Bachelor's thesis

Quantification of nanoplastics in a baby bottle and a reusable water bottle by Fluorescence Microscopy and Atomic Force Microscopy.

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Abstract

Contents

1. Introduction

1.1. Motivation

Plastic is everywhere in our daily lives, but the use of plastic has a problem, the non-biological slow degradation and the environmental harm it causes. It could also have an impact on our own health. When the plastic degrades, it breaks down into tiny particles, microplastics, and nanoplastics. The degradation is stimulated by mechanical wear, heat, UV, and biological factors such as microorganisms and hydrolysis [1]. A schematic display is shown in Figure 1 of the plastic degrading process.

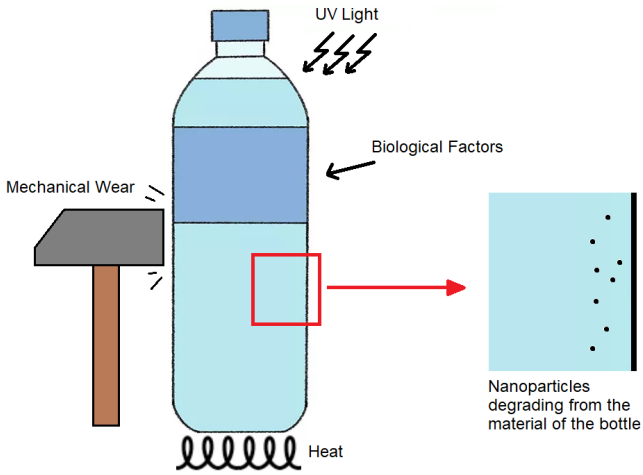

Figure 1: Schematic display of nanoparticles (plastics) breaking down from the bottle's material. The process is sped up by the factors given in the picture

Those nanoplastics can be found everywhere and even in humans, due to consuming products with those particles. The particles originate from the degrading plastic in which the consumer product is packed, such as drinking water from a plastic bottle, which will be the focus.

The smaller the particles, the more harmful. When plastics are microscopic, they can penetrate the cell wall of living organisms. It is known that particles can move through our body from one place to another [2]. This movement mostly occurs through the lymphatic system and bloodstream [3]. Nanoplastics can offer many threats to our health;

- They cause oxidative stress in the intestine and more serious inflammation of the intestine.
- They cause disorders in the production, use, and breaking down of amino acids.
- The integrity of the lipid membranes of the reproduction cells can be disrupted, and the nanoplastics clump together in the reproduction cells and embryos, damaging the embryo's body.
- The movement of the particles through the blood leads to the accumulation of the particles in the brain whilst penetrating the blood-brain barrier.
- Muscle tissue and nerve fibers are damaged by entering the muscle tissue through the epidermis [4].

The health issues that result from the intake of those particles depend on the quantity of the particles. Quantifying nanoplastics is the first step in establishing the dangers to our health. The size and amount of particles can be derived from the quantification. Many studies focus on microplastics, but the knowledge of nanoplastics is still rudimentary due to the complexity of the measurement techniques with decreasing particle size. The motivation of the research is the health risks that nanoplastics induce on the human body and researching the complexity of measurement techniques due to their relatively small size.

1.2. Research goals

Just like described in the research motivation, the measurement techniques' complexity increases with the decreasing size of the particles in the sample. The nanoplastics are hard to detect and quantify, so the research aims to benchmark different measurement techniques with their advantages and disadvantages. The chosen measurement techniques are Fluorescence Microscopy (FM) and Atomic Force Microscopy (AFM), which will be explained in the theoretical background information. The overall goal of the research is to identify the size and quantity of nanoplastics that are found in a baby bottle and a reusable water bottle when consumed. To achieve the goal, a main research question is formulated:

How can the plastic nanoparticles be quantified with Fluorescence Microscopy and Atomic Force Microscopy while the reduced size of the particles increases the complexity of the measuring techniques?

1.3. Theoretical background Information

To reach the research goal, it is necessary to get theoretical background information about the measurement techniques and previous research on the topic.

1.3.1. Fluorescence Microscopy

The first measurement technique used is Fluorescence Microscopy (FM). The basics of Fluorescence Microscopy rely on the sample's absorption and emission of photons by fluorescent dye molecules. The dye molecules absorb a photon whilst in the ground state, which leads to an electron promoting to an excited state. When the excited electron returns to the ground state, a photon is emitted with lower energy and thus, and longer wavelength. The used microscope is the Nikon Eclipse TE2000U [5].

The functionality of a fluorescence microscope is given as a schematic rendering in Figure 2. The excitation light source contains multiple wavelengths, which have to be filtered. The excitation filter filters the wavelengths of the excitation light so that only the desired wavelength that excites a specific dye is let through.

The Dichroic mirror reflects the excitation light to the sample and transmits the light reflected by the sample. The sample is labelled with a dye, a fluorophore that emits light after excitation. The dye emits light with a different wavelength than the excitation light. The emitted light is transmitted through the dichroic mirror and passes an emission filter, which functions like the excitation filter, filtering unwanted wavelengths from the emitted light [6].

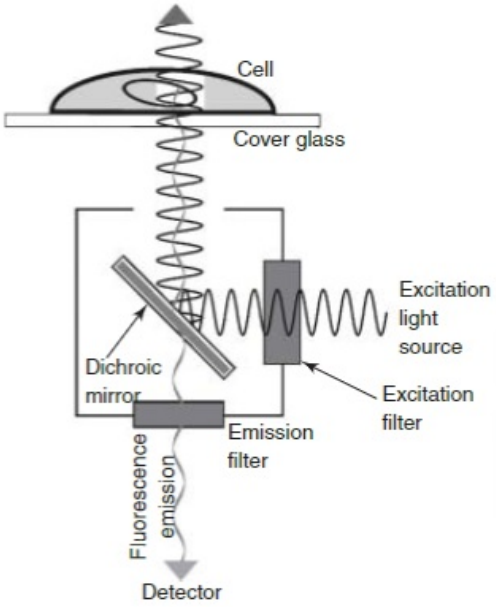

Figure 2: Schematic display of the principle of Fluorescence Microscopy [7].

The detector finally registers the emitted and filtered light, creating an image of the sample labelled with the dye. The dye that is used in the experiments is Nile Red. It has an excitation maximum at a wavelength of 552 nm and an emission maximum at a wavelength of 636 nm [8]. This is seen in Figure 3, the fluorescence spectra of Nile Red.

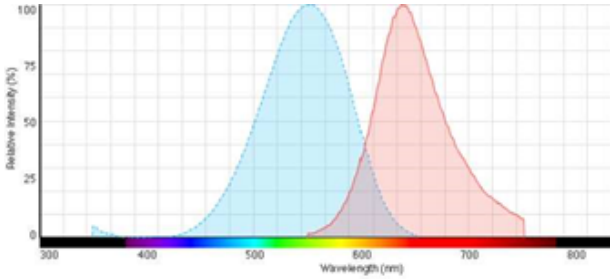

Figure 3: Fluorescence spectra of Nile Red [8].

Nile Red is suitable for staining plastics, which are used in the experiment. Nile red stains any hydrophobic surface. The plastics that can be stained with Nile Red are polyethylene, polypropylene, polystyrene, polycarbonate, polyurethane, and poly(ethylene-vinyl acetate) [9]. Polypropylene and polystyrene are mainly used for the experiments. Nile red will not stain minerals and most organic materials, so mostly plastics will be detected [10].

1.3.2. Atomic Force Microscopy

The second measurement technique used is Atomic Force Microscopy (AFM). AFM is a type of scanning probe microscopy. Information on the height of the sample is obtained by scanning the surface by feeling over it. AFM uses the atomic force between the tip and the surface of the sample. An AFM consists of a tip with a radius of around 10 nanometres, which is connected to a cantilever.

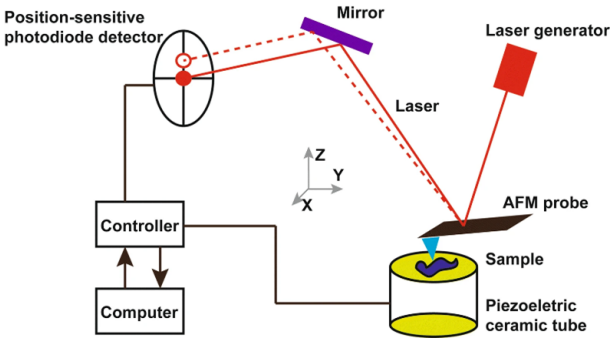

Figure 4: Schematic display of the principle of Atomic Force Microscopy [11].

The tip scans over the sample's surface while a laser shines on the AFM cantilever end. Due to the sample's height difference, the cantilever will bend up. The laser is directed on the cantilever, and the angle of the cantilever determines the angle of reflection, towards a position-sensitive photo-diode detector. Deflection of the laser is a measure of height as seen in Figure 4 [12].
The values of the height of the sample are then constructed as a line cross-section of the sample with nm height resolution. The lines that are measured are constructed to the said 3D image. The samples are dried on the substrate and measured by selecting the recommended mode to use, tapping in air.

In contact mode the tip stays on the sample, giving it the chance to destroy or move the particles. Tapping mode repeatedly taps on the surface so it will not move or squish the particles. Tapping mode is the mode that generates the least amount of shear forces due to the movement of the tip [13]. The vertical resolution of AFM can be very high (0,1 nm), resulting in the possibility of distinguishing particles as small as a few nanometres big [14]. The shortcoming of the AFM is the lateral resolution due to convolution. The resolution could be low (30 nm).

1.3.3. Earlier research and added value

A research (Dunzhu Li et al.,2020) [15] also researched plastics that were released off the side of a baby bottle during infant formula preparation. That research looked at the amount of particles that were released. They used AFM and Raman to detect the particles. Millions of particles were consumed by infants due to the preparation process of the baby bottle. The research used a filter with a pore size of 0,8 µm. This means that the nanoplastics are not taken into account. The added value of the bachelor's thesis is that the aim is the nanoparticles. The other added value is that the thesis looks at volumes instead of particles, which gives a more clear indication of the consumed plastic quantity than the number of particles.

Another research (Wanyi Fu et al.,2020)[16] focussed on nanoplastics and microplastics. It indicates that AFM is a viable way to detect the small nanoparticles, however, the scan size of the AFM is relatively small, so a concentration is not calculated. It also utilises Raman Spectroscopy to qualify the particles which is possible in both liquid and solid samples. The AFM performs well in both liquid and air, so the conditions of the sample stay the same, making it an excellent way of quantifying the nano- and microplastics.

Not many research is done on nanoparticles, so the topic of the research has a lot of added value in that field. Also researching the possibility of quantifying the volume of particles is not done a lot before.

2. Methodolgy

2.1. Approach

to get a clear idea of the approach of the method, the goal, as stated in the introduction, is repeated one more time:

How can the plastic nanoparticles be quantified with Fluorescence Microscopy and Atomic Force Microscopy while the reduced size of the particles increases the complexity of the measuring techniques?

Fluorescence Microscopy and Atomic Force Microscopy are the imaging techniques used to achieve the goal. The advantage of FM is that it can quickly scan a relatively large area. By staining the plastic nanoparticles, the number of particles can be counted. It can also make many measurements per second, allowing an approximation of the particle size by Brownian motion.

The size of the particles can be determined by Brownian motion, but AFM can measure the height of the particles more accurately. Due to the high resolution in the z-direction, the height is determined very precisely. The height difference that AFM can separate is a few nanometers, while the measured particles are in the order of 50 to 500 nanometres. Both of the measurement techniques are used to determine the plastic volume.

2.2. Samples

The various samples that are measured are:

- A validation sample with a known size; 100 nm polystyrene beads, 10.000 x diluted from the stock solution.
- Real-life samples:

  Reusable water bottle; Dopper-bottle sample

  Baby bottle 1, Difrax bottle 1

  Baby bottle 2, Difrax bottle 2; This sample serves as a validation of the previous Difrax sample. This was necessary due to possible pollution of the distilled water used in the previous Difrax sample.

The samples were all individually made. The real-life samples were made by simulating real-life scenarios. The samples were made according to the following procedure:

2.2.1. 100 nm polystyrene beads

The 100 nm beads serve as a mono-disperse validation for size measurements. The concentration of the beads is not relevant. The only important thing is that the sample is measurable by the AFM and the fluorescence microscope. The fluorescence microscope can measure a maximum of around 9000 particles per nanolitre, and it is beneficial for the AFM to have the concentration on the high side due to the slow measurement speed. By optimization of the procedure, the sweet spot of the techniques is calculated. The following dilution factors from the stock solution have been determined:

- AFM: 20.000 times diluted
- FM: 5.000 times diluted

The experiments have been executed with a dilution of 10.000 times, in between the sweet spots. This resulted in a dilution that was still detectable for both of the measurement techniques.

2.2.2. Dopper bottle

The Dopper-bottle sample is made by first cleaning the Dopper-bottle five times with warm water (60 °C) to simulate the warmth from a dishwasher. After cleaning the bottle, 200 ml of room-temperature distilled water is poured. The bottle with the distilled water is put aside for 6 hours to simulate the time a bottle could be in a bag before total consumption. The distilled water is poured into a cleaned glass beaker of 250 ml. The beaker with 200 ml sample is concentrated by evaporation to a total volume of ≈ 400 µl on a hot plate at 160 °C whilst not letting the sample cook to prevent loss of the sample.

2.2.3. Difrax bottle 1

The Difrax bottle, a baby bottle, is to be sterilized. First, grab a two-litre beaker and fill it with milli-Q water. Disassemble the Difrax bottle to the fullest and put it in the water. Warm up the water in the microwave with the Difrax bottle inside. Put the whole bottle under water and let it not peak out. Microwave the beaker until the water boils. This will be around 17 minutes. Take out the beaker and take out the parts of the Difrax bottle. Assemble the bottle again, and it is sterilized. This sterilizes the Difrax bottle like in real life. 200 ml of 80°C distilled water is poured to imitate warm milk poured in the bottle. The whole sample is cooled to room temperature before pouring into a cleaned glass beaker of 250 ml. The beaker with 200 ml sample is concentrated by evaporation to a total volume of ≈ 400 µl on a hot plate at 160 °C whilst not letting the sample cook to prevent loss of the sample.

