(b) EDX spectrum of spot 98.



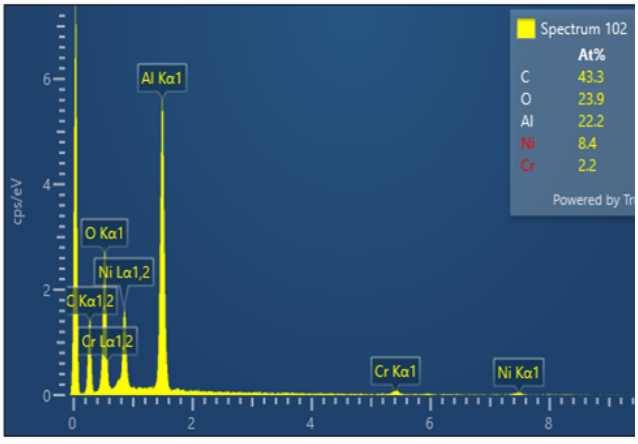
(c) EDX spectrum of spot 99.



(d) EDX spectrum of spot 100.

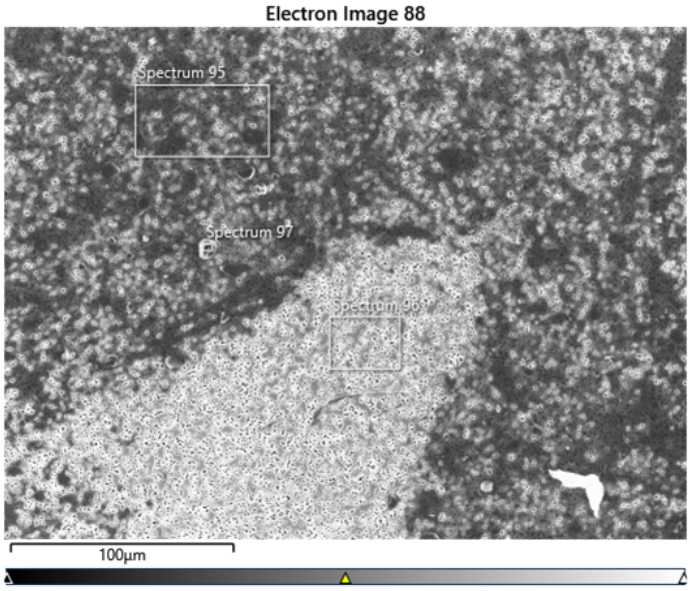


(e) EDX spectrum of spot 101.

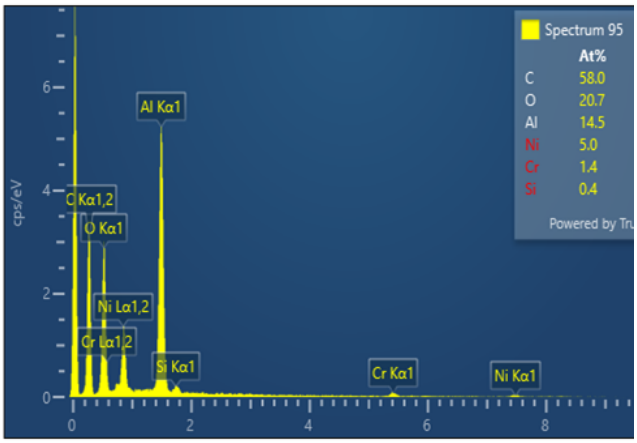


(f) EDX spectrum of spot 102.

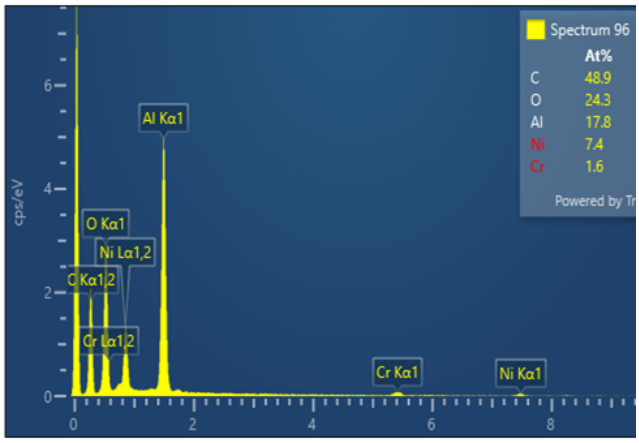
Figure 23: All images and spectra made with EDX of different spots from the electron image 89.



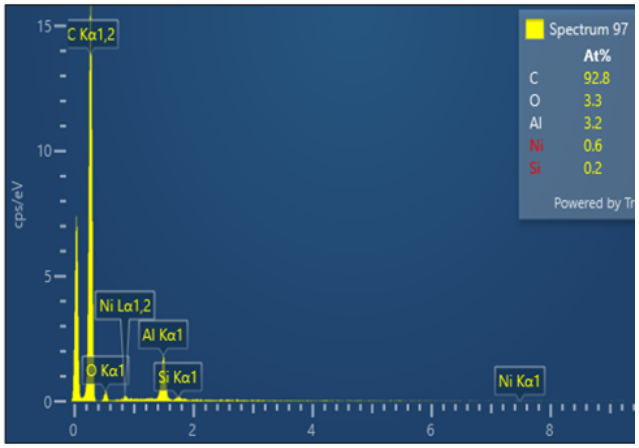
(a) Electron image overview of the different spots where an EDX spectrum is made of. All spots are possible MP's, this image has a smaller magnification.



(b) EDX spectrum of spot 95.



(c) EDX spectrum of spot 96.

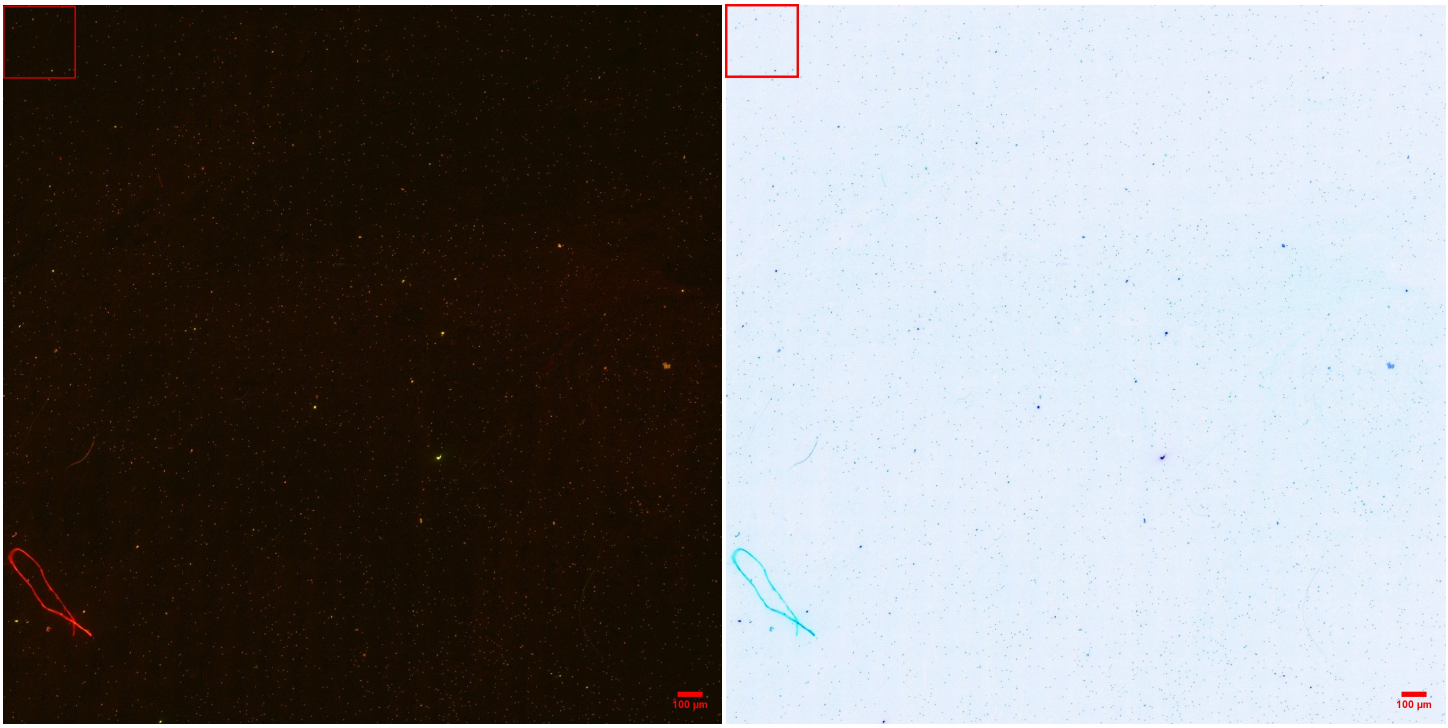


(d) EDX spectrum of spot 97.

Figure 24: All images and spectra made with EDX of different spots from the electron image 88.

D Stitched pictures

D.1 Stitched: beads

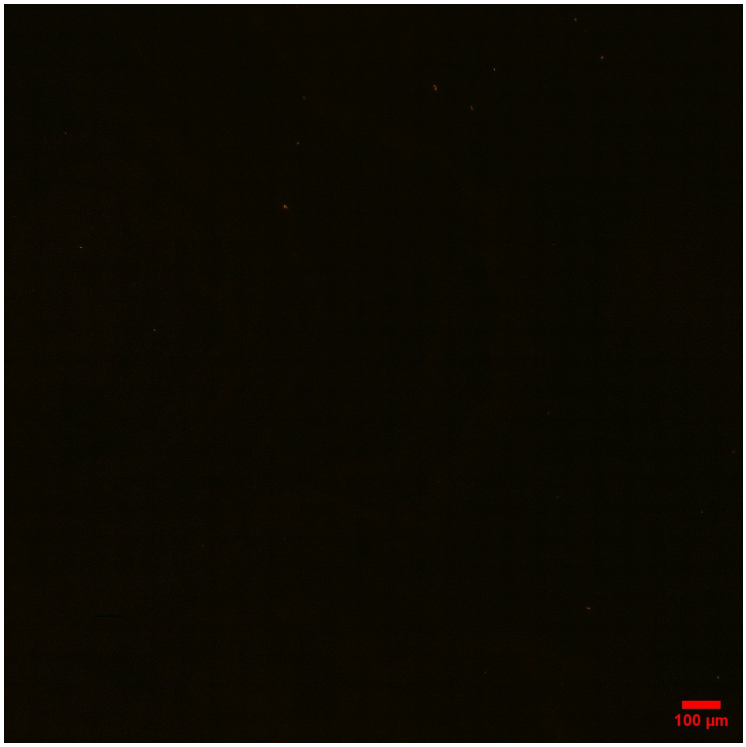


(a) The stitched image of the images made with the microscope for the beads of area 1. The red square is highlighted and is shown in section 4.1.2 (Beads) in figure 7.

(b) The stitched image of the images made with the microscope for the beads of area 1, but then with the colors inverted. The red square is highlighted and is shown in section 4.1.2 (Beads) in figure 7.

