Aalborg University

Quality Control in Microplastic Research

Statistical Approaches and Introduction of the FlowCam as Tool for Recovery Experiments

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STUDENT REPORT

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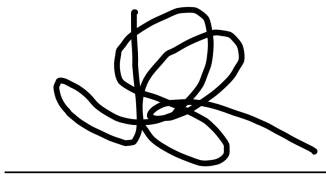
Synopsis:

This thesis describes the problem regarding the topic of microplastics in the environment and discusses the gabs of knowledge that need to be filled. There is neither a defined size on micro-, nano-, or macro particles or a standard method to separate microplastic particles from environmental samples. Thus, scientists all over the globe are working on creating standards to finally being able to compare results and numbers. In focus is the method developed at Aalborg University, especially the density separation for removing the inorganic matter is a cause of concern in regard to loss and contamination. This thesis deals with the quality of the method itself and additional with the quality of the recovery experiment that has been accomplished. Thereby it is tried to bring light into the dark regarding the detection and quantification limits especially with focus on the 'World of Microplastics'. The following parts, chapters ans section will lead the reader through definitions of terms, discussions of possible lacks and errors and assessments of the quality control both in view of the method itself and the accomplished quality assurance. This thesis is meant as a basis for future research at Aalborg University, as a starting point where the basic questions are answered.

Preface

This report is a result of the 4th semester Master thesis of the 'Water and Environmental Engineering' program at Aalborg University. The thesis is written by Lena-Sophie Kuhr in the period from February 2020 to June 2020. It deals with the method development of separating microplastic particles from environmental matrices so as sediment, water and bio organic tissue and the associated quality assurance which is necessary to create a standard method. The outbreak of Covid19 in spring 2020 caused the inevitable lock down of the laboratory in March which consequently resulted in restructuring of this thesis. After the institute reopened for laboratory work under controlled circumstances in the middle of May, the recovery experiment could partly be completed. So after the main report will follow an 'Outlook' which will take up the topic of what was planned for this thesis and can be caught up in future projects.

I wish to thank Professor Jes Vollertsen for stimulating the interest in the topic discussed here, for showing different perspectives and aspects related to the same and for his support and trust in my work. Also I whish to thank Claudia Lorenz for her patience and time for every question through the entire project and the time independent attendance in every situation.



Lena-Sophie Kuhr

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Introduction and Motivation 1.

1.1. Impression of the Plastic Problem

The innovation and fabrication of synthetic and semi-synthetic materials, such as polypropylene, polyethylene and acrylic has, combined with the rapid growth of engineering capabilities in relation to mass production, successfully made plastics to one of the most popular materials in modern times [1]. The advantage of being a functional, light weight, strong and durable material, makes plastics ideal for a variety of applications since the 1950's [2], [3]. Due to its reasonable price, about one third of all the plastics produced is used as consumer packaging material which includes disposable single-use items. Consequently,have conventional materials such as glass, metal and paper successfully been replaced over the last decades [4].

The global plastics production has on average increased by about 9% per year since 1950 and was up to 359 million tonnes (Mt) in 2018 and is based on the yearly summary of 'Plastics Europe' increasing exponentially. This is illustrated on figure 1.1.

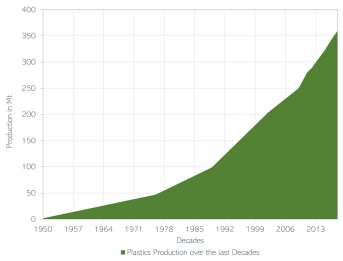

Figure 1.1.: The increasing plastic production since 1950 based on the determinations from [3], [5], [6].

While a part of the plastic waste is recycled or recovered for energy gain, the majority ends up in land fill and litter [6], [7]. However, the beneficial properties that confer plastics as highly desirable manufacturing materials, such as strength and durability, are the same properties which hamper their degradation so it takes decades before they finally decompose [8]. Consequently, the intense consumption and rapid disposal of plastic products is leading to a visible accumulation of plastics dropping and dumping on land fills and by transportation of just these through pathways into the sea [9]. It was found that approximately 8.3 Mt of the produced plastics respectively are lost to the environment and the amount of marine plastics debris is increasing worldwide [7], [10]. Thus, once plastic litter is released in the freshwater environment, they will undergo distribution, transportation and degradation processes [1]. The impact that large plastic debris, known as 'macroplastics', can have on the marine environment has long been the subject of environmental research because the issue of plastics ending up in the oceans and harming marine lifeforms and consequently at one point enter the human food chain has been known since the 1970's [7], [2]. The most common plastic

types, their application and the density [g/cm^3] compared to sea water is shown on table 1.1, based on [11].