2.2.4. Difrax bottle 2

The Difrax bottle has to be sterilized like the Difrax bottle sample 1. The difference is in the process after the sterilization. 200 ml of distilled water is warmed up to 80°C like Difrax sample 1. 100 ml of the distilled water is poured into the Difrax bottle, whilst the other 100 ml is poured into a cleaned glass beaker instantly. The 100 ml in the Difrax bottle is cooled to room temperature like before and put in another cleaned glass beaker. Both beakers with 100 ml sample are concentrated by evaporation to a total volume of ≈ 400 µl on a hot plate at 160 °C whilst not letting the sample cook to prevent loss of the sample.
The Difrax bottle sample is done the second time because of needed validation. The distilled water from the first sample showed particles in the AFM, so the distilled water could be polluted. The second sample and the distilled water were measured to validate this possibility.

2.3. Fluorescence microscopy

The samples for the fluorescence microscopy have to be stained with the fluorophore, Nile Red, with a concentration of 30 nM. The samples are prepared with $1/20^{\text{th}}$ part 600 nM Nile Red and $19/20^{\text{th}}$ part sample.

The sample is not pipetted directly on a microscope slide because of the drift that occurs that way, which messes with the Brownian Motion. To maintain clean Brownian Motion, the sample is enclosed in a sample chamber consisting of two glass surfaces separated by a ring as seen in Figure 5. The volume in between the glass plates is 70 µl.

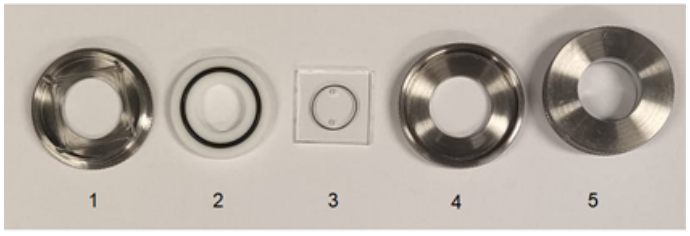

Figure 5: Parts of the metal chamber for the samples. 1,4 = casing. 2 = white pressure ring. 3 = thick glass plane with pipette holes, rubber ring, and a cover slip. 5 = assembled chamber

After preparing the sample in the chamber, the samples were imaged and saved using the 'Labview' software. For each sample, 128 pictures are taken at random places to count the number of particles in the sample.

This was done in triplicate for each sample.

Secondly, for size determination, particles needed to be tracked by making 200 pictures with a frame rate of 50 frames per second. Multiple series at random places were executed in the sample. The 100 nm polystyrene beads sample has a high concentration of particles, so one single measurement was done in triplicate. The other samples were lower in concentration of particles, so 20 measurements were done at random locations in triplicate to get a high enough population.

2.4. Atomic Force Microscopy

The samples for the AFM have to be dried on a substrate. The chosen substrate is MICA due to the atomic flatness of the substrate. Before the sample is pipetted on, a layer of MICA has to be peeled off by tape to secure a new and clean MICA sheet. The MICA sheet is placed in a UV/ozone-radiation chamber for 15 minutes to make the surface more hydrophilic. The idea behind this is a faster sample spread on the MICA. The particles will attach to the MICA faster to secure a more evenly dried sample droplet. The sample is pipetted on the MICA with a volume of 1 µl. After 10 minutes of drying, the sample can be measured. It is not possible to scan the whole drying surface due to time restrictions and the scan size of the AFM. The experiments were done with a scan size of 20 µm by 20 µm. Instead of scanning the whole surface, the surface is divided grid with an X and Y axis. Measurements are done by mapping out the edges and measuring along the axis with 500 µm between the measurements, as shown in Figure 6.

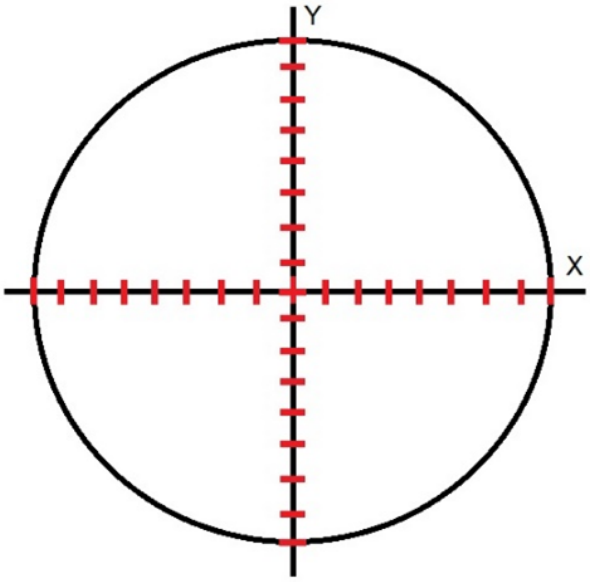

Figure 6: AFM Grid. The red markings mark the measurement coordinates

3. Characterization of methods

The two measurement techniques have their characteristics. In the methodology is explained how the measurements are done, but not what is done with the results to obtain the final usable result. In the characterisation is explained how the measurements are manipulated to ensure an accurate result.
An action that has been done with all the real-life samples is concentrating the samples. Each real-life sample begins with a 100 to 200 ml volume, which has to be concentrated to a volume of ≈ 400 µl. This is done so the density of the particles is higher and the validity of the measurements is better whilst keeping enough volume to execute all the measurements. The concentration factor (CF) is equal to:

$$CF = \frac{ISV}{CSV}$$

Where the initial sample volume is given by ISV and the concentrated sample volume by CSV, this factor is needed to calculate the initial mass of the particles towards the volume of the initial sample. Therefore, the mass of the initial and concentrated samples is measured by measuring the empty canisters' weight and the same canisters' weight plus the sample's weight.

3.1. Fluorescence microscopy

The concentration factor is already explained, but the staining is not. The beads are stained with 1 / 20th Nile Red, increasing the sample volume. To correct this, the amount of beads measured has to be multiplied by 1,053, corresponding to dividing the amount by 19 and multiplying by 20. The multiplier of 1,053 will be called the Staining Correction (SC).

To execute the measurements with fluorescence microscopy, the samples must be stained and concentrated before being detected by Trackpy. This package runs on Python for tracking features in video images [17]. By assigning the features, plastic particles in this case, to a coordinate system, tracking and counting the particles is possible. A frame, analysed by Trackpy, is given as a figure 7.

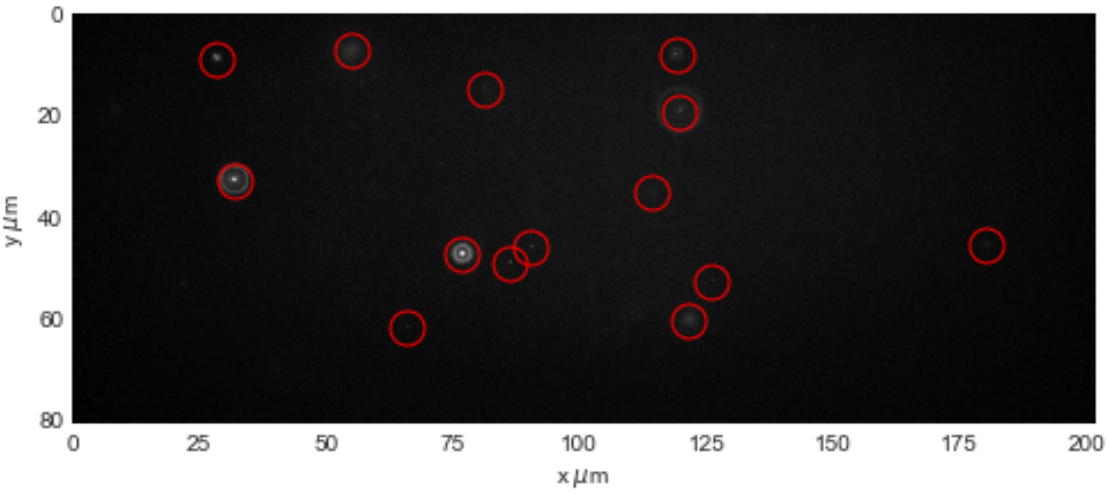

Figure 7: An example of an image by Fluorescence Microscopy, analysed by trackpy. The single particles are encircled to indicate the detection by trackpy.

3.1.1. Counting

The counting of the particles by Fluorescence Microscopy is a simple task. Trackpy is used to detect the particles in the frames. The intensity of fluorescence of the particles needs to reach a threshold to detect a particle. This threshold is manually selected. The images are verified by eye to see if all particles were detected. After the threshold was approved, trackpy detected all the particles properly. 128 frames per sample in triplo were measured to have enough measurements so the Poisson error count would not be too high. To prevent human bias, the stage with the sample will move randomly by moving the stage automatically with a random motion.

3.1.2. Size Determination

Like the counting, the particles are detected by trackpy with the tracking of the particles. The tracking of the particles is needed to determine the size of the particles. This is done by the principle of Brownian motion, the principle of random walk of particles.
"Brownian motion is the random motion of particles suspended in a fluid (a liquid or a gas) resulting from their collision with the fast-moving atoms or molecules in the gas or liquid" [18]. Due to the difference in size, the amount of collisions needed for motion differs for different particle sizes. This indicates that smaller particles move relatively faster than the bigger particles when the same amount of energy from collisions is put in. A path of motion can be constructed by tracking the particles for 200 frames with a frame rate of 50 FPS. An image of those random paths is given in Figure 8:

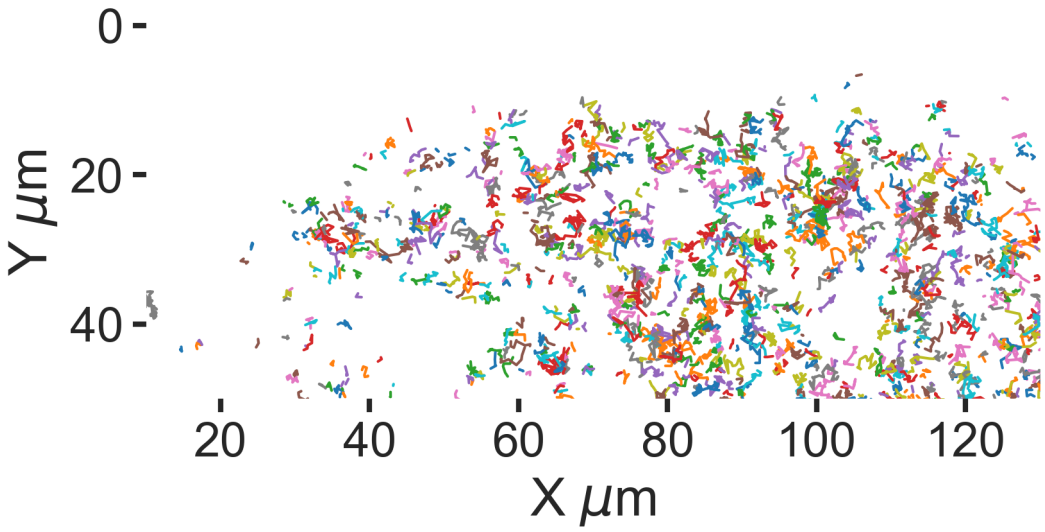

Figure 8: An example of the measured particle tracks for multiple frames. Each different colour is a different particle

This path gives information about the speed of motion and, thus, information about the size of the particles. It is essential that the particles can be measured for multiple frames to avoid noise and unrealistic results due to photobleaching. To calculate the diameter by Brownian Motion, the following formula is used:

$$r = \frac{(k_b \cdot T)}{(6 \cdot \pi \cdot \eta \cdot D)}$$

Where the desired parameter r is the particle radius, k_B is the Boltzmann constant, T is the temperature (K), and η is the viscosity (=0,98e-3 of water at 21°C). D (diffusivity) is calculated by plotting the ensemble mean squared displacement of particles which yields a plot like this:

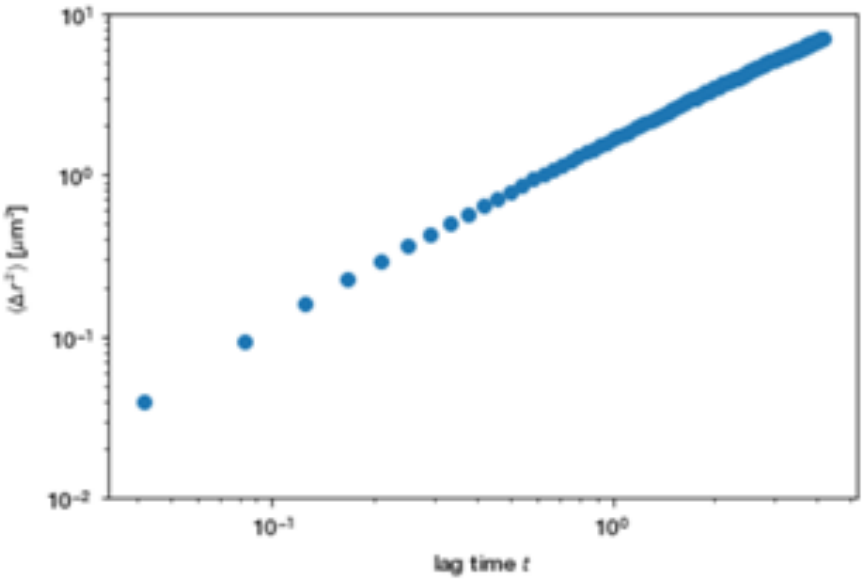

Figure 9: squared displacement of the particles plotted against the lagtime to calculate the diffusivity [19]

This figure shows the fitted plot of all the particles, with the viscosity equalling the slope of the plot. Normally the value of the Δr^2 at lag time = 1 is taken, which is equal to A and equal to the following formula:

$A = 4 \cdot D$

The value of Δr^2 at the lag time = 1 second can not be derived in the measurements because the particles are not measured that long. The solution is to get the value from the first data point and extrapolate it to the lag time of 1 second. The first data point is taken because it contains the most data values in comparison to the following data points which decreases by one data value per data point further in the time.