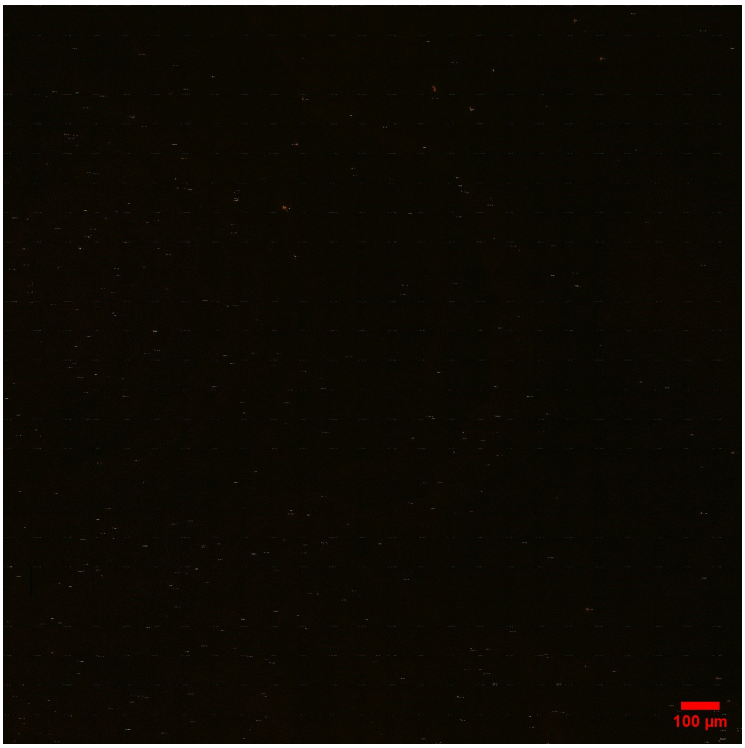
Figure 25: The stitched images of area 1 for the beads.

D.2 Stitched: Blank control



(a) The stitched image of all microscope pictures of the control experiment of area 1.

(b) The stitched image of all microscope pictures of the control experiment of area 1 but then inverted.



(c) The stitched image of all microscope pictures of the control experiment of area 1 after Python analysis.

(d) The stitched image of all microscope pictures of the control experiment of area 1. After Python analysis and inverted.

Figure 26: The stitched images of area 1 of the control experiment from the microscope.

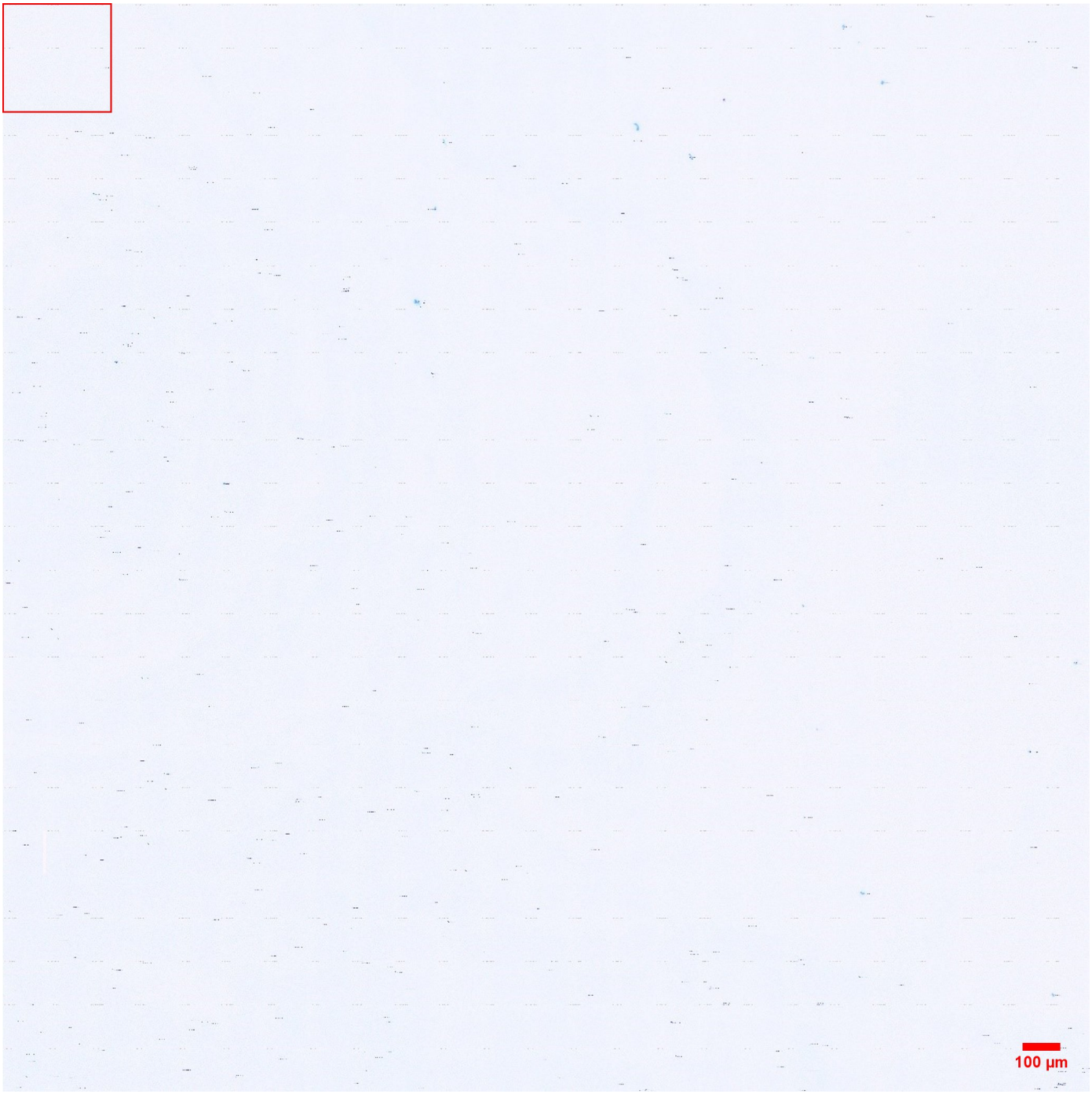
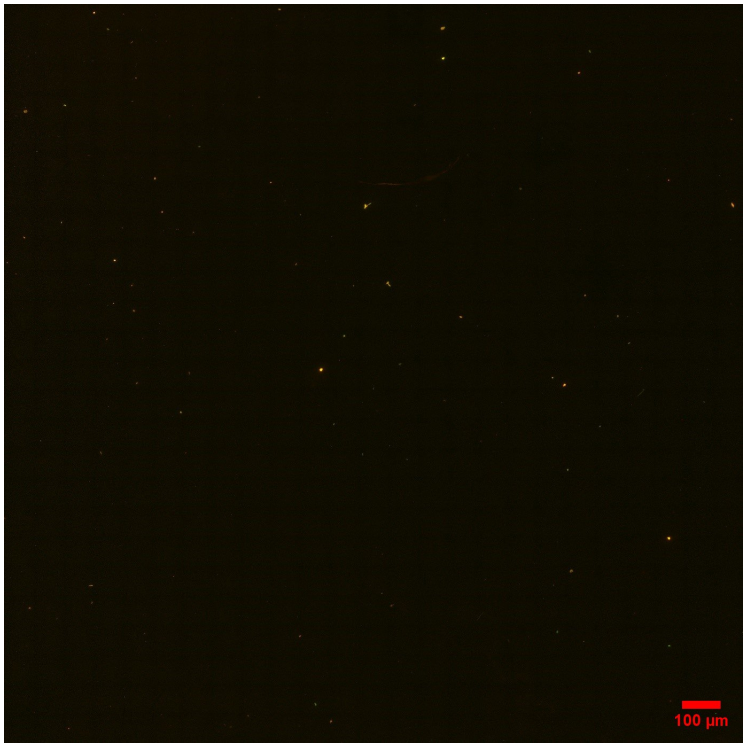


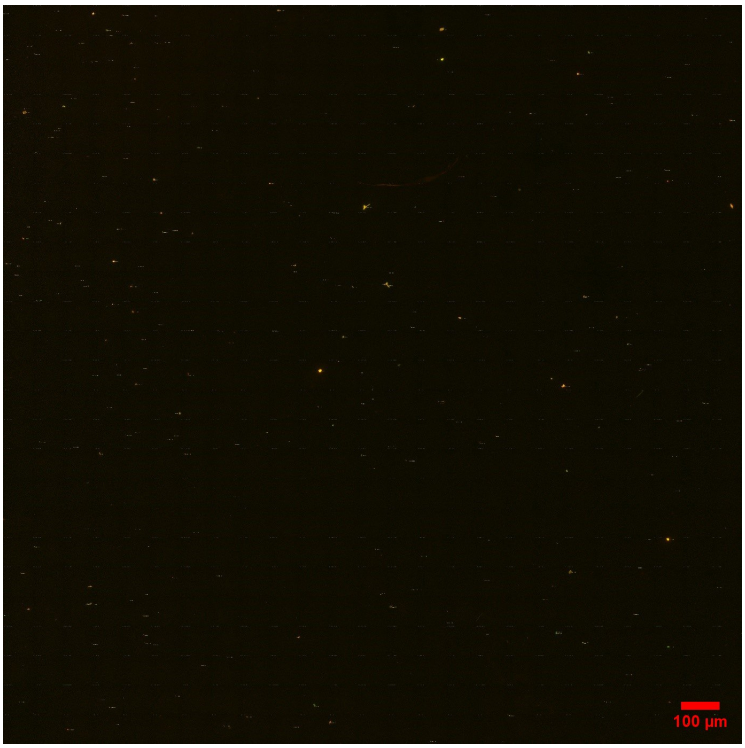
Figure 27: The stitched image of the control experiment of area 1. After Python analysis. The red square is highlighted and is shown in section 4.2.5 (Results: overview) in figure 12a.

D.3 Stitched: 5 minutes of sterilization



(a) The stitched image of all microscope pictures of the first experiment of area 1.

(b) The stitched image of all microscope pictures of the first experiment of area 1 but then inverted.



(c) The stitched image of all microscope pictures of the first experiment of area 1 after Python analysis.

(d) The stitched picture of all microscope pictures of the first experiment of area 1. After Python analysis and inverted.

Figure 28: The stitched images of area 1 of the experiment with 5 minutes of sterilization from the microscope.