Table 1.1.: Common polymere types including use and density compared to sea water, based on Sundt et. al. 2014.

Categories/Types	Percent of market [%]	Application/Use	Density [g/cm^3]
Polyethylene (PE)	29.5	Plastic bags, bottles and pipes	0.91-0.94
Polypropylene (PP)	18.8	Rope, bottle caps, gear, strapping	0.90-0.92
Styrene Rubber (SBR)	-	Roofing felt, car tyres	0.94-1.08
Polystyrene (PS)	7.4	Utensils, containers	1.04-1.09
Polyvinyl chloride (PVC)	10.7	Film, pipe, containers & buoys	1.16-1.30
Polyurethane (PUR)	7.3	Insulation	1.2
Polyethylene Terephtalate (PET)	6.5	Strapping, gear	1.34-1.39
Seawater	-	-	~1.02

The shown differences in density compared to sea water are the reason why plastic material has been found in varying sizes in the entire water column down to 1000 m transported through physical and biological processes[12]. And even deeper in deep water sediments has been found plastic particles, so the deepest water column is not providing protection [13], [14]. According to Crawford and Quinn, 2017, it is widely assumed that more than 50% of all thermoplastics will sink in seawater [8].

Whilst macroplastic debris has been the focus of environmental concern for a couple of decades, tiny plastic fragments, fibres and granules, collectively termed "microplastics", have been considered as a pollutant in their own right since the turn of century [15], [16]. Microplastics have been attributed with numerous size-ranges, varying from study to study and there is still no scientific justification and dedicated boundaries [17]. Officially there is no clear definition of microplastics and the change-over to macro-, and nano scaled plastics due to the size but typically a typical dimension of $1\mu m$ - $5mm$ is used to define microplastic particles as shown in figure 1.2.

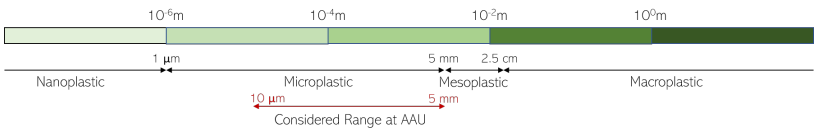

Figure 1.2.: The matter of size definition modified from [18].

To clarify the concept of microplastics they have been defined by

two categories based on their origin, primary and secondary microplastics [2], [7], [19]. Primary microplastic are made and emitted as just these particles. Examples are the abrasive microbeads in face scrubber cosmetics and toothpaste, or plastic raw material granules used in manufacturing. There are three major categories of sources for primary microplastics ending up in the marine environment based on [7] and [1]:

Internationally produced and used as such are these particles part of the human daily life in form of personal care products.

Diffuse sources Inherent by-product of other products or activities as e.g. abrasive blasting media based on plastic beads.

Accidental or unintentional spill Originated from pellets loss from plastic factories and transport.

Secondary microplastics are formed in the marine environment when macroplastic litter is fragmented to smaller and smaller pieces by weathering.

In addition, plastics contain a multitude of chemical additives and harmful substances along with the property of adsorbing organic contaminants from the surrounding media. Since these compounds can transfer to organisms by ingestion, microplastics act as vectors for other organic pollutants and are therefore, a source of exposure for wildlife to these chemicals [20]. Consequently, even if the particles are not visible for the naked eye they still have harmful properties for the environment, the marine wildlife and will at one point irresistible find a way into the human food chain [11].

1.2. Key Knowledge Gaps and Motivation

As mentioned in section 1.1, the focus on microplastic pollution is a relative new environmental challenge. In order to obtain a better understanding of this issue a focus is to develop better elaboration on the definitions and criteria of microplastics as well as on sources, pathways and extraction methods [11], [8].