When a particle is tracked for a small number of frames and, due to random process, has trajectory points whose coordinates lay relatively far from one another, the particle is assumed to be very small. Increasing the number of trajectory points gives a more accurate average of displacement. The average displacement is needed for the Brownian motion. The research has chosen a minimum of 10 frames so that not too many particles get filtered out, but there are enough frames to validate the sizes of the particles.

3.1.3. Volume Calculations

As explained in the methodology and the characterisation, tracking the particles yields a diameter of the particles in the sample due to Brownian motion. Counting gives us an average amount of particles per frame. With the two, a volume per frame can be determined by multiplying the average amount of particles by the average volume of the measured particles. The average volume can be calculated by converting the individual diameters to all the individual volumes and then calculating the average volume per particle.
The frames are not 2D but have a volume. As usual, this volume equals the width times the length times the depth of the frame. The depth is characterized in the past and set at ≈ 3 µm. The width and length can be calculated by the pixel size and amount of pixels in the X and Y direction.
The mass per frame can be calculated with the concentration factor (CF), the staining correction (SC), and by using the density. The following formula is used with ρ being the density of the plastic particles:

$$\text{initial mass per volume of a frame} = \frac{\text{average particles per frame} \cdot \text{average volume of a single bead} \cdot \rho \cdot SC}{CF}$$

Which can be converted to a plastic mass per nl.

3.2. Atomic force microscopy

Before the real-life measurements can be executed, the AFM must be calibrated and tested on known samples. This is to master the AFM as a measurement technique and to set the parameters.

The chosen measurement parameters are as follows:

- scan area of 20 µm by 20 µm
- 256 scan lines (512 lines at one sample, due to no usable measurements)
- scan rate of 1 Hz

The scan area needs to be large enough to depict multiple particles whilst not depicting the particles too small. The resolution and pixel size is due to the number of scan lines equal to 78,1 nm. This is smaller than the 100 nm beads and smaller than the particles from the real-life samples. More scan lines would result in a better resolution, but due to the time constraint of the bachelor's thesis, 256 lines were chosen. The scan rate could be increased, but it resulted in more image errors due to the tip letting loose from the surface and creating artefacts. All the measurements of a single sample with 256 lines took already around 4 hours. The increase of scan lines would increase linearly, so the choice of 256 scan lines was made mostly.

3.2.1. Calibration

During the research, it was noticed that the 100 nm beads did not measure as 100 nm in height under the AFM but as 160 nm in height. In the first instance, it was thought to be an error by the manufacturer of the beads. There was no thought of a miscalibration of the AFM because there would be no reason that the AFM was not well calibrated. Towards the end of the research, two calibration samples were used with a step size of precisely 106 nm and 24,5 nm. This was done to be a hundred per cent sure of the calibration of the AFM. The 3D image of the calibration sample and a 2D view are depicted in Figure 10. The AFM pictures were analysed using the SPIP program.

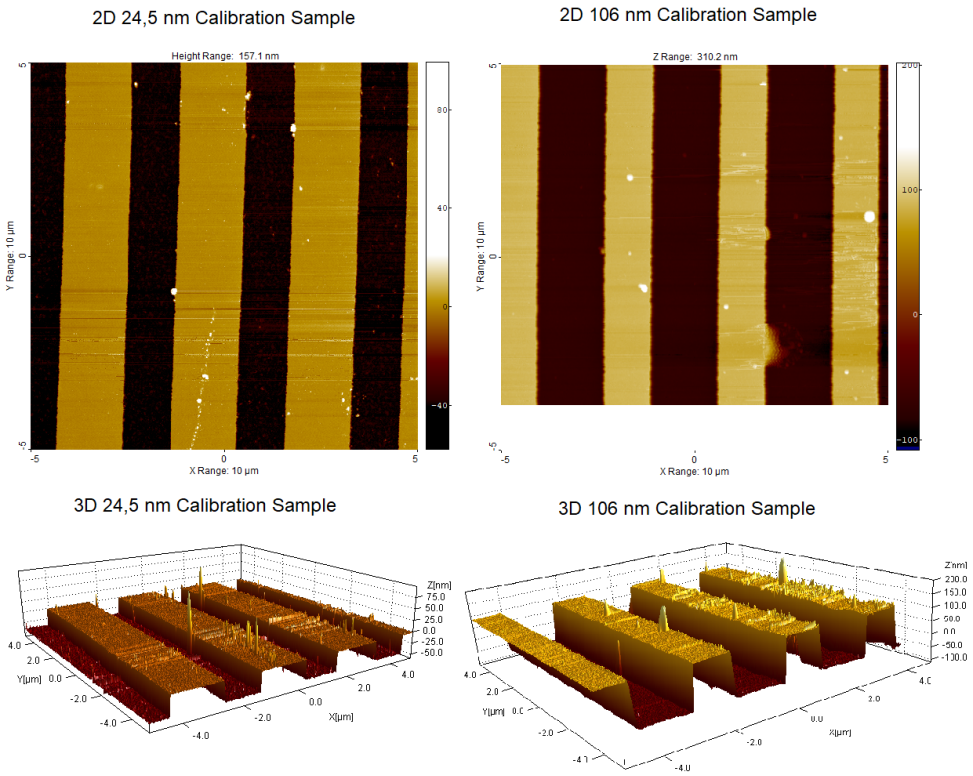

Figure 10: 2D and 3D AFM images of calibration samples with 106 nm and 24,5 nm step sizes. The left side depicts the 24,5 nm sample, and the right side depicts the 106 nm sample

The 2D AFM images are looked at in the form of a horizontal cross-section to determine the height of the step in the pattern. The average of all the horizontal lines was taken to even out the filth spikes in the sample. The following line diagrams were constructed:

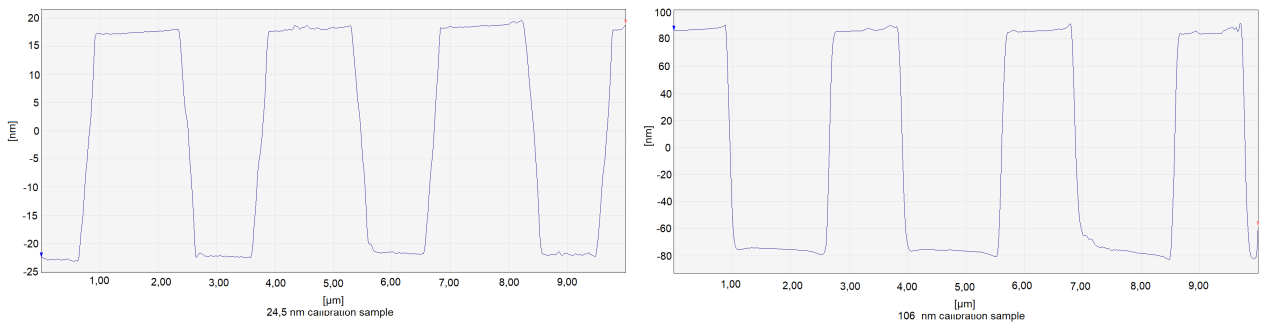

Figure 11: Line diagrams of horizontal cross-sections of the calibration samples. All possible horizontal cross-sections were taken, and the average is plotted as a line diagram

The height of the line diagrams is not equal to the expected step size. The width of the grooves and plateaus is equal to the expected size so no issue there. The line diagrams are converted to a histogram which plots the height of the measurement point to the occurrence, which gives the following histograms:

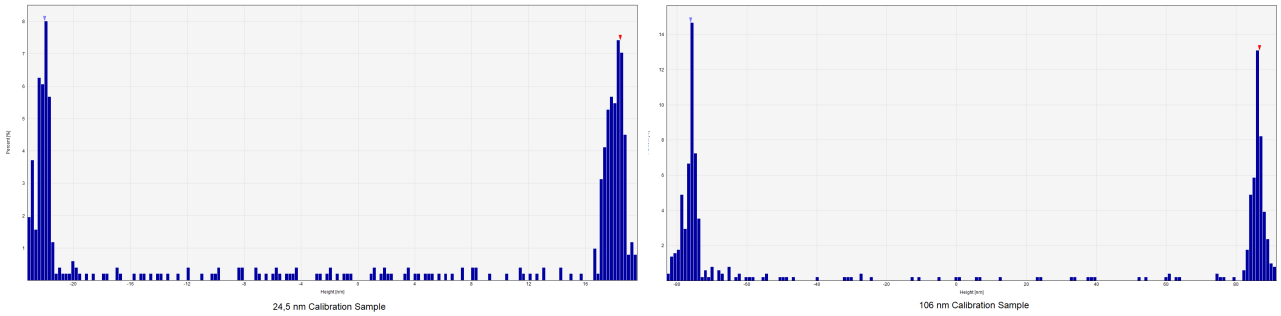

Figure 12: Histograms derived from the line diagrams in Figure 11. The height values are plotted against the occurrence to determine the average gap between the plateaus

The peaks' values were taken to calculate the height of the calibration sample's step. The peaks indicate the most frequent height value of the higher and lower plateau of the calibration sample. The error was derived by using the peaks' full-width half maximum (FWHM) values. The results were as follows:

- 24,5 nm: measured as 40,5 $\pm$ 1,3 nm (1,65 $\pm$ 0,053 times off)
- 106 nm: measured as 162,9 $\pm$ 1,9 nm (1,54 $\pm$ 0,018 times off)

The calibration of the AFM needed to be more accurate, as seen. The problem is that many measurements were done before this realization, so a calibration correction factor (CCF) was used in the results. The factor used is equal to 1,54 $\pm$ 0,018 times, calculated by dividing the measured height of the 106 nm sample by 106 nm. The 24,5 nm sample is ignored on purpose. 106 nm is the order of the measured particles. It seems that the Z-calibration is not fully linear since the 24,5 nm step sample has a mismatch with the 106 nm. The factor divides the measured height of the real-life samples by the factor to get the actual volume.

3.2.2. Reference MICA

Now that the AFM height values are calibrated, the next step in the validation is reference samples. The first reference measurement is a baseline measurement, without a sample dried in, but only the substrate MICA. MICA is commonly used in AFM due to its even surface. This was checked by measuring the MICA alone. This yielded no particles as expected. A few measurements were taken from the dataset and depicted in the following figure:

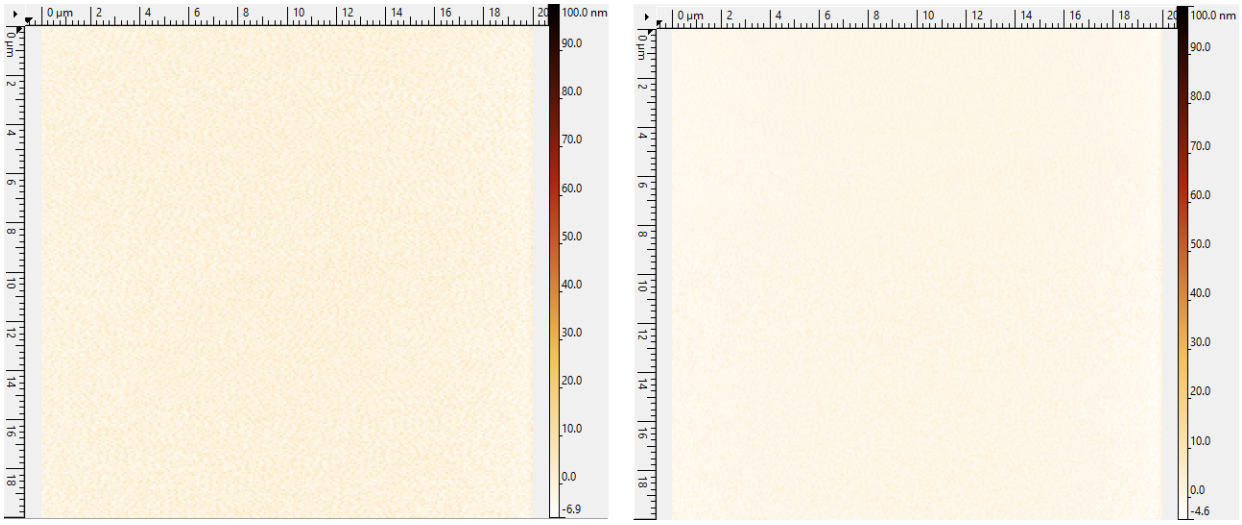

Figure 13: inverted AFM images of MICA, the substrate, without a sample dried in on. No particles were found

In the measurements, the MICA was treated with UV/Ozone radiation. To see if this impacted the MICA, AFM measurements were done on MICA treated with the radiation without a sample pipetted on the substrate. Again, a few measurements are depicted in the following figure:

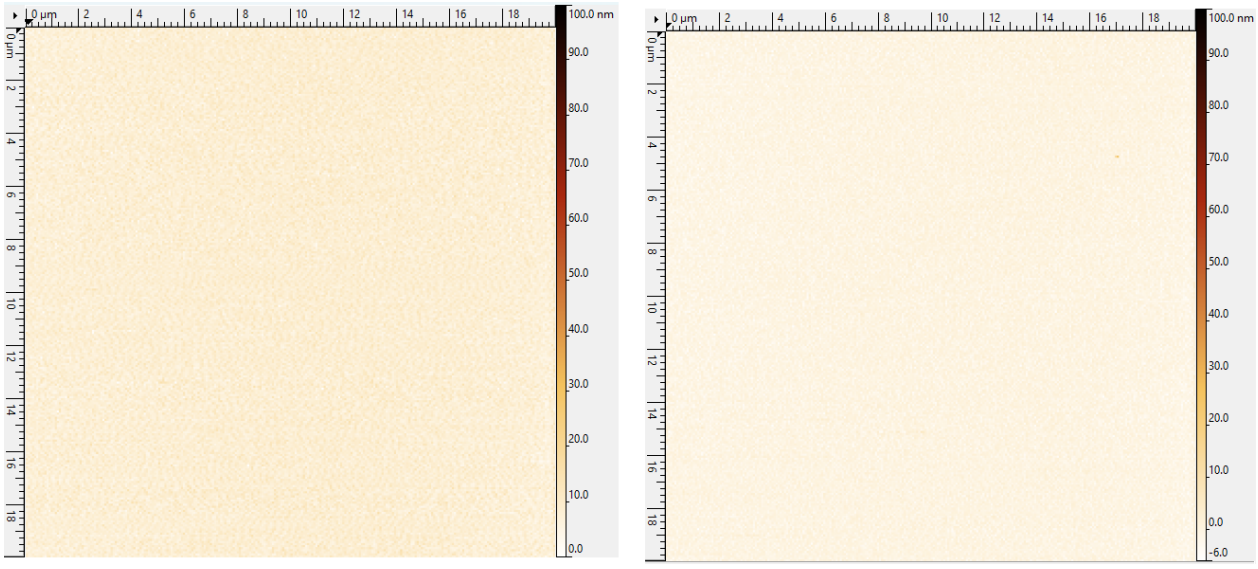

Figure 14: inverted AFM images of MICA with 15 minutes of UV/Ozone radiation, without a sample dried in on. No particles were found

The conclusion that can be drawn from these two measurements is that the MICA is a perfect substrate with a smooth surface and that the UV/Ozone radiation does not impact the substrate in a way that makes it unusable.