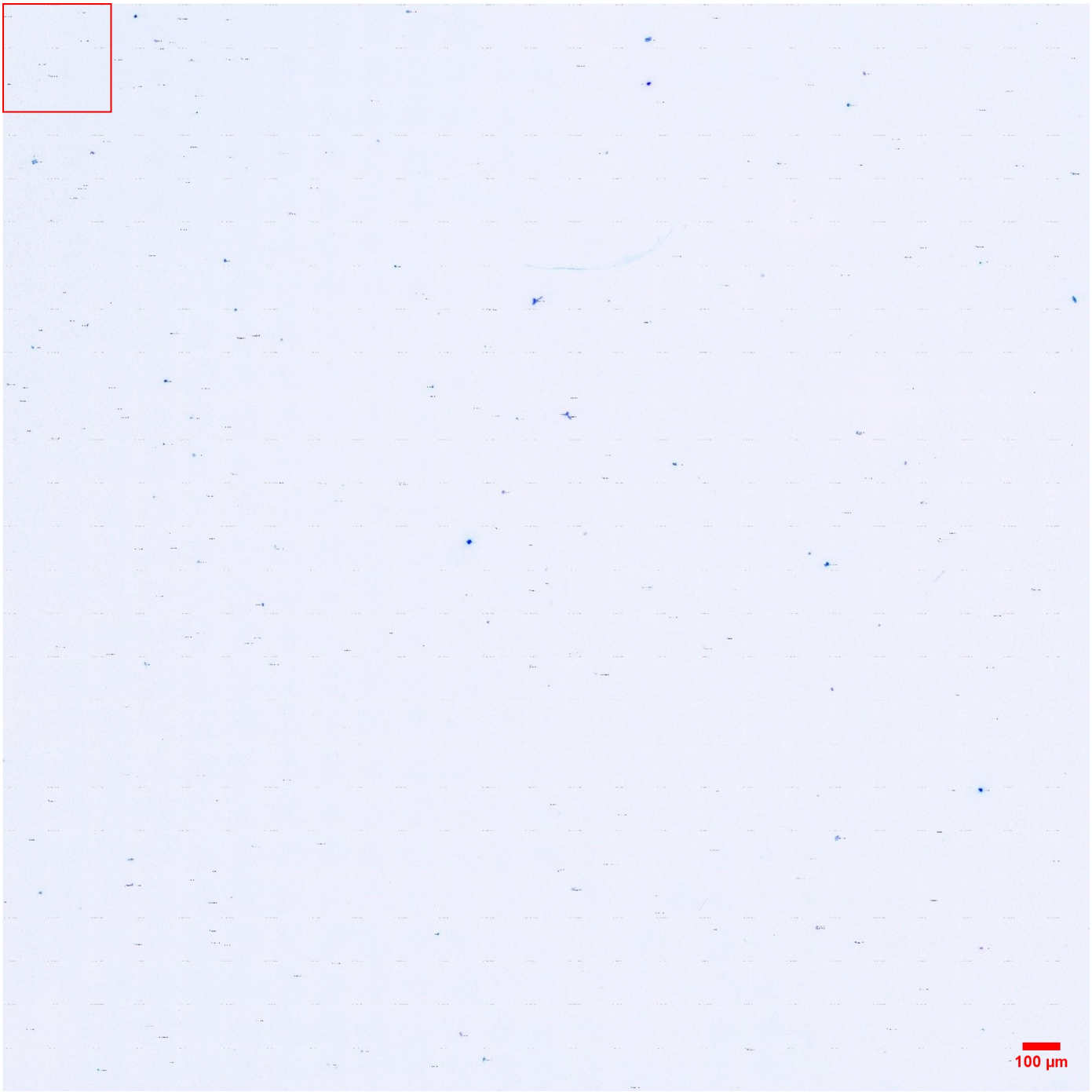
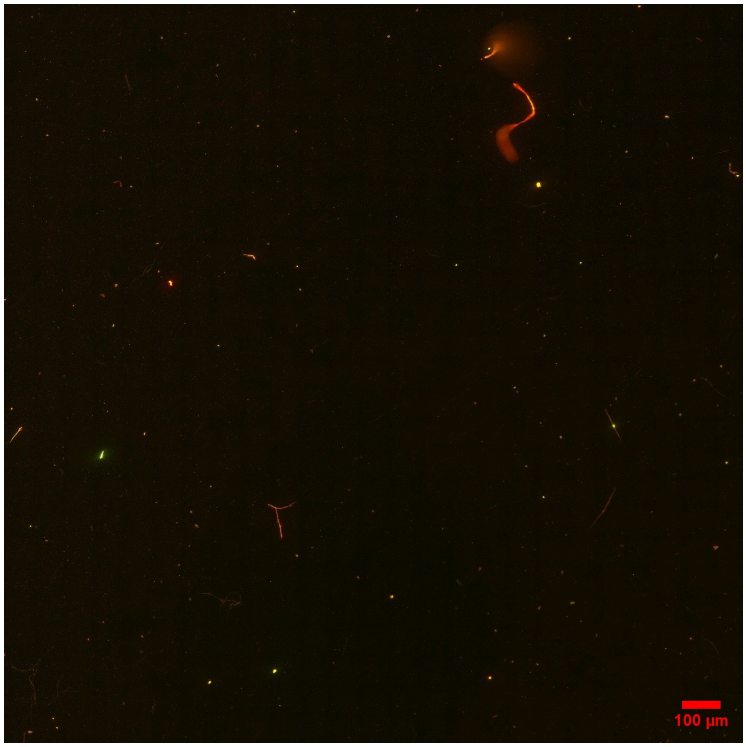
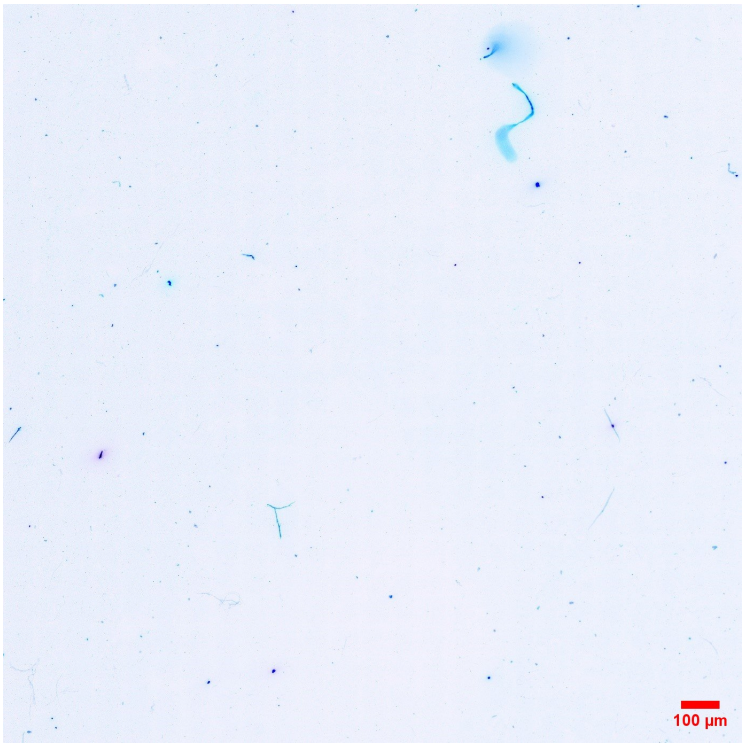


Figure 29: The stitched image of the experiment of 5 minutes of sterilization of area 1. After Python analysis. The red square is highlighted and is shown in section 4.2.5 (Results overview) in figure 12b.

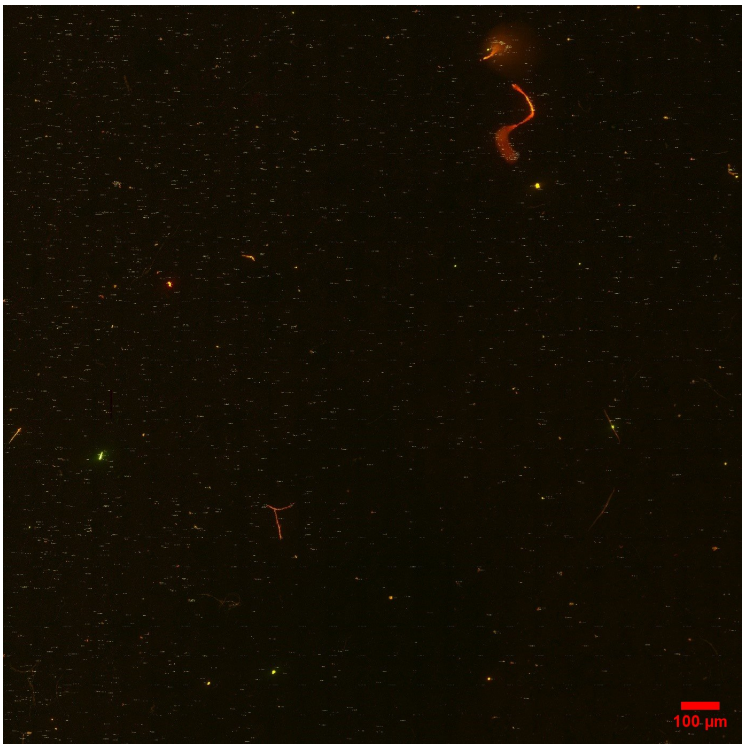
D.4 Stitched: 30 minutes of sterilization



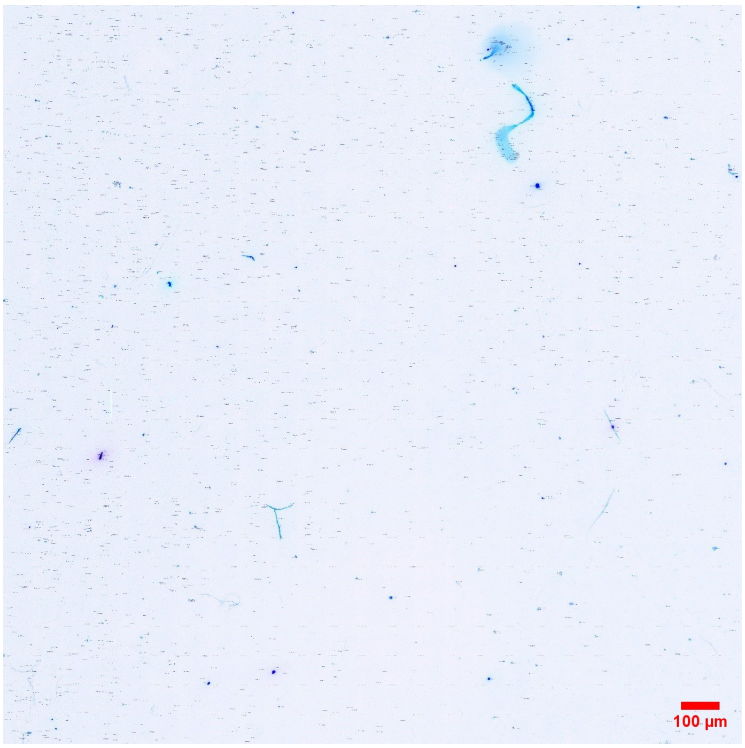
(a) The stitched image of all microscope pictures of the second experiment of area 1.



(b) The stitched image of all microscope pictures of the second experiment of area 1 but then inverted.



(c) The stitched image of all microscope pictures of the second experiment of area 1 after Python analysis.



(d) The stitched image of all microscope pictures of the second experiment of area 1. After Python analysis and inverted.

Figure 30: The stitched images of area 1 of the experiment with 30 minutes of sterilization from the microscope.