Studies all over the world are concentrating on definitions and sources of microplastics but the challenging part is the isolation of microplastics from the chosen matrix which can e.g. be storm- or wastewater (e.g. [21], e.g. [22]), sediments (e.g. [23]) or biota from e.g. filterfeeders like plankton (e.g. [24]) or bivalves (e.g. [25]). However, due to the rapid emergence and development in the field of microplastic research, there is a general lack of standardisation and consistency in the extraction techniques used to extract microplastics from organic and inorganic matter. Nevertheless, there are several techniques commonly used in the laboratory for the separation of microplastics from organic and inorganic matter,

including visual sorting, filtration, sieving, density separation, elutriation, flotation and chemical digestion [8]. The aim is to develop a method which is able to give the best impression of the real situation and thereby consider any kind of contamination or loss of particles. This lead to that quality assurance and control has become increasingly important considering the expanding number of studies on microplastics using different methodologies.

The objective of this study is to give a critical review of what defines a standard method and which aspects have to be taken into account especially in regard to the limit of blanks, detection and quantification and the reason of blank and recovery experiments. Ensuing this study will give a short description of the method developed at Aalborg University in Northern Denmark with focus on quality assurance and control and give an idea of how to apply the essential aspects in laboratory experiments or statistical calculations.

Literature Review

Quality Control and Assurance

2.

As mentioned in chapter 1 has due to the rapid expansion and the exponential increasing amount of plastics and microplastics in the environment (see figure 1.1) arisen a general lack of standardisation and consistency of handling which requires action [8]. Method development and the creation of standards is connected to a number of experimental procedures and repetitions. The requirements for standard methods are pretty high and require a bank of accomplishments and correspondence because the goal is to prevent as many errors as possible and take every reasonable step to keep the measurement process reliable [26]. The necessity of quality control and assurance results in the fact of multiple possibilities to design an experiment [27]. A standard method ensures the chance to compare results regardless of location and influencing factors like e.g. temperature, pH value or possibly other fluctuating and uncontrollable factors. Quality control and assurance of a tested method is generally to provide confidence in regard to the productiveness of the considered method [28].

The development of a method is a long procedure of 'trials and errors' which includes a row of experiments, changing and repetitions before the method can be accredited and accepted as a standard [7], [29]. This procedure is simplified in figure 2.1 and shown more detailed in figure 2.2.

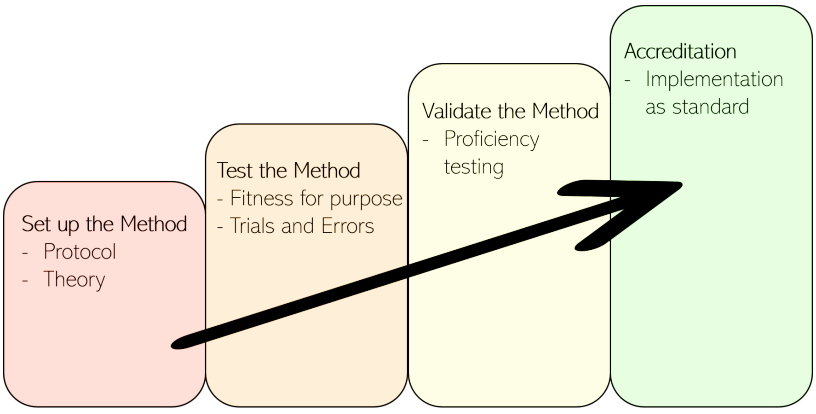

Figure 2.1.: From method development to accreditation as standard method. Modified from [29].

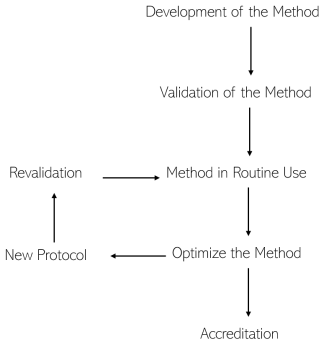

Figure 2.2.: The procedure of method development modified from [29].