3.2.3. 100 nm polystyrene beads

Now that the AFM is calibrated and the substrate and measurement techniques are validated to yield no unforeseen and unwanted particles, a validation sample needs to be tested. The sample described in the methodology is 100 nm polystyrene beads, 10.000x diluted.
The height miscalibration is already discussed, but the lateral resolution of the AFM is limited by tip convolution. Tip convolution occurs due to the finite size and shape of the tip. The tip is mounted on the AFM at an angle, and the sides of the tip also stand at an angle to 'glide' over the sample instead of destroying it. To illustrate tip convolution, it is easy to compare the tip to a V-shape. Due to the width being wider when further away from the actual tip, the tip is pushed upwards by the sample, while the actual end of the tip is not yet touching the particles in the sample. This result is a bigger measured volume than the actual volume depicted in Figure 15.

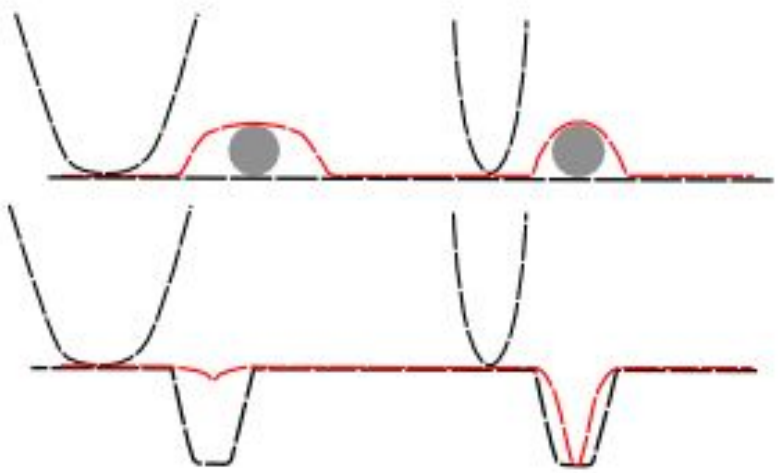

Figure 15: Tip convolution explained over a sample. The narrower the tip (right), the less tip convolution.

It is seen that a narrower tip, with a steeper angle, creates less tip convolution. On tapping mode, the shape of the tip is more or less similar so the tip has to be generated one time. SPIP must generate the tip to correct the tip convolution. The result of a generated tip is depicted in Figure 16.

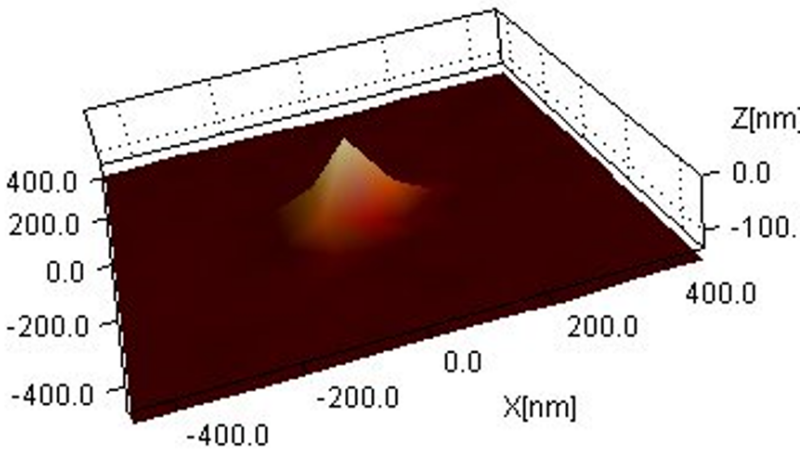

Figure 16: Auto-generated 3D image of an AFM tip

Tip deconvolution is usually done with samples with a higher resolution than the measured samples in this report. This is because it is not possible to deconvolute the images with fewer measurement points. To explain this further, figure 17 depicts the deconvolution and the effect on the volume of the measured 100 nm particles with a scan size of 20 µm.

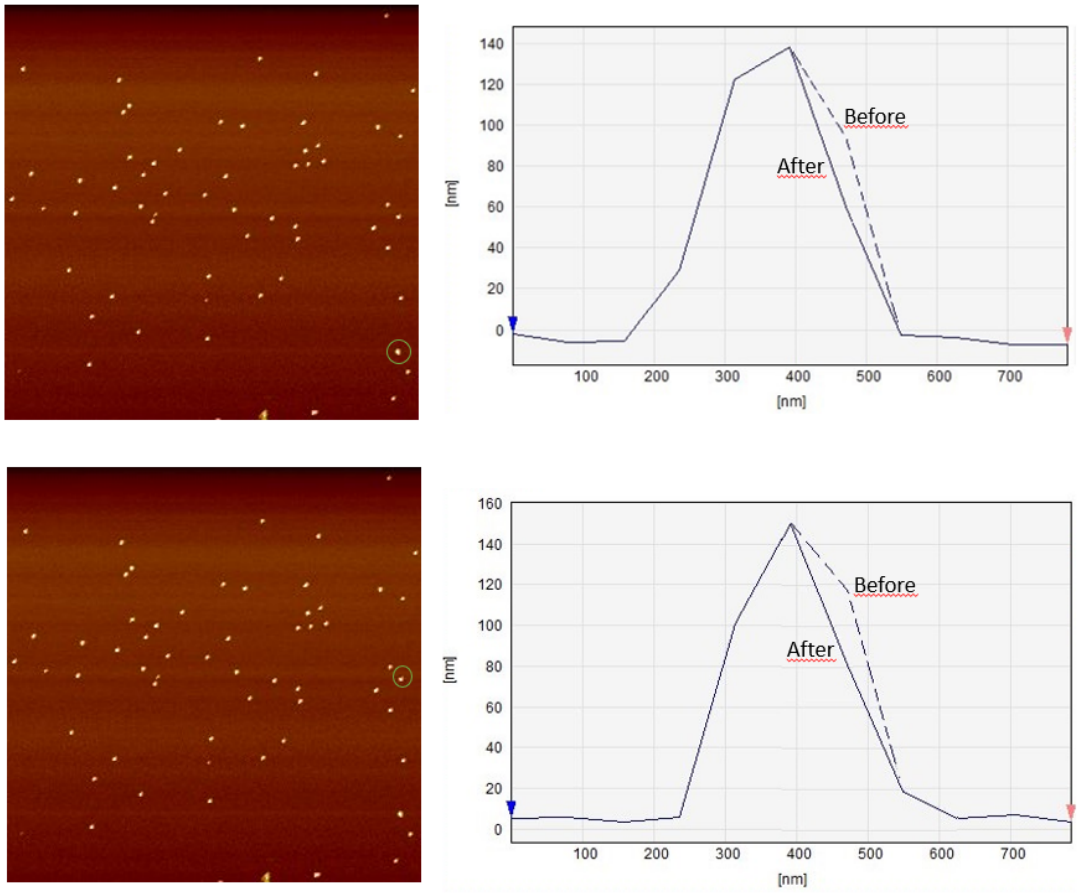

Figure 17: Tip deconvolution with the measured particles. The chosen particles are circled on the left, and a horizontal cross-section was made before and after the deconvolution to indicate the change in measured heights

The tip deconvolution impacts the measured height of the sample and, thus, the volume, but it is not the impact that is expected when deconvoluting the AFM images. This is because of the earlier mentioned few measurement points. To compare the results to the effect of tip deconvolution that is desirable, single 100 nm beads are measured. To show the effect, the 2D images, including the horizontal cross-section line diagram, are depicted in Figure 18.

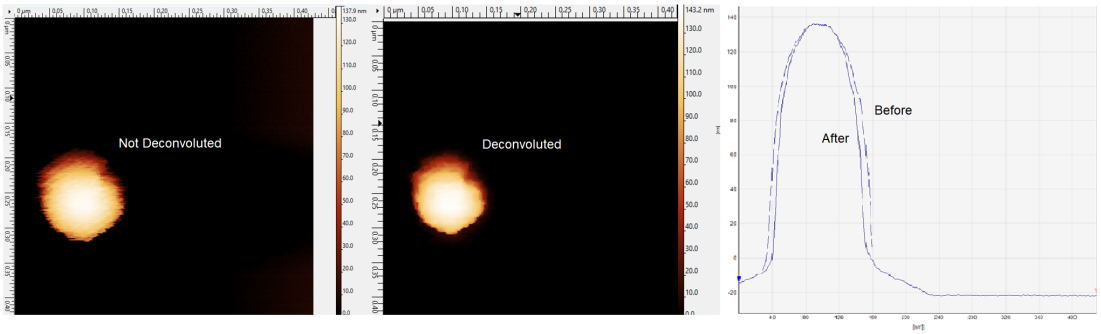

Figure 18: Tip deconvolution made clear with the 100 nm beads measured with a higher resolution (scan size = 428 nm . Left: raw image. Middle: raw image deconvoluted. Right: horizontal cross-section, which depicts the result of the deconvolution. The measured volume is narrower whilst the height (with high resolution) stays equal.

Tip deconvolution is impossible with too few measurement points; thus, deconvolution is chosen not to be executed on the AFM images. To correct for the tip convolution, the single particles are looked at from Figure 18, the zoomed-in, not deconvoluted, particles. The volume of the particles is measured by taking the following steps:

1. setting the background height to 0
2. clean up the image by removing the tilt and possible artefacts like spikes or horizontal line artefacts.
3. converting the AFM image to an XY matrix with the Z-values (height) on the elements of the list
4. factoring in a threshold in height so filth is filtered out
5. obtaining the volume by multiplying the individual heights by the pixel surface area.

A problem with this approach is that the particles are round, and there is a volume of the sphere which is measured but not present. Figure 19 schematically presents this problem.

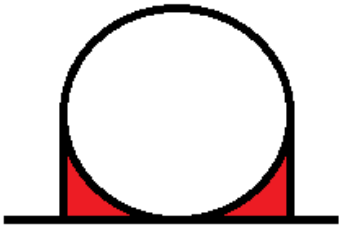

Figure 19: the measured volume of a bead (sphere) with the volume in red that is measured but not present

To correct for this volume, the measured volume is multiplied by 0,8, which ensures that the red volume is not taken into account when calculating the volume of the bead. The value is explained in the appendix C: AFM volume reduction (0,8 times). This factor is only used for the 100 nm PS beads sample.
The volume of a bead in the 20 µm scan size measurements is not equal to the volume of the beads when measured up close. This is because of the low resolution from the 20 µm scan size measurements. The beads are measured with a bigger volume when the resolution is lower. To correct for this issue, the Lateral Resolution Correction Factor (LRCF), is introduced. To calculate the LRCF, the volume of the single bead measurements is compared to the 20 µm scan size measurements. 4 measurements with a clear distinction between the particles were counted by hand, with a total of 327 100 nm polystyrene beads. The following table shows the calculations and the LRCF used for the rest of the real-life results:

20µm measurements						Single beads	
measurement	particles counted (by hand)	Volume (total) (m^3)	Error (m^3)	Volume (per particle)	Error (m^3)	Volume	Error (m^3)
1	55	1,51E-19	1,79E-21	2,75E-21	3,26E-23	8,17E-22	9,68E-24
2	59	1,58E-19	1,88E-21	2,68E-21	3,18E-23	8,98E-22	1,06E-23
3	88	2,94E-19	3,48E-21	3,34E-21	3,96E-23	9,46E-22	1,12E-23
4	125	3,93E-19	4,66E-21	3,14E-21	3,73E-23	7,23E-22	8,57E-24
5	N/A	N/A	N/A	N/A	N/A	8,83E-22	1,05E-23
Average		2,49098E-19	2,95178E-21	2,97891E-21	3,53E-23	8,53492E-22	1,0114E-23

LRCF = 3,49025989

Figure 20: The calculation of the LRCF. The individual volumes of the 20 µm scan size measurements are compared to single beads volumes to get a difference factor in the volumes

The LRCF equals 3,49, so the measured volume of the 20 µm scan size measurements has to be divided by this factor to ensure the actual volume.

3.2.4. Volume Calculations

Many corrections in the volume were needed beforehand to ensure an accurately measured volume by the AFM with a relatively low resolution compared to the measured beads.
This sub-chapter serves as a recap of all upper paragraphs of the AFM characterisation. The volume of the beads can be calculated by using the following steps/formula:

1. Put a threshold on the height values of the AFM images
   if height <threshold, height = 0
   if height >threshold, nothing happens
2. Concentrated plastic volume per frame = $(\frac{\sum heights}{LRCF})\cdot$ pixel surface area

The pixel surface area is calculated by calculating the scan area in square meters and dividing that by the number of pixels
The concentrated plastic volume per frame can be averaged per frame. The total volume in the pipetted sample can then be calculated with the following formula:

Total concentrated plastic volume = Average concentrated plastic volume per frame · number of possible frames

with:
number of possible frames = $\frac{\text{total dried surface area}}{\text{surface area frame}}$

The total dried surface area is the area that the 1 μl droplet spread out to when dried in on the MICA. The total concentrated plastic volume is calculated per pipetted sample volume. This only has to be converted to a mass and divided by the CF to get the initial mass per pipetted sample. This can be rewritten to the wanted units. The following formula is used for the last calculations:

Mass plastic per pipetted sample = $\frac{\text{The total concentrated plastic volume}\cdot\rho}{CF}$
Where ρ is equal to the density, and CF is equal to the concentration factor explained in the first paragraph of the characterisation.