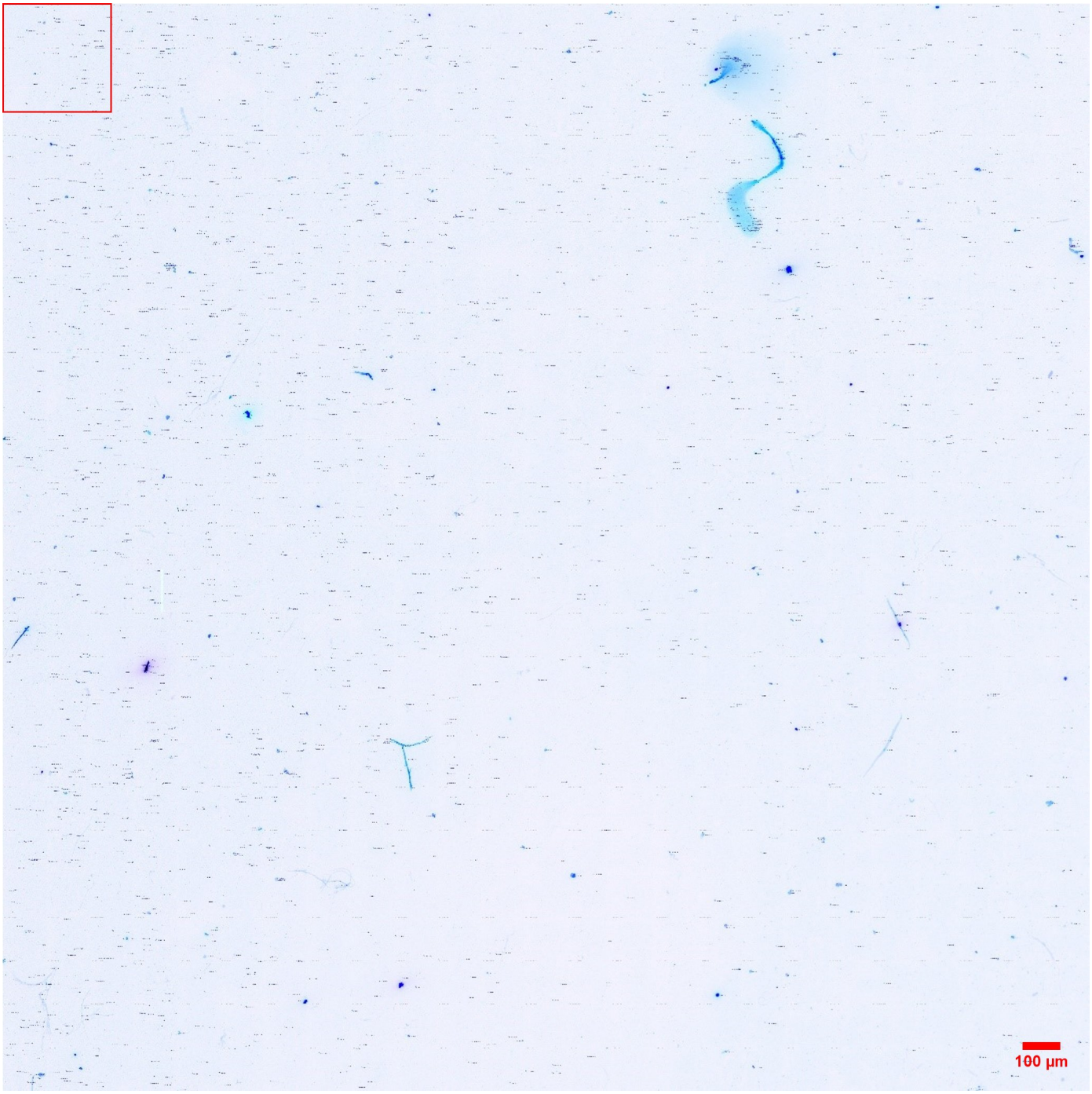
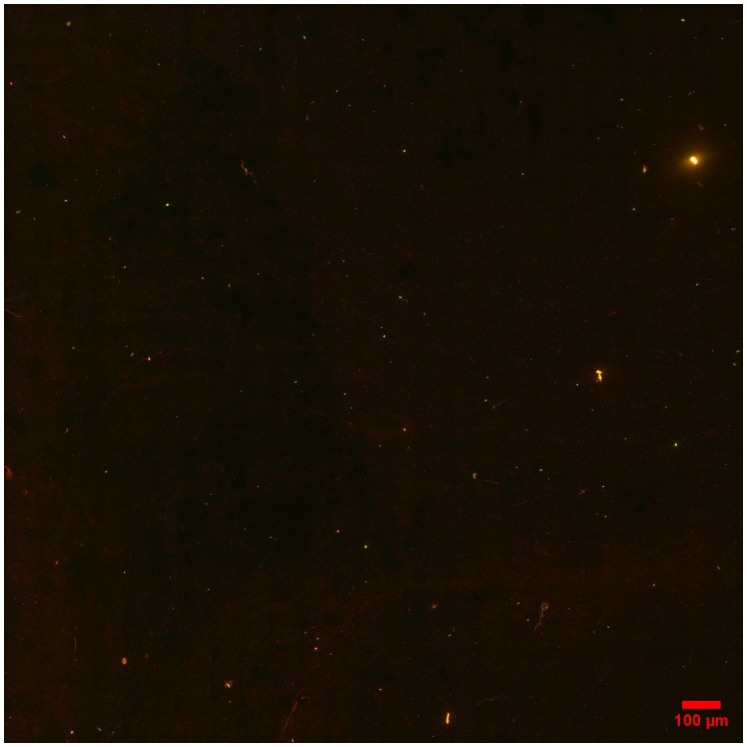
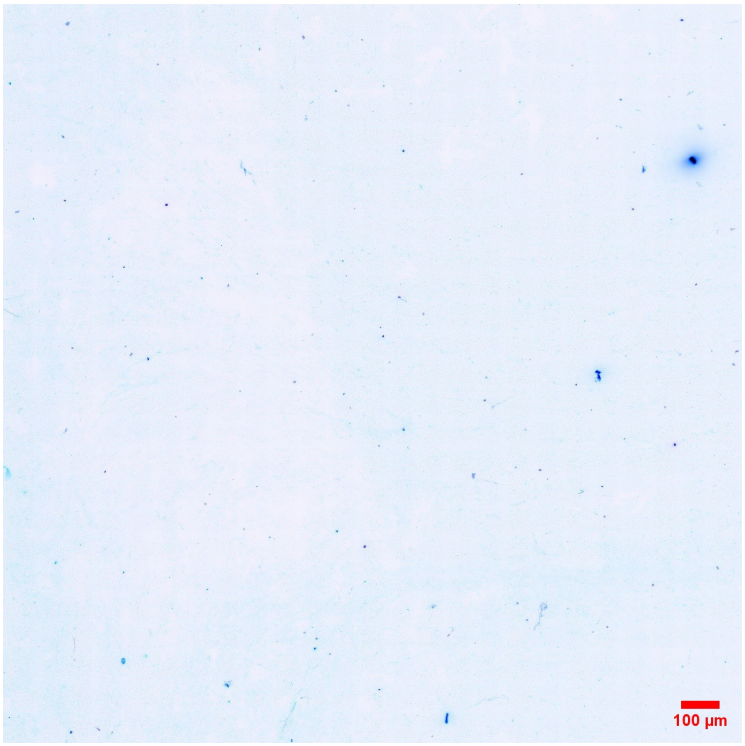


Figure 31: The stitched image of the experiment of 30 minutes of sterilization of area 1. After Python analysis. The red square is highlighted and is shown in section 4.2.5 (Results overview) in figure 12c.

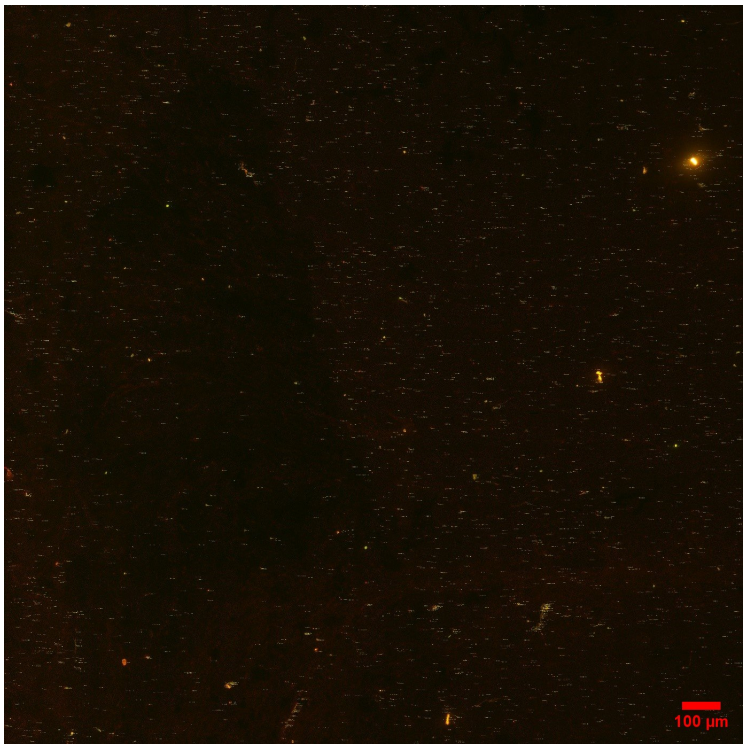
D.5 Stitched: no sterilization or heating



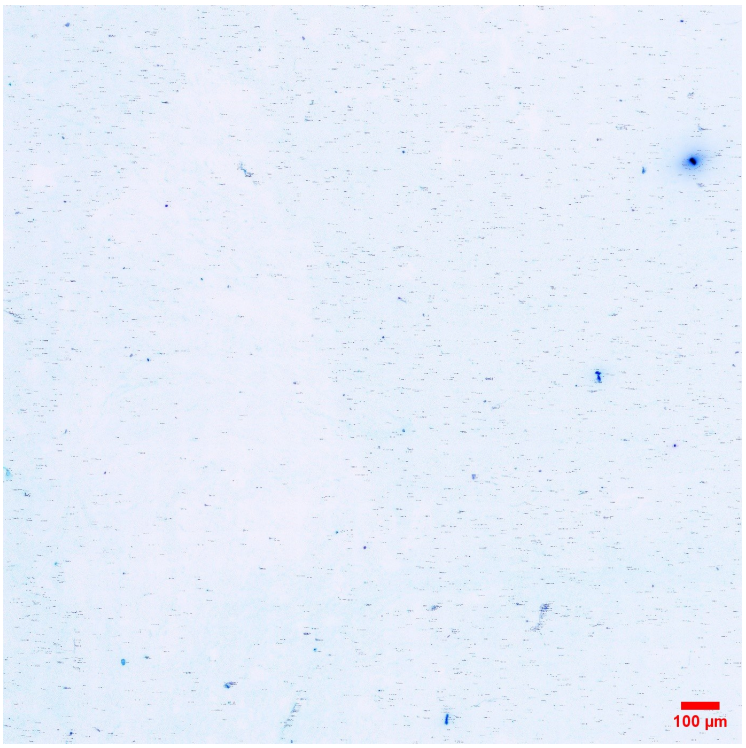
(a) The stitched image of all microscope pictures of the third experiment of area 1.



(b) The stitched image of all microscope pictures of the third experiment of area 1 but then inverted.