For the 100 nm beads sample, a factor of 0,8 is multiplied by the height as explained in appendix C. The height of the particles is also divided by the CCF of 1,54 $\pm$ 0,018 times at all the samples except the Dopper sample due to bad calibration of the AFM. The Dopper sample was measured after the calibration of the AFM.

When the average concentrated plastic volume per frame is known the following formula can be used
Initial mass volume per nl = $\frac{\text{Average concentrated plastic volume per frame}\cdot\frac{\text{total dried surface area}}{\text{surface area frame}}\cdot\rho}{CF}\cdot\frac{\text{Pipetted volume}[\mu l]}{\text{amount of µl in a nl}}$

4. Sample results

In this chapter, the results of the measurements are explained. The methodology and characterization of the methods indicate how the measurements are executed, and the results of those measurements are given per sample.

4.1. Distilled water - blank

Before beginning with the results of the samples, a blank sample was executed, distilled water. The distilled water from the Difrax 2 sample has been used, so the way of preparing the samples is the same as the blank, except that the pure distilled water did not go into the Difrax bottle.
As expected, almost no particles showed up. The Fluorescence Microscopy counted 101 particles in a total of 384 frames, whilst the distilled water was concentrated from 92,94 ml to 0,31 ml. Tracking with this low amount of particles is not possible. To support this claim, Fluorescence Microscopy, as well as AFM images, are depicted below:

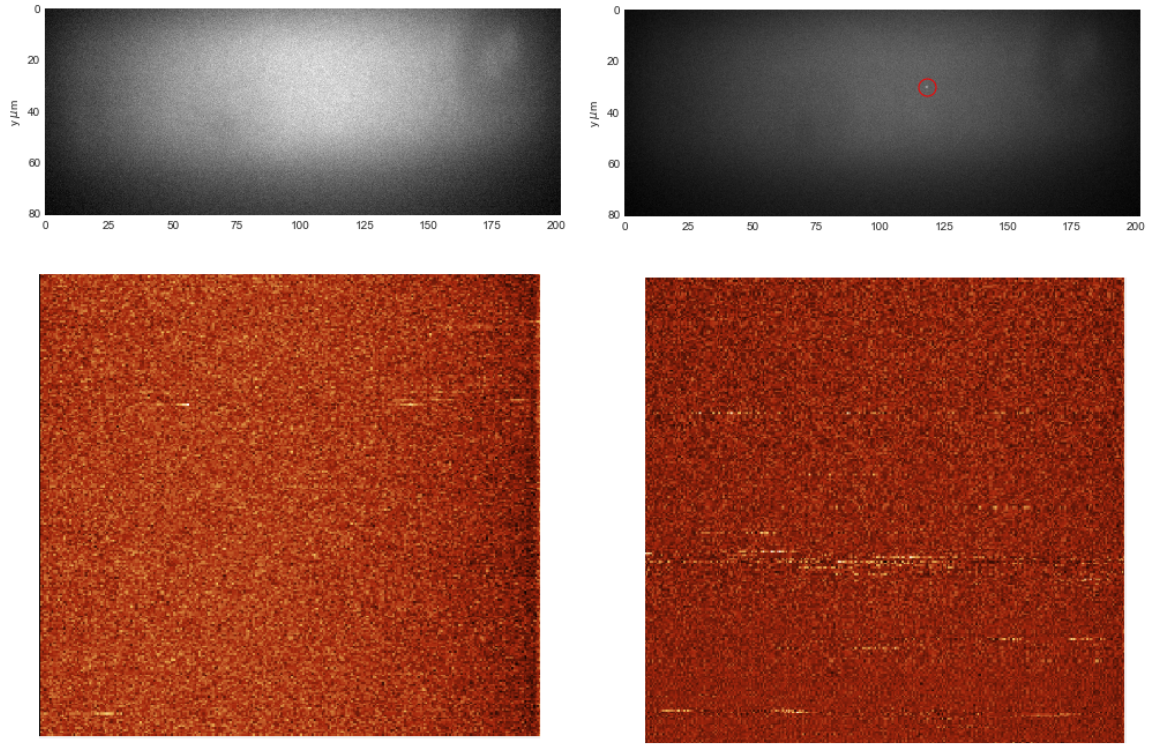

Figure 21: two examples of the analyzed Fluorescence Microscopy measurements by Trackpy and two examples of AFM images, empty

4.2. 100 nm polystyrene beads

The results begin with the results from the 100 nm polystyrene beads validation sample.

4.2.1. Fluorescence Microscopy

Given:
- SC = 1,053
- ρ = 1,05 g/cm^3
- CF = 1
- Volume of a frame: 3,84 $\cdot 10^4 \mu m^3$
  - measurement XY size = 816 x 317 pixels
  - pixel-size = 3,45 micrometres
  - height of the measurement = 3 micrometres
  - binning = 2
  - magnification = 31 times

Needed:
- average volume of a particle
- average amount of particles per frame

The trackpy software easily detected the particles. To show this, a few images of the analysis are given below:

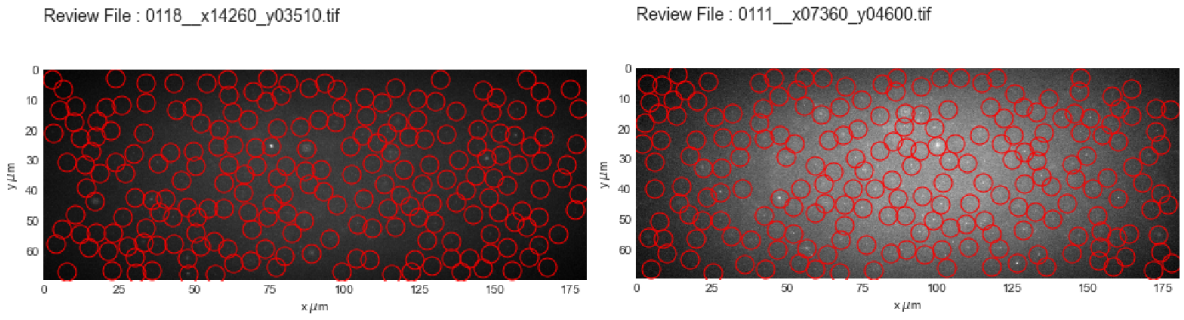

Figure 22: two examples of the analyzed Fluorescence Microscopy measurements by Trackpy. Although there are many particles, they are individually detectable

4.2.1.1 Tracking

The following numbers are derived from the Fluorescence Microscopy images, with a trajectory length >10. By tracking the particles, the size of the particles can be derived by the principle of Brownian motion. The measured diameters are put into an occurrence histogram with n=622:

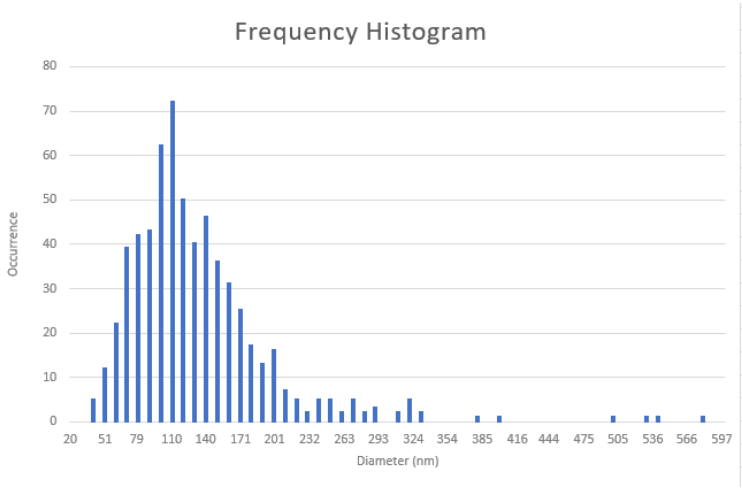

Figure 23: The diameters of the measured particles plotted out against the occurrence

A peak is seen at the 100 nm diameter, which is as expected because the beads are 100 nm. To calculate the average volume of the beads, the individual diameters were converted to the individual volumes and the following occurrence histogram is plotted:

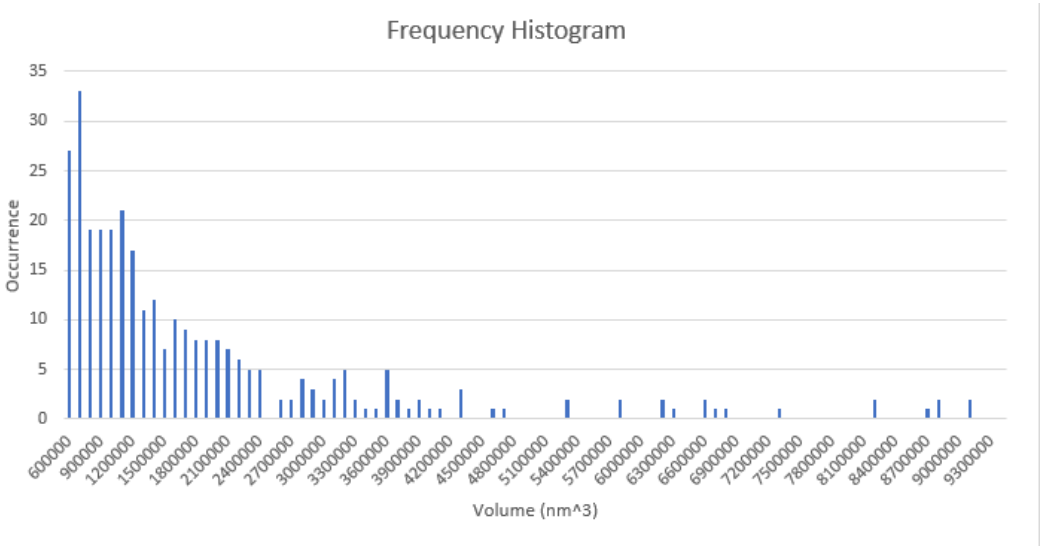

Figure 24: The Volumes of the measured particles plotted out against the occurrence

The average volume of a particle is calculated to be: $\mathbf{1,90 \cdot 10^6 nm^3}$, which correlates to an average diameter of 153,57 nm.

4.2.1.2 Counting

The following numbers are derived from the Fluorescence Microscopy images, with 128 measurements in triplo: The average amount of particles per frame = **215,63 ± 1,30 particles**.

With all this information, the total mass per volume can be calculated with the formula from the characterization:

initial mass per volume of a frame $= \frac{\text{average particles per frame} \cdot \text{average volume of a single bead} \cdot \rho \cdot SC}{CF}$

initial mass per volume of a frame $= \frac{215{,}63 \pm 1{,}30 \cdot 1{,}90 \cdot 10^{-}15 cm^3 \cdot 1{,}05 \cdot 1{,}053}{1}$ gram per 3,84 $\cdot 10^4 \mu m^3$ sample

This has to be converted to a mass volume per nl, which gives the following formula:

initial mass per nanolitre $= \frac{215{,}63 \pm 1{,}30 \cdot 1{,}90 \cdot 10^{-}15 cm^3 \cdot 1{,}05 \cdot 1{,}053}{1} \cdot \frac{1{,}0 \cdot 10^6}{3{,}84 \cdot 10^4} = 1,18 \cdot 10^{-}11 \pm 7,09 \cdot 10^{-}14$ grams per nanolitre
1 nanolitre contains 1 million cubic micrometres so the amount of frames is calculated per nanolitre by dividing the 2.

4.2.2. Atomic Force Microscopy

Given:
- $\rho = 1{,}05/cm^3$
- CF = 1
- Total dried surface area
  edge $= 2,74 \cdot 10^4 \mu m^2$ The width of the edge is equal to 1,43 μm, the average of 34 measurements of the 3 found edges
  middle $= 2,91 \cdot 10^7 \mu m^2$ The surface area is calculated by averaging the radius of the edges to the middle and calculating the area with the average radius.
- surface area of a single frame $= 400 \mu m^2$
- scan size $= 20$ µm x 20 µm
- lines = 256
- pixel surface area $= \frac{400}{256 \cdot 256} = 6,10 \cdot 10^{-}3 \mu m^2$

Needed:
- average volume per frame

The results from the 100 nm beads are different from the real-life samples. The factor of 0,8 which the end volume is multiplied by due to the spherical form, is only done with the 100 nm beads sample. The drying on the MICA of the 100 nm beads is also different. Due to containing more particles, a lot of particles have shifted to the side, leaving a ring, like a coffee stain effect. To calculate the total mass per volume, the surface area of the AFM is split into two areas. The edges have a lot more particles and to show that, figure 25 depicts a line diagram of the number of particles plotted against the X-axis of the dried droplet:

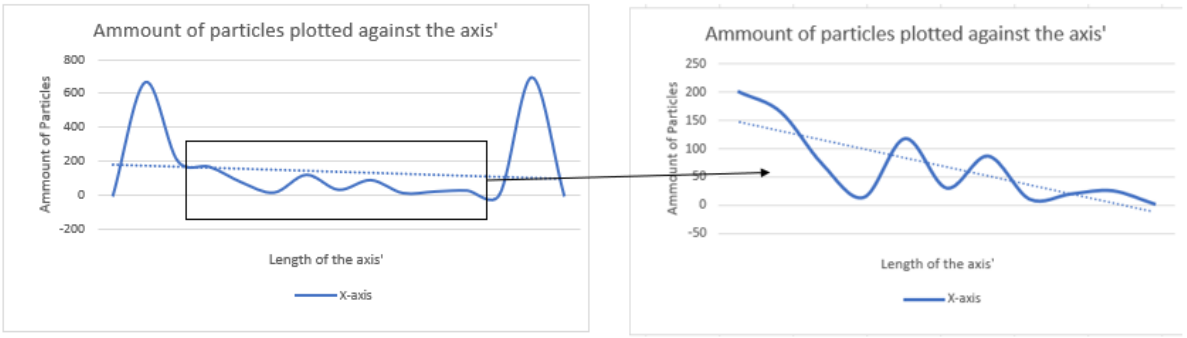

Figure 25: A line diagram of the number of particles plotted against the X-axis of the dried droplet. The middle of the diagram shows no clear increase of the particle count to the sides, so the middle is averaged over the whole surface

It is said that the particles shift to the side when drying the sample on the MICA. To show this the following Figure depicts the clustering of those particles:

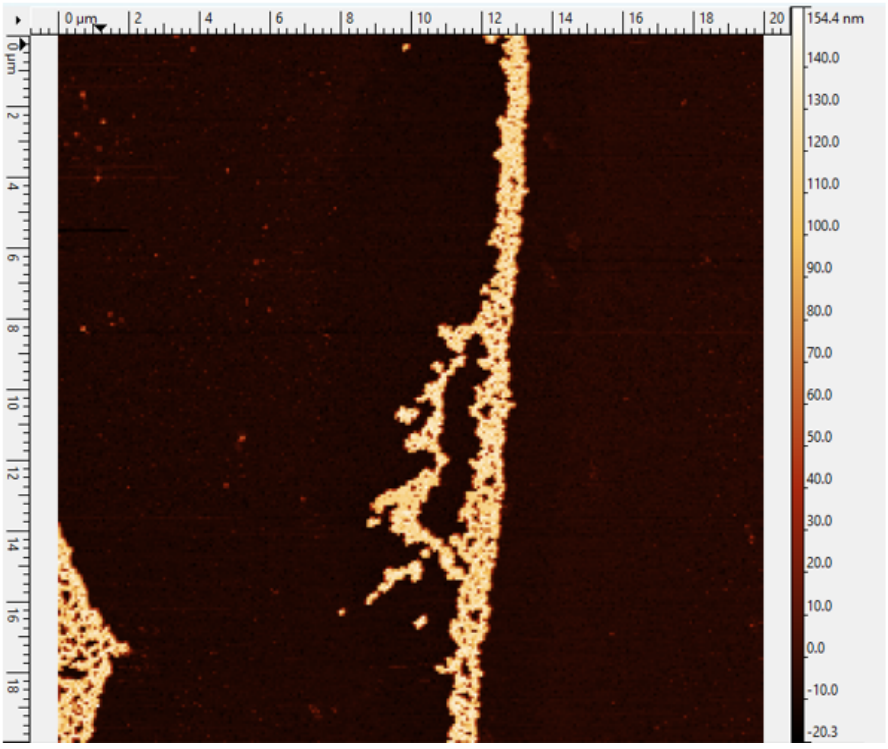

Figure 26: AFM image of the edge with the 100 nm polystyrene beads dried in on MICA

The other measurements are averaged in volume to calculate the total mass per volume. Two measurements are depicted below:

The average concentrated plastic volume per frame is equal to:

- edge: $3,28 \cdot 10^{-}12cm^3$
- middle $2,21 \cdot 10^{-}13cm^3$

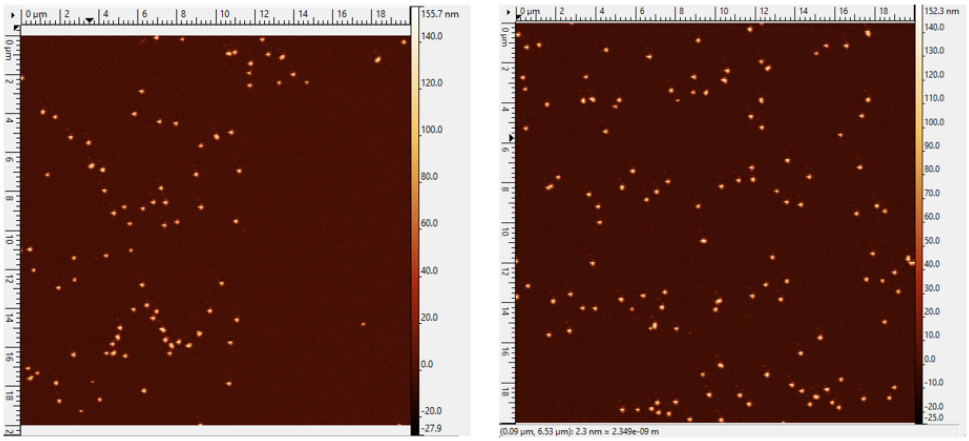

Figure 27: Two AFM images of the 100 nm polystyrene beads

To calculate the mass per nl, the following formula is used:

$$\text{Initial mass volume per nl} = \frac{\text{Average concentrated plastic volume per frame} \cdot \frac{\text{total dried surface area}}{\text{surface area frame}} \cdot \rho}{CF} \cdot \frac{\text{Pipetted volume}[\mu l]}{\text{amount of µl in a nl}}$$

Which will be split in two for the individual surfaces:

$$\text{Initial mass volume per nl (edge)} = \frac{3{,}28 \cdot 10^{-}12 \cdot \frac{2{,}74 \cdot 10^{4}}{400} \cdot 1{,}05}{1} \cdot \frac{1}{1000}$$

$$\text{Initial mass volume per nl (middle)} = \frac{2{,}21 \cdot 10^{-}13 \cdot \frac{2{,}91 \cdot 10^{7}}{400} \cdot 1{,}05}{1} \cdot \frac{1}{1000}$$

The results of the formulas have to be averaged, which is done by combining the formulas:

$$\text{Initial mass volume per nl} = \frac{3{,}28 \cdot 10^{-}12 \cdot \frac{2{,}74 \cdot 10^{4}}{400} \cdot 1{,}05 + 2{,}21 \cdot 10^{-}13 \cdot \frac{2{,}91 \cdot 10^{7}}{400} \cdot 1{,}05}{1} \cdot \frac{1}{1000}$$

$= 4,90 \cdot 10^{-}12$ grams per nl

4.3. Dopper bottle

The first real-life sample is the Dopper sample, a bottle that many students use daily.

4.3.1. Fluorescence Microscopy

Given:
- SC = 1,053
- ρ = 0,91 g/cm^3
- CF = $\frac{203{,}92}{0{,}570} = 357,8$
- Volume of a frame: 4,97 $\cdot 10^4 \mu m^3$
  - measurement XY size = 912 x 367 pixels
  - pixel-size = 3,45 micrometres
  - height of the measurement = 3 micrometres
  - binning = 2
  - magnification = 31 times

Needed:
- average volume of a particle
- average amount of particles per frame

The trackpy software easily detected the particles. To show this, a few images of the analysis are given below:

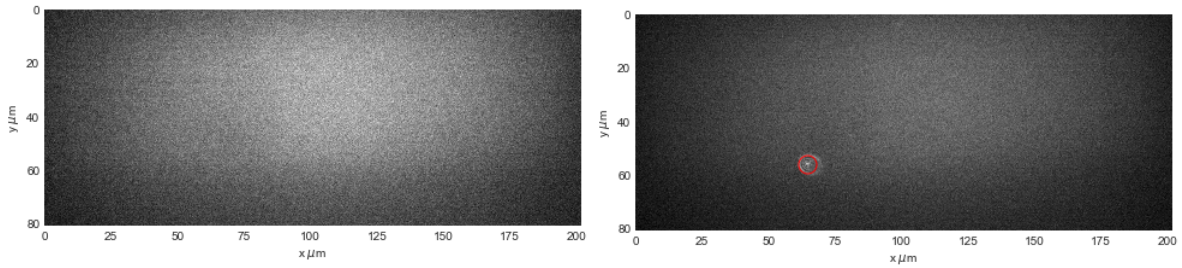

Figure 28: two examples of the Dopper sample of the analyzed Fluorescence Microscopy measurements by Trackpy

4.3.1.1 Tracking

Like the distilled water sample, the Dopper sample has almost no particles. Figure 25 shows two frames, one with a single particle and one without particles. Tracking with so few particles is not possible due to a lack of data. The average volume of a particle can not be calculated by Fluorescence Microscopy.

4.3.1.2 Counting

In the 384 frames, 156 particles were counted. This leads to an average of **0,41 ± 0,06** particles per frame. The mass per volume cannot be calculated because of the lack of information on the diameter due to no possible tracking. The number of particles per frame can be calculated with the following formula:

$$\text{particles per nl} = \frac{\text{average particles per frame} \cdot SC}{CF} \cdot \frac{\text{1 nl in } \mu m^3}{\text{Volume per frame}}$$

which gives:

$$\text{particles per nl} = \frac{0{,}41 \pm 0{,}06 \cdot 1{,}053}{357{,}8} \cdot \frac{1{,}0 \cdot 10^6}{4{,}97 \cdot 10^4} = 2,4 \cdot 10^-2 \pm 3,5 \cdot 10^-3 \text{ particles per nl}$$

4.3.2. Atomic Force Microscopy

Given:

- $\rho = 0{,}91/cm^3$
- CF = 357,8
- Total dried surface area $= 1,77 \cdot 10^7 \mu m^2$ (diameter of 4750 micrometres)
- surface area of a single frame $= 400 \mu m^2$
- scan size $= 20$ µm x 20 µm
- lines = 256
- pixel surface area $= \frac{400}{256 \cdot 256} = 6,10 \cdot 10^-3 \mu m^2$

Needed:

- average volume per frame

The results of the AFM of the Dopper bottle do not utilize the 0,8 factor volume reduction, because the particles are not assumed to be spherical. All the results are averaged to get an average volume per 400 squared micrometres. Two AFM images of the Dopper bottle are shown below:

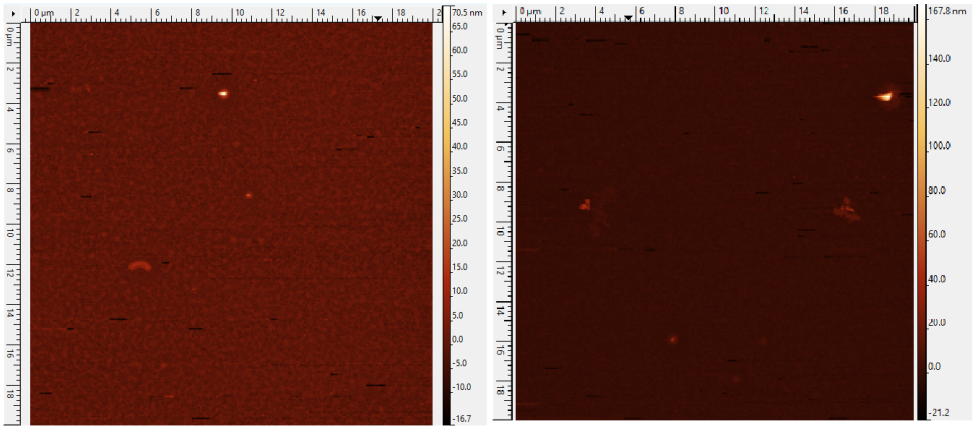

Figure 29: Two AFM images of the Dopper sample

Not a lot of particles are shown, which correlates to the few particles in the Fluorescence Microscopy images. The average volume of plastic per frame is equal to $1,11 \cdot 10^-14 cm^3$ per frame. The following formula for the total mass per nl is used:

$$\text{Initial mass volume per nl} = \frac{\text{Average concentrated plastic volume per frame} \cdot \frac{\text{total dried surface area}}{\text{surface area frame}} \cdot \rho}{CF} \cdot \frac{\text{Pipetted volume}[\mu l]}{\text{amount of µl in a nl}}$$

which gives:

$$\text{Initial mass volume per nl} = \frac{1{,}11 \cdot 10^-14 \cdot \frac{1{,}77 \cdot 10^7}{400} \cdot 0{,}91}{357{,}8} \cdot \frac{1}{1000}$$

$= 5,51 \cdot 10^-7 \cdot 10^-18$ gram per nanolitre

4.4. Difrax bottle 1

The second real-life sample is a baby bottle, the Difrax bottle 1 sample.

4.4.1. Fluorescence Microscopy

Given:
- SC = 1,053
- ρ = 0,91 g/cm^3
- CF = $\frac{195}{0,32} = 609.4$
- Volume of a frame: 3,84 $\cdot 10^4 \mu m^3$

  measurement XY size = 816 x 317 pixels
  pixel-size = 3,45 micrometres
  height of the measurement = 3 micrometres
  binning = 2
  magnification = 31 times

Needed:
- average volume of a particle
- average amount of particles per frame

The trackpy software easily detected the particles. To show this, a few images of the analysis are given below:

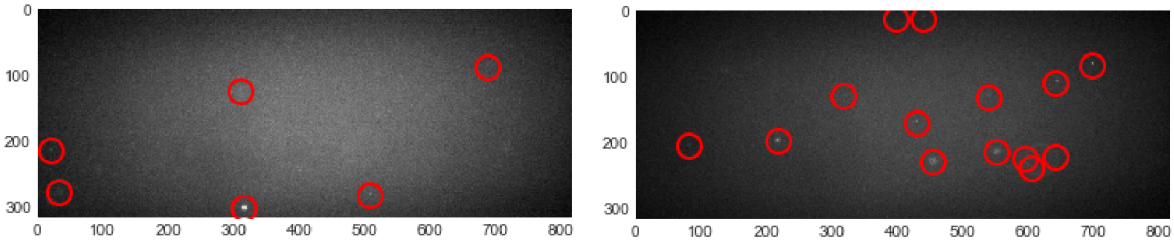

Figure 30: two examples of the analyzed Fluorescence Microscopy measurements by Trackpy. The sample is the Difrax bottle 1 sample

4.4.1.1 Tracking

The following numbers are derived from the Fluorescence Microscopy images, with a trajectory length >10. By tracking the particles, the size of the particles can be derived by the principle of Brownian motion. The measured diameters are put into an occurrence histogram with n=708:

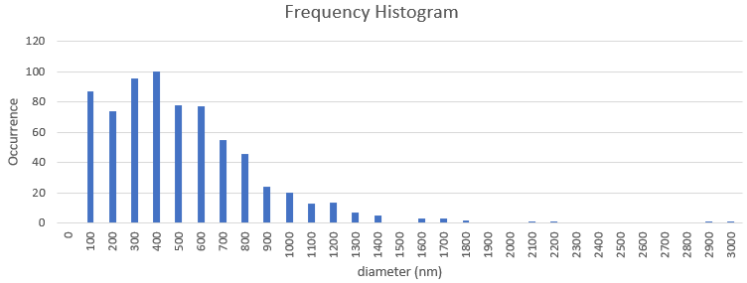

Figure 31: The diameters of the measured Difrax particles plotted out against the occurrence