(c) The stitched image of all microscope pictures of the third experiment of area 1 after Python analysis.



(d) The stitched image of all microscope pictures of the third experiment of area 1. After Python analysis and inverted.

Figure 32: The stitched images of area 1 of the experiment without heating the bottle from the microscope.

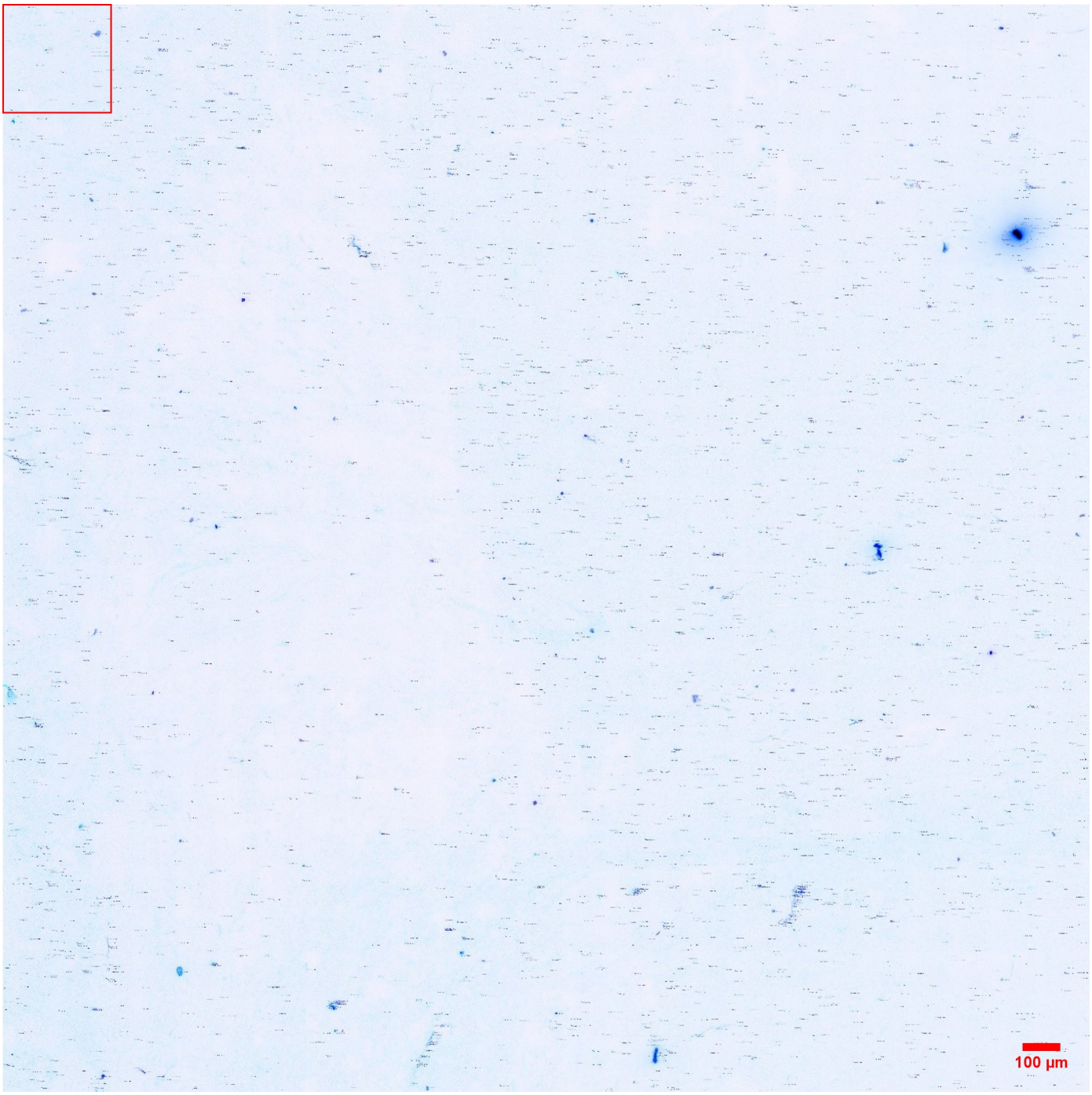


Figure 33: The stitched image of the experiment of no sterilization of area 1 after Python analysis. The red square is highlighted and is shown in section 4.2.5 (Results overview) in figure 12d.

E Protocols

E.1 Protocol: blank control

How the control experiment is done to check the water is clean.

1. Put the filter set-up on the kitchen scale.
2. Assemble the filter set-up.
3. Pour 170 ml of distilled water through the filter set-up.
4. Put the plug in the filter.
5. Make the Nile red solution.
6. Put the Nile red solution on the filter.
7. Wait 30 minutes.
8. Let the Nile red solution run through the filter.
9. Let the filter air dry.
10. Put the filter in a glass petri dish for storage or place it on the object slide under the microscope for analysis.

E.2 Protocol: beads

1. Fill a clean glass beaker with Milli-Q.
2. Rinse the baby bottle 3 times with 25 ℃ Milli-Q.
3. Submerge the (uncaped) baby bottle in the Milli-Q in the glass beaker.
4. Put the beaker with Milli-Q in the microwave to heat the water.
5. Heat the Milli-Q until it boils and let it boil for 5 minutes.
6. Take out the bottle and let it air dry, for 30 minutes with the opening covered.
7. Put 170 ml distilled water in the baby bottle.
8. Put in the dilution with the beads.
9. Heat the bottle in the microwave until the water is 70℃.
10. Let it turn (with the cap on) on a shaker for 60 seconds at 140 rpm.
11. Let the water cool down to 37℃.
12. Let the water from the bottle run through the aluminum filter.
13. Make the Nile red staining solution.
14. Put the Nile red solution on the filter and wait for 30 minutes.
15. Let the Nile red run through the filter and let the filter air dry.
16. Put the filter in a glass petri dish for storage or place it on the object slide under the microscope for analysis.

E.3 Protocol: 5 minutes of sterilization

The article used as a reference is the article of D. Li, et al. [4]

1. Fill a clean glass beaker with Milli-Q.
2. Rinse the baby bottle 3 times with 25 ℃ Milli-Q.
3. Submerge the (uncaped) baby bottle in the Milli-Q in the glass beaker.
4. Put the beaker with Milli-Q in the microwave to heat the water.
5. Heat the Milli-Q until it boils and let it boil for 5 minutes.
6. Take out the bottle and let it air dry, for 30 minutes with the opening covered.
7. Put 170 ml distilled water in the baby bottle.
8. Heat the bottle in the microwave until the water is 70℃.
9. Let it turn (with the cap on) on a shaker for 60 seconds at 140 rpm.
10. Let the water cool down to 37℃.
11. Let the water from the bottle run through the aluminum filter.
12. Make the Nile red staining solution.
13. Put the Nile red solution on the filter and wait for 30 minutes.
14. Let the Nile red run through the filter and let the filter air dry.
15. Put the filter in a glass petri dish for storage or place it on the object slide under the microscope for analysis.

E.4 Protocol: 30 minutes of sterilization

1. Fill a clean glass beaker with Milli-Q.
2. Rinse the baby bottle 3 times with 25 ℃ Milli-Q.
3. Submerge the (uncapped) baby bottle in the Milli-Q in the glass beaker.
4. Put the beaker with Milli-Q in the microwave to heat the water.
5. Heat the Milli-Q until it boils and let it boil for 30 minutes.
6. Take out the bottle and let it air dry, for 30 minutes with the opening covered.
7. Put 170 ml distilled water in the baby bottle.
8. Heat the bottle in the microwave until the water is 70℃.
9. Let it turn (with the cap on) on a shaker for 60 seconds at 140 rpm.
10. Let the bottle cool down until the water is 37℃.
11. Let the water from the bottle run through the aluminum filter.
12. Make the Nile red staining solution.

13. Put the Nile red solution on the filter and wait for 30 minutes
14. Let the Nile red run through the filter and let the filter air dry.
15. Put the filter in a glass petri dish for storage or place it on the object slide under the microscope for analysis.

E.5 Protocol: no sterilization or heating

1. Fill a clean glass beaker with Milli-Q.
2. Rinse the baby bottle 3 times with 25 ℃ Milli-Q.
3. Put 170 ml distilled water in the baby bottle.
4. Let it turn (with the cap on) on a shaker for 60 seconds at 140 rpm.
5. Let the bottle rest for 45 minutes.
6. Let the water from the bottle run through the aluminum filter.
7. Make the Nile red staining solution.
8. Put the Nile red solution on the filter and wait for 30 minutes.
9. Let the Nile red run through the filter and let the filter air dry.
10. Put the filter in a glass petri dish for storage or place it on the object slide under the microscope for analysis.

E.6 Protocol: FT-IR experiment

1. Fill a clean glass beaker with Milli-Q.
2. Rinse the baby bottle 3 times with 25 ℃ Milli-Q.
3. Submerge the (uncaped) baby bottle in the Milli-Q in the glass beaker.
4. Put the beaker with Milli-Q in the microwave to heat the water.
5. Heat the Milli-Q until it boils and let it boil for 5 minutes.
6. Take out the bottle and let it air dry, for 30 minutes with the opening covered.
7. Put 170 ml distilled water in the baby bottle.
8. Do this for 6 baby bottles.
9. Heat 2 bottles to 70℃ and set aside for 20 minutes.
10. After 20 minutes heat 2 other bottles and set aside for 20 minutes.
11. Fill the last 2 bottles with the water from the first 2 bottles and repeat the past 2 steps until 30 liters have been through the bottles.
12. Let the water from the bottles run through the aluminum filter, make sure to leave around 20 ml of water above the filter.
13. Pipette the water above the filter up and put it in a petri dish.

14. Let the water damp out of the petri dish.
15. Scrape the residue from the bottom of the petri dish into a pile.
16. Put the powder on the FT-IR.

F Scripts

F.1 Python-script for microplastics

```
import numpy as np
import cv2
import os
import warnings
warnings.simplefilter(action='ignore', category=FutureWarning)
#warnings.simplefilter(action='ignore', category=RuntimeWarning)
import pandas as pd
from pathlib import Path
import seaborn as sns
sns.set_theme(style="whitegrid")
import wx
import wx.lib.agw.multidirdialog as MDD
import matplotlib.pyplot as plt

from base64 import b64encode
from io import BytesIO
from IPython.display import HTML

#from matplotlib_scalebar.scalebar import ScaleBar

Default_Folder =r'D:\240417_controle'
folder_path=r'-'


min_contour_area    = 20      #Filter on minimun contour size
#intensity_threshold = 40     #Manual absolute intensity
   tresholdingif set to 0 fraction is used
intensity_threshold = 0       #Manual absolute intensity
   tresholding if set to 0 fraction is used
fraction=.3                  #intensity is a fraction of the peak
   intensity for each image
bins=201

open_chrome     =True
write_screens   =True         #creatye file copies with contours
Show_images     =False
Annotate        =True         #write stats in image
print_filenames =True
DelOverExposed  =False
go_histo        =False
sort_df         =False
```

```
39
40 def GUI_select_Multiple_folders(message):
41     """ A function to select a multiple folders via a GUI,
42         It starts at the current working directory of the process
              os.getcwd()
43     """
44     app = wx.App(False, clearSigInt=False)
45     dialog = MDD.MultiDirDialog(None, title="Choose Multiple
          folders to procces", defaultPath=Default_Folder,
46                           agwStyle=MDD.DD_MULTIPLE|MDD.
                                DD_DIR_MUST_EXIST)
47
48     if dialog.ShowModal() == wx.ID_OK:
49         paths = dialog.GetPaths()
50
51         for indx, pathGUI in enumerate(paths):
52             #this part replaces corrects Root folder 'Path (E:)'
                   to 'E:'
53             fp=pathGUI.split(os.sep)
54             s=fp[0]
55             fp[0]=s[s.find("(")+1:s.find(")")]+'\\'
56             pathGUI =os.path.join(*fp)
57             #paths[indx]=pathGUI.replace('\\', '/')
58
59
60     dialog.Destroy()
61     app.Destroy()
62     return paths
63
64
65 def GUI_select_folder(message):
66     """ A function to select a PTU filename via a GUI,
67         It starts at the current working directory of the process
68     """
69     wildcard = "(*.tif)| *.tif"
70     app = wx.App(False, clearSigInt=False)
71     path = wx.FileSelector(message='Select you the folder',
          default_path=Default_Folder, default_extension='*.tif',
          wildcard=wildcard)
72     directory,filename=os.path.split(path)
73     app.Destroy()
74     print('Selected file folder: '+path)
75     return directory
76
77
78
79 def ResizeWithAspectRatio(image, width=None, height=None, inter=
       cv2.INTER_AREA):
80     dim = None
```

```
    (h, w) = image.shape[:2]

    if width is None and height is None:
        return image
    if width is None:
        r = height / float(h)
        dim = (int(w * r), height)
    else:
        r = width / float(w)
        dim = (width, int(h * r))

    return cv2.resize(image, dim, interpolation=inter)



def create_tumb(image):
    fig, ax = plt.subplots(figsize=(2, 2))
    ax.imshow(cv2.cvtColor(image, cv2.COLOR_BGR2RGB))
    ax.axis('off')
    plt.close(fig)

    img = BytesIO()  # create Bytes Object
    fig.savefig(img) # Save Image to Bytes Object
    encoded = b64encode(img.getvalue())  # Encode object as
       base64 byte string
    decoded = encoded.decode('utf-8')    # Decode to utf-8
    return f'<img src="data:png;base64,{decoded}">' # Return HTML
        tag



results_df = pd.DataFrame(columns=['File', 'Spot', 'X', 'Y', '
   Size', 'Tumbnail', 'Hue', 'Extent', 'Aspect Ratio', 'RG','Mean
    Intensity'])

histo_df   = pd.DataFrame()
binedges=np.linspace(0.2,1.6, bins)
histo_df = pd.concat([histo_df, pd.DataFrame({'GR bins': binedges
   })], axis=1, ignore_index=False)


#folder_path=GUI_select_Multiple_folders('select_files')[0]
folder_path=GUI_select_Multiple_folders('select_files')[0]



s_path =Path(folder_path)
Title_name=s_path.parts[-2]+'|'+s_path.parts[-1]

```

```
125
126 if not os.path.exists(folder_path+'//Python_analyzed'):
127     os.mkdir(folder_path+'//Python_analyzed')
128
129 #fig, ax = plt.subplots(tight_layout=True)
130
131     # Iterate through all files in the folder
132
133 for filename in os.listdir(folder_path):
134     if filename.endswith(('.TIF','.tif')):  # Adjust the file
        extensions as needed
135         file_path = os.path.join(folder_path, filename)
136
137         #file_path=r'C:\Users\Molenaarr\Desktop\20240119-01_Tif
            \1080.png'
138         # Read the image
139         RGB_image = cv2.imread(file_path, cv2.IMREAD_COLOR)
140         #RGB_image = cv2.imread(file_path, cv2.IMREAD_UNCHANGED)
141
142         hsv       = cv2.cvtColor(RGB_image, cv2.
            COLOR_BGR2HSV_FULL)
143         #hsv       = cv2.cvtColor(RGB_image, cv2.
            COLOR_BGR2HLS_FULL)
144
145         hsv_hue   = hsv[:,:,0]
146         hsv_sat   = hsv[:,:,1]
147         hsv_val   = hsv[:,:,2]
148         #over_exp  = cv2.threshold(hsv_val, 253, 255, cv2.
            THRESH_BINARY)[1]
149
150         RG_ratio  = (RGB_image[:,:,1]+0.1)/(RGB_image[:,:,2]+0.1)
151
152         #np.mean(image)
153         if intensity_threshold==0:
154             intensity_threshold= int((fraction*(np.max(hsv_val)-
                np.mean(hsv_val))+np.mean(hsv_val)))
155         #intensity_threshold=80
156
157         # Apply threshold to identify bright spots
158         _, thresholded = cv2.threshold(hsv_val,
            intensity_threshold, 255, cv2.THRESH_BINARY)
159
160         # Find contours of bright spots
161         contours, _ = cv2.findContours(thresholded, cv2.
            RETR_EXTERNAL, cv2.CHAIN_APPROX_SIMPLE)
162
163         # Filter small contours (potential noise)
164         filtered_contours = [cnt for cnt in contours if cv2.
            contourArea(cnt) > min_contour_area]
```

```
165
166
167         blank_image = np.zeros(hsv_val.shape, np.uint8)
168         mask_contour = hsv_val > intensity_threshold
169
170
171         if go_histo:
172             histo= ax.hist(RG_ratio[mask_contour], bins=binedges,
                        alpha=0.5)[0]
173             ax.set_title('colour histogram')
174             ax.set_xlabel('RG')
175             ax.set_ylabel('counts')
176
177             histo_df = pd.concat([histo_df, pd.DataFrame({f'{
                    filename}': histo.T})], axis=1, ignore_index=False
                    )
178
179
180         font = cv2.FONT_HERSHEY_COMPLEX_SMALL
181
182
183         # Iterate through each bright spot
184         for i, cnt in enumerate(filtered_contours):
185
186             RGB_image_with_contours = cv2.drawContours(RGB_image.
                    copy(), filtered_contours, -1, (200, 200, 200), 1)
187
188             # Create blank image
189             mask_blank = np.zeros(hsv_val.shape, np.uint8)
190
191             # Draw contour in the mask
192             cv2.drawContours(mask_blank, [cnt], -1, (255, 255,
                    255), -1)
193
194             # Create a mask to select pixels inside the figure
195             mask_contour = mask_blank == 255
196
197             # Calculate centroid (X, Y) of the bright spot
198             M = cv2.moments(cnt)
199             cx = int(M['m10'] / M['m00'])
200             cy = int(M['m01'] / M['m00'])
201
202             # Calculate mean intensity of the bright spot
203             mean_intensity   = np.mean( hsv_val[mask_contour])
204             mean_hue         = np.mean( hsv_hue[mask_contour])
205             RG               = np.mean(RG_ratio[mask_contour])
206
207             x,y,w,h = cv2.boundingRect(cnt)
208
```

```
209             # Get the size of the bright spot
210             size = cv2.contourArea(cnt)
211             aspect_ratio = float(w)/h
212             extent = size/w*h
213             equi_diameter = np.sqrt(4*size/np.pi)
214             border=10
215
216             if any(hsv_val[mask_contour]>254) and DelOverExposed:
217                 #keep overexposed out of the results and indicate
                        them in red
218                 print(f'Overexposed {filename} particle {i}')
219                 annote_colour=(0,0,240)
220
221             else:
222                 # Append the results to the DataFrame
223                 # results_df =  results_df.concat({
224                 #     'File': filename,
225                 #     'Spot': i + 1,
226                 #     'X': cx,
227                 #     'Y': cy,
228                 #     'Size': size,
229                 #     'Mean Intensity': mean_intensity,
230                 #     'Hue': mean_hue,
231                 #     'Aspect Ratio': aspect_ratio,
232                 #     'RG': RG,
233                 #     'Tumbnail' :  create_tumb(
                        RGB_image_with_contours[np.clip(cy-int(h/2)-
                        border,0,1535):np.clip(cy+int(h/2)+border,0,
                        1535),np.clip(cx-int(w/2)-border,0,1535):np.
                        clip(cx+int(w/2)+border,0,1535)]),
234                 #     'Extent': extent}, ignore_index=True)
235
236                 Insert_df =  pd.DataFrame({
237                     'File': filename,
238                     'Spot': i + 1,
239                     'X': cx,
240                     'Y': cy,
241                     'Size': size,
242                     'Mean Intensity': mean_intensity,
243                     'Hue': mean_hue,
244                     'Aspect Ratio': aspect_ratio,
245                     'RG': RG,
246                     'Tumbnail' :  create_tumb(
                            RGB_image_with_contours[np.clip(cy-int(h
                            /2)-border,0,1535):np.clip(cy+int(h/2)+
                            border,0, 1535),np.clip(cx-int(w/2)-border
                            ,0,1535):np.clip(cx+int(w/2)+border
                            ,0,1535)]),
247                     'Extent': extent}, index=[0])
```

```
248
249                 results_df = pd.concat([results_df, Insert_df],
                        ignore_index=True)
250
251                 annote_colour=(0,240,240)
252
253
254         # Optionally, you can display the image with marked
                bright spots
255
256             if Annotate:
257                 #b=0   #initial offset
258                  #annotate xy boundary conditions
259                  if cx>=(RGB_image.shape[0]-180):
260                      ox=cx-190
261                  else:
262                      ox=cx+20
263
264                  if cy>=(RGB_image.shape[0]-100):
265                      oy=cy-20
266                  else:
267                      oy=cy+20
268
269                  RGB_image = cv2.putText(RGB_image, f'p{i+1} GR {
                        RG:.2g}, H {mean_hue:.2g}', (ox,oy), font, 1,
270                       annote_colour, 1, cv2.LINE_AA, False)
271
272
273         fname=  os.path.join(folder_path+'\Python_analyzed',
                filename)
274
275
276
277         if write_screens:
278
279             if print_filenames:
280                  RGB_image = cv2.putText(RGB_image, f'{filename}',
                         (36,36), font, 1,
281                       (80, 80, 80), 1, cv2.LINE_AA, False)
282
283             RGB_image_with_contours = cv2.drawContours(RGB_image.
                    copy(), filtered_contours, -1, (0, 240, 240), 1)
284             cv2.imwrite(fname,RGB_image_with_contours)
285
286
287             if Show_images:
288                  resized = ResizeWithAspectRatio(
                        RGB_image_with_contours, width=600)
289                  cv2.imshow("Image with Bright Spots", resized)
```

```
                cv2.waitKey(50)


            # RGB_image = cv2.putText(RGB_image, f'F{filename
                [0:4]}|p{i+1} GR {RG:.2f}', (ox,oy), font, 1,
            #       (0, 240, 240), 1, cv2.LINE_AA, False)

cv2.destroyAllWindows()
# Print the results DataFrame
print(results_df)


if sort_df:
    results_df=results_df.sort_values('Hue', ascending=False)

html_df=results_df.to_html(escape=False)
text_file = open(f'{folder_path}\_Report.html', "w")
text_file.write(html_df)
text_file.close()


binedges=np.linspace(0,80, 81)
fig, ax = plt.subplots(tight_layout=True)
histo= ax.hist(results_df['Hue'], bins=binedges, alpha=0.5)[0]
ax.set_title('colour histogram')
ax.set_xlabel('Hue')
ax.set_ylabel('counts')
ax.xaxis.grid(True, "minor", linewidth=.25)
ax.yaxis.grid(True, "minor", linewidth=.25)
fig.savefig(folder_path+'\_RG_histo.png', dpi=600)



sns.set(rc={'figure.figsize':(10,6)})

g=sns.relplot(data=results_df,x='RG', y='Mean Intensity', hue='
    Hue', size='Size', palette='hsv', hue_norm=(0,255),sizes=(10,
    200))
#g=sns.relplot(data=results_df,x='Hue', y='index', hue='Hue',
    size='Size', palette='hsv', hue_norm=(0,255),sizes=(10, 200))
Xmin=0.1
Xmax=1.6
g.set(xscale="linear", yscale="linear")
#g.set(xscale="linear", yscale="linear")
g.fig.suptitle('Particle analysis scatter \n   source |   '+
    s_path.parts[-2]+' | '+s_path.parts[-1], size=11,x=0.18, y
    =1.04, ha='left')
g.ax.xaxis.grid(True, "minor", linewidth=.25)
```

```
g.ax.yaxis.grid(True, "minor", linewidth=.25)

g.ax.text(Xmax*0.65,185,f' \u03bcRG {results_df.loc[:,"RG"].mean
    ():.3g}    {results_df.loc[:,"RG"].std():.3g}')
g.ax.axvline(x = 0.439, color = 'powderblue', label = 'Nylon',
    linewidth=4, alpha=0.3)
g.ax.axvline(x = 0.8,   color = 'powderblue', label = 'PMMA',
    linewidth=4, alpha=0.3)
g.ax.axvline(x = 0.8,   color = 'powderblue', label = 'LDPE',
    linewidth=4, alpha=0.3)
g.ax.axvline(x = 1.18,  color = 'powderblue', label = 'HDPE',
    linewidth=4, alpha=0.3)
g.ax.axvline(x = 0.5,  color = 'powderblue', label = 'PET',
    linewidth=6, alpha=0.3)
g.ax.axvline(x = 0.74,  color = 'powderblue', label = 'PS',
    linewidth=4, alpha=0.3)
g.ax.axvline(x = 0.664, color = 'powderblue', label = 'PP',
    linewidth=2, alpha=0.3)
g.ax.axvline(x = 1.30,  color = 'powderblue', label = 'PE',
    linewidth=2, alpha=0.3)

g.set_ylabels('Intensity [ bit ]')
g.set_xlabels('GR ratio [ - ]')
g.ax.set_xlim(Xmin,Xmax)
g.ax.set_ylim(50,200)


g.savefig(folder_path+'\_particle_colour_scatter.png', dpi=600)


results_df.to_csv(folder_path+'\_Particle_Analysis_df.dat', sep='
    ,', index_label='index',float_format='%.3g')
print(f"Total particles counted {(len(results_df['Size'])):.0f}
    with a size are larger than {min_contour_area} pixel")
print(f"Total particles coverage {(np.sum(results_df['Size'])
    *0.119):.2E} um2 ")
print(f"Average GR= {results_df.loc[:,'RG'].mean():.3f} ")
average_size = results_df['Size'].mean()
print(f"Average size of all particles found: {average_size:.2f}
    pixels")
#Calculate the average size of all particles in micrometers
average_size_um = results_df['Size'].mean() * 0.119  # Assuming
    the size is in pixels and converted to micrometers

# Print or display the average size
print(f"Average size of all particles: {average_size_um:.2f}
    micrometers")
histo_df.to_csv(folder_path+'\_Particle_histo_df.dat', sep=',',
    index_label='index',float_format='%.3g')
```

```
366
367 print('Dataframe Report\n'+f'{folder_path}\_Report.html'+'\n')
368
369 if open_chrome:
370     import webbrowser
371     filename=(f'{folder_path}\_Report.html')
372     webbrowser.open_new_tab(filename)
```