To calculate the average volume of the beads, the individual diameters were converted to the individual volumes, and the following occurrence histogram is plotted:

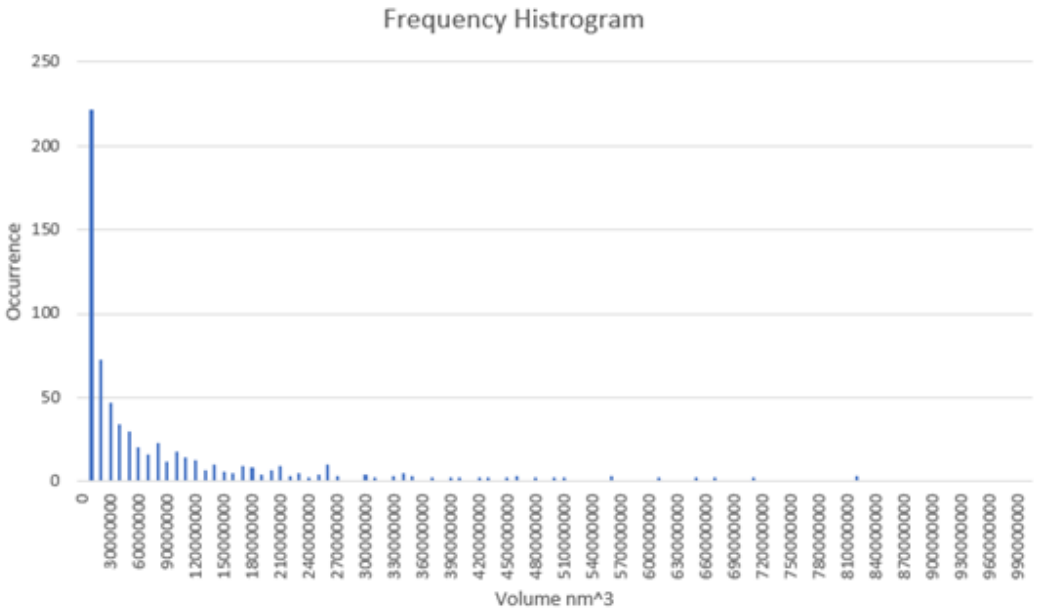

Figure 32: The Volumes of the measured Difrax particles plotted out against the occurrence

The average volume is calculated to be: $\mathbf{1,90 \cdot 10^8 nm^3}$, which correlates to an average diameter of 713,12 nm.

4.4.1.2 Counting

The following numbers are derived from the Fluorescence Microscopy images, with a total of 128 measurements in triplo: The average amount of particles per frame $= \mathbf{11,68 \pm 0,30}$ particles per frame
With all this information, the total mass per volume can be calculated.

initial mass per volume of a frame $= \frac{\text{average particles per frame} \cdot \text{average volume of a single bead} \cdot \rho \cdot SC}{CF}$

initial mass per volume of a frame $= \frac{11{,}68 \pm 0{,}30 \cdot 1{,}90 \cdot 10^{-}13 cm^3 \cdot 0{,}91 \cdot 1{,}053}{609{,}4}$ gram per 3,84 $\cdot 10^4 \mu m^3$ sample

This has to be converted to a mass volume per nl, which gives the following formula:

initial mass per nanolitre $= \frac{11{,}68 \pm 0{,}30 \cdot 1{,}90 \cdot 10^{-}13 cm^3 \cdot 0{,}91 \cdot 1{,}053}{609{,}4} \cdot \frac{1{,}0 \cdot 10^6}{3{,}84 \cdot 10^4} = 9,03 \cdot 10^{-}14 \pm 2,32 \cdot 10^{-}15$ grams per nanolitre

4.4.2. Atomic Force Microscopy

Given:
- $\rho = 0{,}91/cm^3$
- CF = 609,4
- Total dried surface area $= 3,01 \cdot 10^7 \mu m^2$ (diameter of 6190,5 micrometres)
- surface area of a single frame $= 400 \mu m^2$
- scan size $= 20$ µm x 20 µm
- lines $= 512$
- pixel surface area $= \frac{400}{512 \cdot 512} = 1,53 \cdot 10^{-}3 \mu m^2$

Needed:
- average volume per frame

The results of the AFM of the Difrax bottle 1 do not utilize the 0,8 volume reduction, because the particles are not assumed to be spherical. All the results are averaged to get an average volume per 400 squared micrometres. Two AFM images of the Difrax bottle 1 are shown below:

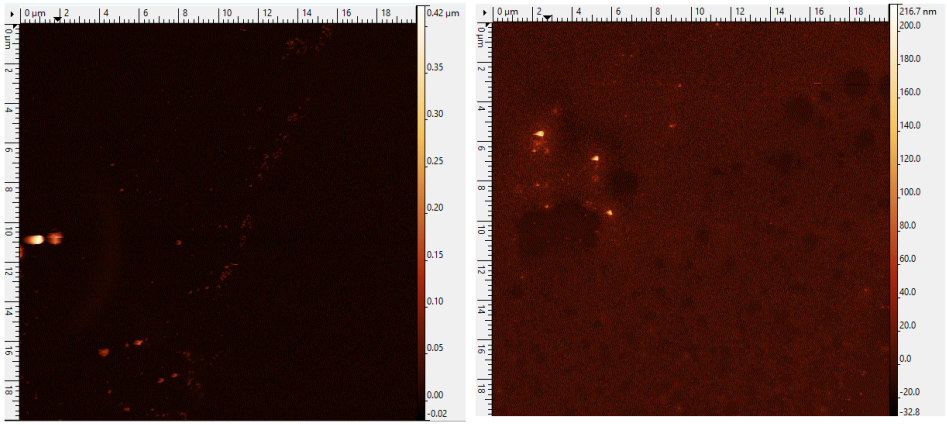

Figure 33: Two AFM images of the Difrax bottle 1 sample

The average plastic volume per frame is equal to $\mathbf{1,35 \cdot 10^{-}13 m^3}$
The following formula for the total mass per nl is used:

$$\text{Initial mass volume per nl} = \frac{\text{Average concentrated plastic volume per frame} \cdot \frac{\text{total dried surface area}}{\text{surface area frame}} \cdot \rho}{CF} \cdot \frac{\text{Pipetted volume}[\mu l]}{\text{amount of µl in a nl}}$$

which gives:

$$\text{Initial mass volume per nl} = \frac{1{,}35 \cdot 10^{-}13 \cdot \frac{3{,}01 \cdot 10^7}{400} \cdot 0{,}91}{609{,}4} \cdot \frac{1}{1000}$$
$$= 4,36 \cdot 10^{-}15 \text{ gram per nanolitre}$$

4.5. Difrax bottle 2

The third real-life sample is the second baby-bottle sample, the Difrax bottle 2 sample.

4.5.1. Fluorescence Microscopy

Given:
- SC = 1,053
- ρ = 0,91 g/cm^3
- CF = $\frac{95{,}35}{0{,}410} = 232,6$
- Volume of a frame: 4,97 $\cdot 10^4 \mu m^3$

  measurement XY size = 912 x 367 pixels
  pixel-size = 3,45 micrometres
  height of the measurement = 3 micrometres
  binning = 2
  magnification = 31 times

Needed:
- average volume of a particle
- average amount of particles per frame

The trackpy software easily detected the particles. To show this, a few images of the analysis are given below:

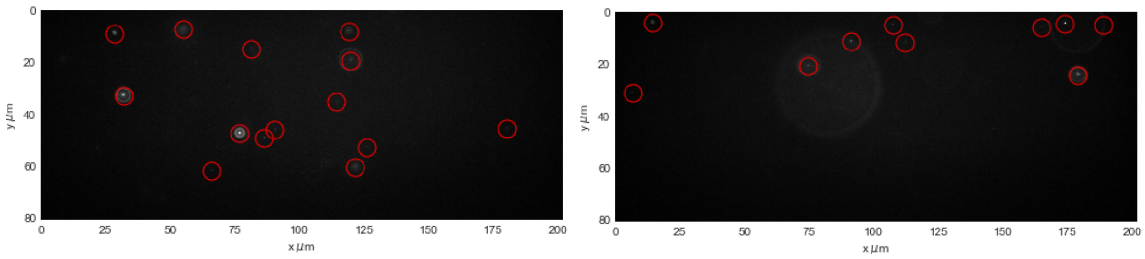

Figure 34: two examples of the analyzed Fluorescence Microscopy measurements by Trackpy. The sample is the Difrax bottle 2 sample

4.5.1.1 Tracking

The following numbers are derived from the Fluorescence Microscopy images, with a trajectory length >10. By tracking the particles, the size of the particles can be derived by the principle of Brownian motion. The measured diameters are put into an occurrence histogram with n=1284:

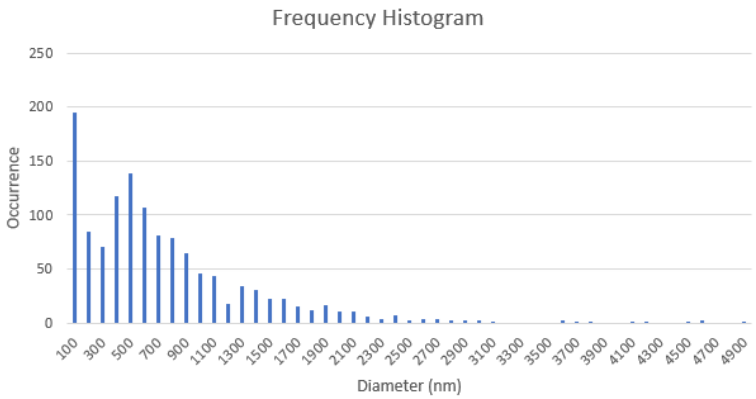

Figure 35: The diameters of the measured Difrax particles plotted out against the occurrence

To calculate the average volume of the beads, the individual diameters were converted to the individual volumes, and the following occurrence histogram is plotted:

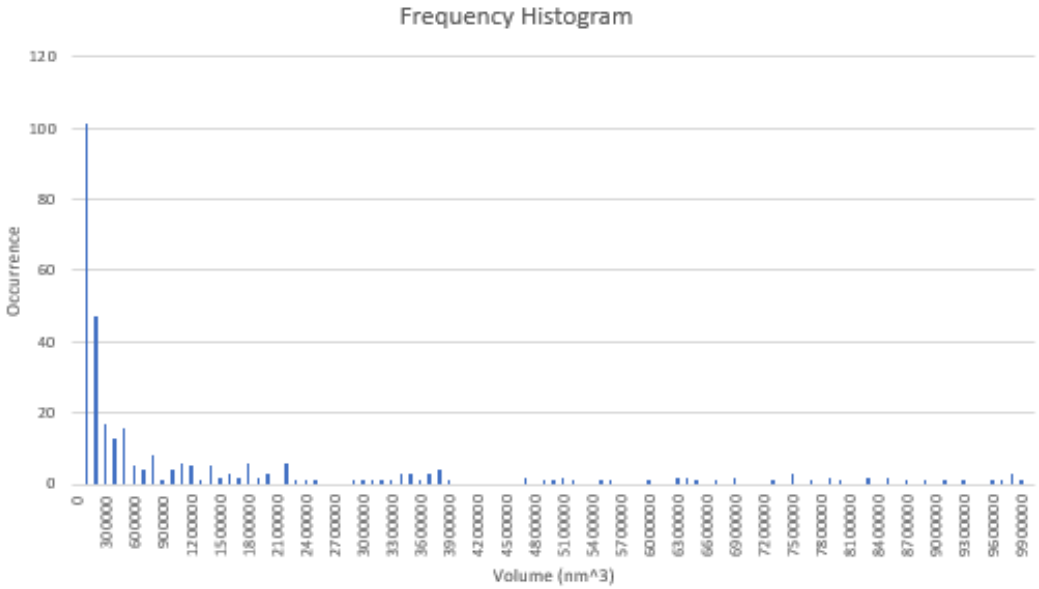

Figure 36: The Volumes of the measured Difrax particles plotted out against the occurrence

The average volume is calculated to be: $\mathbf{8,90 \cdot 10^8}$ cubic nm, which correlates to an average diameter of 1195,11 nm.

4.5.1.2 Counting

The following numbers are derived from the Fluorescence Microscopy images, with a total of 128 measurements in triplo: The average amount of particles per frame $\mathbf{8,30 \pm 0,26}$ particles per frame
With all this information, the total mass per volume can be calculated.

initial mass per volume of a frame $= \frac{\text{average particles per frame} \cdot \text{average volume of a single bead} \cdot \rho \cdot SC}{CF}$

initial mass per volume of a frame $= \frac{8{,}30 \pm 0{,}26 \cdot 8{,}90 \cdot 10^{-}13 cm^3 \cdot 0{,}91 \cdot 1{,}053}{232{,}6}$ gram per 4,97 $\cdot 10^4 \mu m^3$ sample

This has to be converted to a mass volume per nl, which gives the following formula:

initial mass per volume of a frame $= \frac{8{,}30 \pm 0{,}26 \cdot 8{,}90 \cdot 10^{-}13 cm^3 \cdot 0{,}91 \cdot 1{,}053}{232{,}6} \cdot \frac{1{,}0 \cdot 10^6}{4{,}97 \cdot 10^4} = 6,16 \cdot 10^{-}13 \pm 1,91 \cdot 10^{-}14$ grams per nanolitre

4.5.2. Atomic Force Microscopy

Given:
- $\rho = 0{,}91/cm^3$
- CF = 232,6
- Total dried surface area $= 8,30 \cdot 10^6 \mu m^2$ (diameter of 3250 micrometres)
- surface area of a single frame $= 400 \mu m^2$
- scan size $= 20$ µm x 20 µm
- lines = 256
- pixel surface area $= \frac{400}{256 \cdot 256} = 6,10 \cdot 10^{-}3 \mu m^2$

Needed:
- average volume per frame

The results of the AFM of the Difrax bottle 2 do not utilize the 0,8 volume reduction, because the particles are not assumed to be spherical. All the results are averaged to get an average volume per 400 squared micrometres. Two AFM images of the Difrax bottle 2 are shown below:

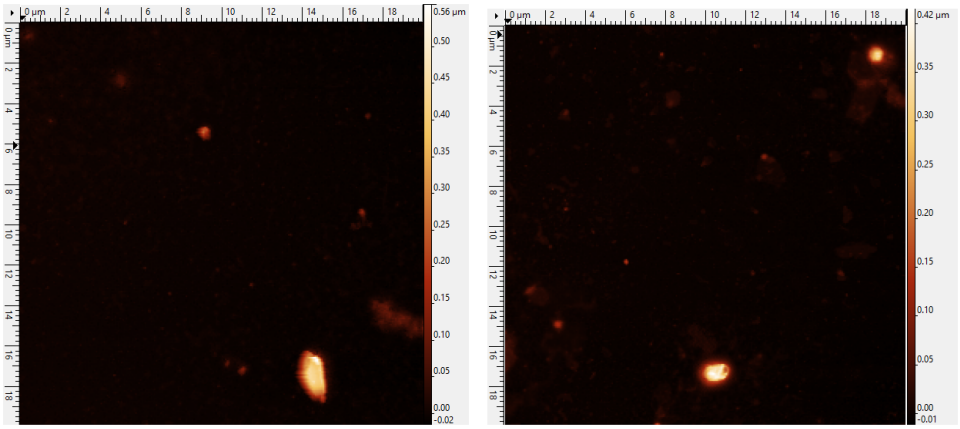

Figure 37: Two AFM images of the Difrax bottle 2 sample

The average volume of plastics per frame is equal to $\mathbf{4,77 \cdot 10^{-}13 m^3}$
The following formula for the total mass per nl is used:

$$\text{Initial mass volume per nl} = \frac{\text{Average concentrated plastic volume per frame} \cdot \frac{\text{total dried surface area}}{\text{surface area frame}} \cdot \rho}{CF} \cdot \frac{\text{Pipetted volume}[\mu l]}{\text{amount of µl in a nl}}$$

which gives:

$$\text{Initial mass volume per nl} = \frac{4{,}77 \cdot 10^{-}13 \cdot \frac{8{,}30 \cdot 10^{6}}{400} \cdot 0{,}91}{232{,}6} \cdot \frac{1}{1000}$$

$= 4,44 \cdot 10^{-}12$ gram per nanolitre

4.6. overview of the results

To get a clear overview of the results, the resulting mass per nanolitres are given in the following table:

	Mass per nanolitre [gram]	
	FM	**AFM**
100 nm PS beads	$1{,}18 \cdot 10^{-11} \pm 7{,}09 \cdot 10^{-14}$	$4{,}90 \cdot 10^{-12}$
Dopper	N/A	$5{,}51 \cdot 10^{-16}$
Difrax 1	$9{,}03 \cdot 10^{-14} \pm 2{,}32 \cdot 10^{-15}$	$4{,}36 \cdot 10^{-15}$
Difrax 2	$6{,}16 \cdot 10^{-13} \pm 1{,}91 \cdot 10^{-14}$	$4{,}44 \cdot 10^{-12}$

Figure 38: All the resulting mass per nanolitres with the different samples and measurement techniques

5. Discussion

The measurements that were executed could be better. There are a few factors that need to be discussed. The techniques self have some discussion points and de characterization and methodology as well.

5.1. Volume instead of counting

In general, the mass per volume is calculated. This means that the mass has to be calculated from a resulting volume. The problem with working with volumes of particles rather than the number of particles is that a single particle with a big volume can increase the average by a lot. When working with diameters instead of volumes, a doubling in the diameter is negligible compared to ten 'normal' diameters. When looking at volume, this is different. The fact that volume puts the radius to the third power increases the volume a lot when the radius increases.

This would not be such an issue when working with the AFM because the big outliers are not frequent. When working with Fluorescence Microscopy, this can be an issue. FM works with the principle of Brownian motion, and this way has some issues.

The frames of the FM are in 2D, so for Brownian Motion, only the motion in the X and Y direction is captured, and the motion in the Z direction is not taken into account. When the particles move a lot in the Z direction, the movement is not registered, resulting in the particle being very big and disturbing the results. The trajectory length is the way to improve this. When a particle moves a lot in the Z direction, it can become out of focus quickly, so tracking the particle longer eliminates some of those particles. Not every particle is eliminated, so it is not the perfect way to measure the diameter of the particles.

Another way to solve this is to increase the FPS. The FPS is currently at 50 frames per second, but the result can not be influenced badly when increased to 75.

It is seen in the overview of the results that the FM has greater mass' per litre with the Difrax 1 and 100 nm beads sample. This can be explained by the error in Brownian Motion, as discussed above. The Difrax bottle 2 sample also has much more mass per litre than the rest. Some big particles can also explain this. The concentration factor also plays a role here because it is concentrated from 100 ml and not 200 ml, making the impact of a single big particle even bigger.

5.2. AFM correction

Due to the time constraint of a bachelor assignment, the measurements on the AFM could not take forever. With a scan size of 20 µm, a scan speed of 1 Hz, and a total of 256-512 lines, it is possible that the AFM scanned too fast. If the AFM scans too fast, the tip can overshoot when encountering a big particle suddenly, resulting in a higher height than the actual height. It is also possible that the tip loses contact with the surface due to a fast scan speed. When this happens, horizontal error lines occur on the image. This is corrected, but it is not perfect.

The AFM has many correction factors. A factor of 0,8 for the volume correction of the beads, a lateral resolution correction factor, and a calibration correction factor. The calibration factor would not be necessary if the calibration was done initially. Unfortunately, the calibration issue was found at the end of the research, and due to time, the measurements could not be done again.

5.3. Amount of measurements

The amount of measurements with the FM sits at 384 when counting and 60 when tracking, resulting in enough particles being tracked and counted for valid results. The AFM, however, only measures around 20 - 25 frames per sample. More than 99 percent of the sample is not measured due to the time constraint. It is one of the disadvantages of the AFM, the slow scan speed. The number of measurements could be higher to get more valid results. When filth is measured, it can be hard to distinguish from particles in real-life samples, so the impact it can make is relatively significant with a few scans.

5.4. Wear of the AFM

The tip of the AFM works fine when just newly placed, but when the tip has scanned many surfaces, the tip begins to wear out. After a day of scanning, the tip does not have the same properties as at the beginning of the day. It would have been better to change the tip more times. The tip is changed too little (around four times in total when measuring) not to consider the tip wear.

5.5. Drying on MICA

All the samples are dried on MICA for the AFM measurements. The 100 nm beads show a clear edge of the drying, indicating a clear surface area for the AFM to scan. The other samples do not have the same amount of particles and do not show an edge like that. It is clear when the particles stop showing and when they are on the MICA, making it possible to calculate a surface area, but not as clear as the 100 nm beads sample. This can be resolved by drying the samples on the heat plate longer, but that can not be monitored accurately. The whole sample may evaporate. It also ensures not enough volume for the FM images to be executed.

The assumption with the AFM calculations was made that the samples dried homogeneously. This is done to make it possible to calculate a mass per volume, but the assumption is not valid. Homogeneous drying on the MICA is not possible, making the results not a hundred percent valid.

5.6. The weather

The AFM is dependent on the weather. When the air is dry, the tip can lose contact with the surface more often. This can be solved by breathing warm air on the sample, which can give more particles, making it impossible. A way to avoid losing contact is by lowering the driving amplitude, which is the cantilever's oscillation amplitude and tip.

5.7. Human error with Fluorescence Microscopy

When analyzing the FM data in Trackpy, the particles can be detected by the minimal signal value. This signal value is given by the researcher, making it possible for a human error along the way. Therefore the Poisonian error count is introduced but is not a hundred percent valid.

When tracking the particles with Trackpy, the minimal Trajectory length is ten frames. This is done by trial and error and does not have a source which it is based on. The number of particles tracked per frame differs from eight to a hundred. The low amount of particles tracked does not have a high validity per frame. Therefore 60 frames are tracked per sample, but the impact of a single frame can still be significant.

5.8. AFM error value

There is chosen not to include an error value for the AFM results. This is because the error with the few measurements could be as high as 100% with the lower mass volume samples.

6. Conclusion

In this chapter, the research question is concluded and a recommendation for further research is given.

6.1. conclusion

To get to the conclusion, the research question is repeated one more time:

How can the plastic nanoparticles be quantified with Fluorescence Microscopy and Atomic Force Microscopy while the reduced size of the particles increases the complexity of the measuring techniques?

As seen in the results, and discussed in the discussion, it is possible to detect nanoparticles in validation and real-life samples. The reduced size of the particles makes it not impossible to detect the particles but makes outliers have more impact when calculating volumes. The two measurement techniques have their advantages and disadvantages. AFM is way better in calculating and detecting the diameters (height) of the particles and FM can scan a lot of surfaces in a relatively small amount of time. Together they could be used to calculate the diameter with the AFM and the occurrence with the FM, which would possibly yield better results, but with questionable viability.

All the samples yield comparable results between the two measurement techniques. The overall conclusion that can be drawn is that the FM yields results with a higher mass per volume than the AFM, due to the issue with bigger particles by Brownian motion, explained in 5.1. The FM yields almost constant results that are 10 times higher than the results of the AFM, making the 2 comparable. Both measurement techniques can be used for quantifying the nanoparticles in the samples. The Dopper and Difrax bottle are made from the same material and the main difference in the preparation of the samples is the heat that is used in the Difrax sample. It can be concluded that the heat yields a lot more particles degrading from the bottle (around ten to almost a thousand times more).

6.2. Further research

For further research, the following aspects could be looked at:

- The qualification of the particles, with the use of Raman spectroscopy. This would eliminate filth from the plastic particles and yield better results
- The impact of heat on the release of particles can be researched. The Difrax bottle could be used with different water temperatures.
- More and longer measurements on the AFM to give it more validity

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Appendices

Appendix A: Abbreviations and Symbols

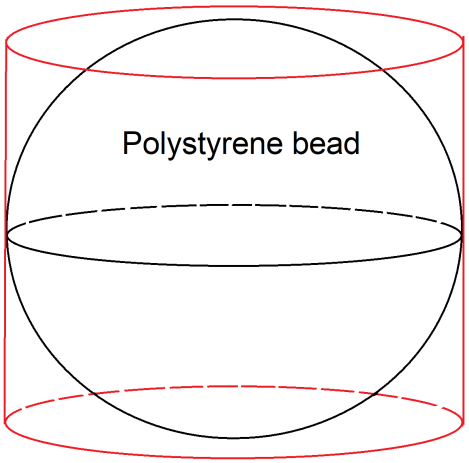
Abbreviations and Symbols	Meaning	Unit
AFM	Atomic Force Microscopy	
CCF	Calibration Correction Factor	
CF	Concentration Factor	
CSV	Concentrated Sample Volume	L
D	Diffusivity	m^2/s
FM	Fluorescence Microscopy	
FPS	Frames Per Second	#
ISV	Initial Sample Volume	L
k_b	Boltzmann Constant	$m^2 \cdot kg \cdot s^{-2} \cdot K^{-1}$
LRCF	Lateral Resolution Correction Factor	
NR	Nile Red	
PS	Polystyrene	
r	Radius	m
SC	Staining Correction	
T	Temperature	K
Δr^2	Squared Displacement	m
η	Viscosity	$kg \cdot m^{-1} \cdot s^{-1}$
ρ	Density	$g \cdot cm^{-3}$

Appendix B: Programming scripts

The script used to determine the volume of the particles from the AFM images is given below:

```
clc
close all
clear all
%% Loading TXT file (a matrix of the AFM image, with the height as the elements)
A = readmatrix('Particle 1 corrected');
A_threshold = readmatrix('Particle 1 corrected'); %loaded in 2 times to do itterations on one whilst not altering the other
amount_lines = 256; %amount of measured lines
FOV_width = 428e-9; %in meters
FOV_length = 428e-9; %in meters

%% Convert matrix to grayscale image whilst holding the FOV and pixelcount --> original image plot
I = mat2gray(A);
size = size(I);

%% Threshold to remove noise
figure()
threshold_noise = 5e-08; %threshold for the noise. Is dependend on the AFM image.
indices = find((A_threshold)<threshold_noise); %find al indices with values under the threshold
A_threshold(indices) = [0]; %indices which were found converting to zero
I_threshold = mat2gray(A_threshold); % Matrix after threshold convert to grayscale image
imshowpair(I,I_threshold,'montage') %original grey scale image showed next to the thresholded grey scale image to compare

%% Histogram plotting to detect noise. Height plotted against the occurence. Histogram determines the threshold used in line 17
figure()
nbins = 100;
subplot (1,2,1), h = histogram(A,nbins);

% show histogram after the threshold, so al values under the threshold are 0
nbins = 100;
subplot (1,2,2), h = histogram(A_threshold,nbins);
xlim([threshold_noise,4e-7]) %x limit chosen to be 4e -7, so all the values would be present
ylim([0,25000]) %idem (line 30)

%% Raw volume calculation
Sum_heights=sum(A_threshold,'all'); %in meters
Pixel_size = (FOV_width/amount_lines) * (FOV_length/amount_lines); %in meter^2
Volume = Sum_heights * Pixel_size; %in meters ^3

%% Volume correctie
CCF = 1.537;  %correction factor for the calibration
CCF_error = 0.018;
Volume_CCF = Volume / CCF;
Volume_error = Volume / (CCF-CCF_error) - Volume_CCF;
%% Volume bepaling
Volume_correct = Volume_CCF *0.8 %'Extra' measured volume what needs to be removed (factor of 0,8) --> Resulting volume
Volume_correct_error = Volume_error * 0.8 %error margin calculation --> Resulting error of the volume
```

Appendix C: AFM volume reduction (0,8 times)



Volume sphere : $V = \frac{4}{3}\pi r^3$

Volume cylinder : $V = \pi r^2 h = \pi r^2 2r = 2\pi r^3$

Difference = $\frac{2}{3}\pi r^3$

Only the bottom half is measured, so half of the difference is measured too much

$(\frac{4}{3}\pi r^3 + \frac{1}{2} * \frac{2}{3}\pi r^3) * X = \frac{4}{3}\pi r^3$

$X = 0,8$