F.2 Python-script for beads

```
1  import numpy as np
2  import cv2
3  import os
4  import warnings
5  warnings.simplefilter(action='ignore', category=FutureWarning)
6  #warnings.simplefilter(action='ignore', category=RuntimeWarning)
7  import pandas as pd
8  from pathlib import Path
9  import seaborn as sns
10 sns.set_theme(style="whitegrid")
11 import wx
12 import wx.lib.agw.multidirdialog as MDD
13 import matplotlib.pyplot as plt
14
15 from base64 import b64encode
16 from io import BytesIO
17 from IPython.display import HTML
18
19 #from matplotlib_scalebar.scalebar import ScaleBar
20
21 Default_Folder =r'D:\Experiments\Sophie_Koopmans\240502_beads_1'
22 folder_path=r'-'
23
24
25 min_contour_area    = 20      #Filter on minimun contour size
26 #intensity_threshold = 40     #Manual absolute intensity
      tresholdingif set to 0 fraction is used
27 intensity_threshold = 0       #Manual absolute intensity
      tresholding if set to 0 fraction is used
28 fraction=.3                  #intensity is a fraction of the peak
      intensity for each image
29 bins=201
30
31 open_chrome     =True
32 write_screens   =True         #creatye file copies with contours
33 Show_images     =False
34 Annotate        =True         #write stats in image
35 print_filenames =True
36 DelOverExposed  =False
37 go_histo        =False
```

```
38 sort_df          =False
39
40 def GUI_select_Multiple_folders(message):
41     """ A function to select a multiple folders via a GUI,
42         It starts at the current working directory of the process
              os.getcwd()
43     """
44     app = wx.App(False, clearSigInt=False)
45     dialog = MDD.MultiDirDialog(None, title="Choose Multiple
          folders to procces", defaultPath=Default_Folder,
46                           agwStyle=MDD.DD_MULTIPLE|MDD.
                                DD_DIR_MUST_EXIST)
47
48     if dialog.ShowModal() == wx.ID_OK:
49         paths = dialog.GetPaths()
50
51         for indx, pathGUI in enumerate(paths):
52             #this part replaces corrects Root folder 'Path (E:)'
                  to 'E:'
53             fp=pathGUI.split(os.sep)
54             s=fp[0]
55             fp[0]=s[s.find("(")+1:s.find(")")]+'\\'
56             pathGUI =os.path.join(*fp)
57             #paths[indx]=pathGUI.replace('\\', '/')
58
59
60     dialog.Destroy()
61     app.Destroy()
62     return paths
63
64
65 def GUI_select_folder(message):
66     """ A function to select a PTU filename via a GUI,
67         It starts at the current working directory of the process
68     """
69     wildcard = "(*.tif)| *.tif"
70     app = wx.App(False, clearSigInt=False)
71     path = wx.FileSelector(message='Select you the folder',
          default_path=Default_Folder, default_extension='*.tif',
          wildcard=wildcard)
72     directory,filename=os.path.split(path)
73     app.Destroy()
74     print('Selected file folder: '+path)
75     return directory
76
77
78
79 def ResizeWithAspectRatio(image, width=None, height=None, inter=
       cv2.INTER_AREA):
```

```
    dim = None
    (h, w) = image.shape[:2]

    if width is None and height is None:
        return image
    if width is None:
        r = height / float(h)
        dim = (int(w * r), height)
    else:
        r = width / float(w)
        dim = (width, int(h * r))

    return cv2.resize(image, dim, interpolation=inter)



def create_tumb(image):
    fig, ax = plt.subplots(figsize=(2, 2))
    ax.imshow(cv2.cvtColor(image, cv2.COLOR_BGR2RGB))
    ax.axis('off')
    plt.close(fig)

    img = BytesIO()  # create Bytes Object
    fig.savefig(img) # Save Image to Bytes Object
    encoded = b64encode(img.getvalue())  # Encode object as
       base64 byte string
    decoded = encoded.decode('utf-8')    # Decode to utf-8
    return f'<img src="data:png;base64,{decoded}">' # Return HTML
        tag



results_df = pd.DataFrame(columns=['File', 'Spot', 'X', 'Y', '
   Size', 'Tumbnail', 'Hue', 'Extent', 'Aspect Ratio', 'RG','Mean
    Intensity'])

histo_df   = pd.DataFrame()
binedges=np.linspace(0.2,1.6, bins)
histo_df = pd.concat([histo_df, pd.DataFrame({'GR bins': binedges
   })], axis=1, ignore_index=False)


#folder_path=GUI_select_Multiple_folders('select_files')[0]
folder_path=GUI_select_Multiple_folders('select_files')[0]



s_path =Path(folder_path)
Title_name=s_path.parts[-2]+'|'+s_path.parts[-1]
```

```
124
125
126 if not os.path.exists(folder_path+'//Python_analyzed'):
127     os.mkdir(folder_path+'//Python_analyzed')
128
129 #fig, ax = plt.subplots(tight_layout=True)
130
131     # Iterate through all files in the folder
132
133 for filename in os.listdir(folder_path):
134     if filename.endswith(('.TIF','.tif')):  # Adjust the file
        extensions as needed
135         file_path = os.path.join(folder_path, filename)
136         print(filename)
137         #file_path=r'C:\Users\Molenaarr\Desktop\20240119-01_Tif
            \1080.png'
138         # Read the image
139         RGB_image = cv2.imread(file_path, cv2.IMREAD_COLOR)
140         #RGB_image = cv2.imread(file_path, cv2.IMREAD_UNCHANGED)
141
142         hsv       = cv2.cvtColor(RGB_image, cv2.
            COLOR_BGR2HSV_FULL)
143         #hsv       = cv2.cvtColor(RGB_image, cv2.
            COLOR_BGR2HLS_FULL)
144
145         hsv_hue   = hsv[:,:,0]
146         hsv_sat   = hsv[:,:,1]
147         hsv_val   = hsv[:,:,2]
148         #over_exp  = cv2.threshold(hsv_val, 253, 255, cv2.
            THRESH_BINARY)[1]
149
150         RG_ratio  = (RGB_image[:,:,1]+0.1)/(RGB_image[:,:,2]+0.1)
151
152         #np.mean(image)
153         if intensity_threshold==0:
154             intensity_threshold= int((fraction*(np.max(hsv_val)-
                np.mean(hsv_val))+np.mean(hsv_val)))
155         #intensity_threshold=80
156
157         # Apply threshold to identify bright spots
158         _, thresholded = cv2.threshold(hsv_val,
            intensity_threshold, 255, cv2.THRESH_BINARY)
159
160         # Find contours of bright spots
161         contours, _ = cv2.findContours(thresholded, cv2.
            RETR_EXTERNAL, cv2.CHAIN_APPROX_SIMPLE)
162
163         # Filter small contours (potential noise)
164         filtered_contours = [cnt for cnt in contours if cv2.
```

```
        contourArea(cnt) > min_contour_area]


    blank_image = np.zeros(hsv_val.shape, np.uint8)
    mask_contour = hsv_val > intensity_threshold


    if go_histo:
        histo= ax.hist(RG_ratio[mask_contour], bins=binedges,
            alpha=0.5)[0]
        ax.set_title('colour histogram')
        ax.set_xlabel('RG')
        ax.set_ylabel('counts')

        histo_df = pd.concat([histo_df, pd.DataFrame({f'{
            filename}': histo.T})], axis=1, ignore_index=False
            )


    font = cv2.FONT_HERSHEY_COMPLEX_SMALL


    # Iterate through each bright spot
    for i, cnt in enumerate(filtered_contours):

        RGB_image_with_contours = cv2.drawContours(RGB_image.
            copy(), filtered_contours, -1, (200, 200, 200), 1)

        # Create blank image
        mask_blank = np.zeros(hsv_val.shape, np.uint8)

        # Draw contour in the mask
        cv2.drawContours(mask_blank, [cnt], -1, (255, 255,
            255), -1)

        # Create a mask to select pixels inside the figure
        mask_contour = mask_blank == 255

        # Calculate centroid (X, Y) of the bright spot
        M = cv2.moments(cnt)
        cx = int(M['m10'] / M['m00'])
        cy = int(M['m01'] / M['m00'])

        # Calculate mean intensity of the bright spot
        mean_intensity    = np.mean( hsv_val[mask_contour])
        mean_hue = np.mean(hsv_hue[mask_contour])
       # Filter the DataFrame based on the condition


```

```
208             # Open the HTML file containing particles with Hue >
                    18 in Chrome
209
210
211
212             # Count the total number of particles with Hue bigger
                    than 18
213
214             #print(total_particles_above_18)
215
216             #print(f"Total particles with Hue bigger than 18: {
                    total_particles_above_18}")
217             RG                = np.mean(RG_ratio[mask_contour])
218
219             x,y,w,h = cv2.boundingRect(cnt)
220
221             # Get the size of the bright spot
222             size = cv2.contourArea(cnt)
223             aspect_ratio = float(w)/h
224             extent = size/w*h
225             equi_diameter = np.sqrt(4*size/np.pi)
226             border=10
227
228             if any(hsv_val[mask_contour]>254) and DelOverExposed:
229                 #keep overexposed out of the results and indicate
                        them in red
230                 print(f'Overexposed {filename} particle {i}')
231                 annote_colour=(0,0,240)
232
233             else:
234                 # Append the results to the DataFrame
235                 # results_df =  results_df.concat({
236                 #     'File': filename,
237                 #     'Spot': i + 1,
238                 #     'X': cx,
239                 #     'Y': cy,
240                 #     'Size': size,
241                 #     'Mean Intensity': mean_intensity,
242                 #     'Hue': mean_hue,
243                 #     'Aspect Ratio': aspect_ratio,
244                 #     'RG': RG,
245                 #     'Tumbnail' :  create_tumb(
                            RGB_image_with_contours[np.clip(cy-int(h/2)-
                            border,0,1535):np.clip(cy+int(h/2)+border,0,
                            1535),np.clip(cx-int(w/2)-border,0,1535):np.
                            clip(cx+int(w/2)+border,0,1535)]),
246                 #     'Extent': extent}, ignore_index=True)
247
248                 Insert_df =  pd.DataFrame({
```

```
                    'File': filename,
                    'Spot': i + 1,
                    'X': cx,
                    'Y': cy,
                    'Size': size,
                    'Mean Intensity': mean_intensity,
                    'Hue': mean_hue,
                    'Aspect Ratio': aspect_ratio,
                    'RG': RG,
                    'Tumbnail' :  create_tumb(
                       RGB_image_with_contours[np.clip(cy-int(h
                       /2)-border,0,1535):np.clip(cy+int(h/2)+
                       border,0, 1535),np.clip(cx-int(w/2)-border
                       ,0,1535):np.clip(cx+int(w/2)+border
                       ,0,1535)]),
                    'Extent': extent}, index=[0])

            results_df = pd.concat([results_df, Insert_df],
               ignore_index=True)

            annote_colour=(0,240,240)


        # Optionally, you can display the image with marked
           bright spots

             if Annotate:
                #b=0   #initial offset
                 #annotate xy boundary conditions
                 if cx>=(RGB_image.shape[0]-180):
                     ox=cx-190
                 else:
                     ox=cx+20

                 if cy>=(RGB_image.shape[0]-100):
                     oy=cy-20
                 else:
                     oy=cy+20

                 RGB_image = cv2.putText(RGB_image, f'p{i+1} GR {
                    RG:.2g}, H {mean_hue:.2g}', (ox,oy), font, 1,
                      annotate_colour, 1, cv2.LINE_AA, False)


        fname=  os.path.join(folder_path+'\Python_analyzed',
           filename)
```

```
289         if write_screens:
290
291             if print_filenames:
292                 RGB_image = cv2.putText(RGB_image, f'{filename}',
                        (36,36), font, 1,
293                         (80, 80, 80), 1, cv2.LINE_AA, False)
294
295             RGB_image_with_contours = cv2.drawContours(RGB_image.
                copy(), filtered_contours, -1, (0, 240, 240), 1)
296             cv2.imwrite(fname,RGB_image_with_contours)
297
298
299             if Show_images:
300                 resized = ResizeWithAspectRatio(
                        RGB_image_with_contours, width=600)
301                 cv2.imshow("Image with Bright Spots", resized)
302                 cv2.waitKey(50)
303
304
305         # RGB_image = cv2.putText(RGB_image, f'F{filename
                [0:4]}|p{i+1} GR {RG:.2f}', (ox,oy), font, 1,
306         #       (0, 240, 240), 1, cv2.LINE_AA, False)
307
308 cv2.destroyAllWindows()
309 # Print the results DataFrame
310 print(results_df)
311
312
313 if sort_df:
314     results_df=results_df.sort_values('Hue', ascending=False)
315
316 html_df=results_df.to_html(escape=False)
317 text_file = open(f'{folder_path}\_Report.html', "w")
318 text_file.write(html_df)
319 text_file.close()
320
321 particles_between_18_and_30 = results_df[(results_df['Hue'] > 18)
     & (results_df['Hue'] < 30) & (results_df['Aspect Ratio'] <
    1.08) & (results_df['Aspect Ratio'] > 0.92) & (results_df['
    Size'] > 600)]
322 html_df_between_18_and_30 = particles_between_18_and_30.to_html(
    escape=False)
323 text_file_between_18_and_30 = open(f'{folder_path}\
    _Report_Between_18_and_30.html', "w")
324 text_file_between_18_and_30.write(html_df_between_18_and_30)
325 text_file_between_18_and_30.close()
326 total_particles_between_18_and_30 = len(
    particles_between_18_and_30)
327
```

```
328
329
330
331 binedges=np.linspace(0,80, 81)
332 fig, ax = plt.subplots(tight_layout=True)
333 histo= ax.hist(results_df['Hue'], bins=binedges, alpha=0.5)[0]
334 ax.set_title('colour histogram')
335 ax.set_xlabel('Hue')
336 ax.set_ylabel('counts')
337 ax.xaxis.grid(True, "minor", linewidth=.25)
338 ax.yaxis.grid(True, "minor", linewidth=.25)
339 fig.savefig(folder_path+'\_RG_histo.png', dpi=600)
340
341
342
343
344 sns.set(rc={'figure.figsize':(10,6)})
345
346 g=sns.relplot(data=results_df,x='RG', y='Mean Intensity', hue='
      Hue', size='Size', palette='hsv', hue_norm=(0,255),sizes=(10,
      200))
347 #g=sns.relplot(data=results_df,x='Hue', y='index', hue='Hue',
      size='Size', palette='hsv', hue_norm=(0,255),sizes=(10, 200))
348 Xmin=0.1
349 Xmax=1.6
350 g.set(xscale="linear", yscale="linear")
351 #g.set(xscale="linear", yscale="linear")
352 g.fig.suptitle('Particle analysis scatter \n   source |   '+
      s_path.parts[-2]+' | '+s_path.parts[-1], size=11,x=0.18, y
      =1.04, ha='left')
353 g.ax.xaxis.grid(True, "minor", linewidth=.25)
354 g.ax.yaxis.grid(True, "minor", linewidth=.25)
355
356 g.ax.text(Xmax*0.65,185,f' \u03bcRG {results_df.loc[:,"RG"].mean
      ():.3g}    {results_df.loc[:,"RG"].std():.3g}')
357 # g.ax.axvline(x = 0.439, color = 'powderblue', label = 'Nylon',
      linewidth=4, alpha=0.3)
358 # g.ax.axvline(x = 0.8,   color = 'powderblue', label = 'PMMA',
      linewidth=4, alpha=0.3)
359 # g.ax.axvline(x = 0.8,   color = 'powderblue', label = 'LDPE',
      linewidth=4, alpha=0.3)
360 # g.ax.axvline(x = 1.18,  color = 'powderblue', label = 'HDPE',
      linewidth=4, alpha=0.3)
361 # g.ax.axvline(x = 0.5,  color = 'powderblue', label = 'PET',
      linewidth=6, alpha=0.3)
362 # g.ax.axvline(x = 0.74,  color = 'powderblue', label = 'PS',
      linewidth=4, alpha=0.3)
363 # g.ax.axvline(x = 0.664, color = 'powderblue', label = 'PP',
      linewidth=2, alpha=0.3)
```

```
364 # g.ax.axvline(x = 1.30,  color = 'powderblue', label = 'PE',
        linewidth=2, alpha=0.3)
365
366 g.set_ylabels('Intensity [ bit ]')
367 g.set_xlabels('GR ratio [ - ]')
368 g.ax.set_xlim(Xmin,Xmax)
369 g.ax.set_ylim(50,200)
370
371
372 g.savefig(folder_path+'\_particle_colour_scatter.png', dpi=600)
373
374
375 results_df.to_csv(folder_path+'\_Particle_Analysis_df.dat', sep='
        ,', index_label='index',float_format='%.3g')
376 print(f"Total particles counted {(len(results_df['Size'])):.0f}
        with a size are larger than {min_contour_area} pixel")
377 print(f"Total particles coverage {(np.sum(results_df['Size'])
        *0.119):.2E} um2 ")
378 print(f"Average GR= {results_df.loc[:,'RG'].mean():.3f} ")
379 print(f"particles_between_18_and_30: {particles_between_18_and_30
        }")
380 average_size = results_df['Size'].mean()
381 #print(f"Average size of all particles found: {average_size:.2f}
        pixels")
382 # Calculate the average size of all particles in micrometers
383 #average_size_um = results_df['Size'].mean() * 0.119  # Assuming
        the size is in pixels and converted to micrometers
384
385 # Print or display the average size
386 #print(f"Average size of all particles: {average_size_um:.2f}
        micrometers")
387 histo_df.to_csv(folder_path+'\_Particle_histo_df.dat', sep=',',
        index_label='index',float_format='%.3g')
388 print('Dataframe Report\n'+f'{folder_path}\_Report.html'+'\n')
389
390 if open_chrome:
391     import webbrowser
392     filename=(f'{folder_path}\_Report.html')
393     webbrowser.open_new_tab(filename)
394     webbrowser.open_new_tab(f'file://{folder_path}/
            _Report_Between_18_and_30.html' )
```