The first step of method development is an idea and a general set-up/protocol of the accomplishment followed by testing and validating the procedure in daily routine. Necessary changes are made and the method is tested and validated again until it gives

satisfying results in every aspect [7]. After this procedure has been accomplished by several laboratories and institutes which agree in a consensus about the quality the method finally can be accredited as standard. Part of the validation process is the establishment of quality assurance by looking on the effectiveness and how close it can get to reality.

The limit of blanks (decision level, LoB), the limit of detection (LoD) and the limit of quantification (LoQ) ([30], [31]) are the most common parameters when looking at a quality assurance and are going to be described more detailed in the following section.

2.1. Limit of Detection, Quantification & Blanks

The limit of blank, limit of detection and the limit of quantification (LoB, LoD and LoQ) are terms used to describe the smallest concentration that can be reliably measured by an analytical procedure or experiment [30]. The definitions are according to Armbruster and Pry, 2008 ([30]) described as follows:

LoB "is estimated by measuring replicates of a blank sample calculating the mean result and the standard deviation (SD) and is calculated by following equation."

$$LoB = Mean_{Blank} + 1.645(SD_{Blank}) \tag{2.1}$$

LoD "is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. LoD is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte and is defined by following equation."

$$LoD = LoB + 1.645(SD_{lowconcentrationsample}) \tag{2.2}$$

LoQ "is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LoQ may be equivalent to the LoD or it could be at a much higher concentration."

Thereby has to be mentioned that the value of 1.645 is defined by the author. It is chosen differently from literature to literature and has no connection to the accomplished calculations in this thesis.

More detailed are these three parameters giving information about **how much/many** of the considered concentration/particles are

originated from the sample itself as how much is caused by contamination. **The limit** where it is possible to find concentration/-particles in the sample and **the limit** of identifying just these.

The dependent relationship between these three parameters is visualized on figure 2.3 which shows that if one limit shifts to the left or right it has an influence on minimum one of the two other.

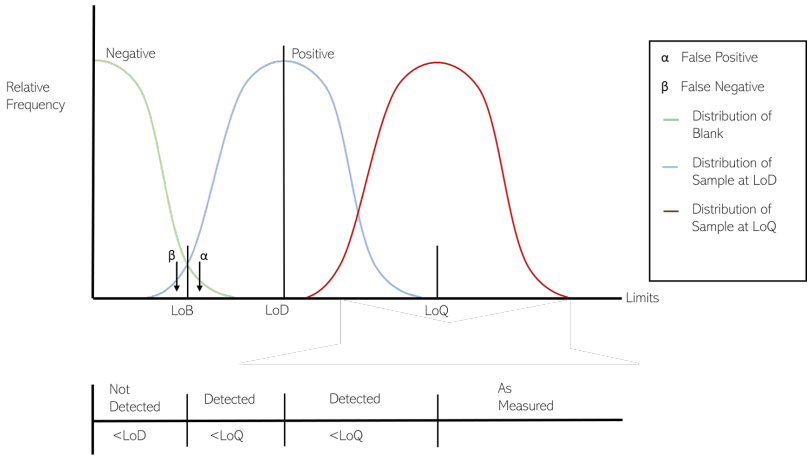

Figure 2.3.: The correlation between LoB, LoD and LoQ modified from [30]

The figure shows directly the denotation of 'false positive' and 'false negative' errors (α and β). In fact 'false positive' results are for example particles measured in an environmental sample which are considered as particles appertaining to the native sample but originated from maybe airborne contamination. To put in a nutshell, particles that are considered as 'positive' but in fact are 'negative'.

'False negative' on the contrary are the particles that are considered as negative because they are below the LoB but in reality belong to the positive part. Thus, is the estimation of LoB, LoD and LoQ as decisive parameters inevitable to assess the extent of quality of the considered method. Practically is this possible by realizing blank and recovery experiments which importance is explained in detail in section 2.2.

2.2. The Reason of Blanks and Recovery Experiments

The blank level of a sample is connected with the detection limit as visible on figure 2.3. In principle the implementation of a blank experiment is the same procedure as the considered method just with Milli-Q water instead of an analyte. The reason is in the concept of finding the 'false positive' in a sample (the α-error), the particles considered as originated from the environmental sample but in reality results from contamination. So a blank sample can produce

an analytical signal which shows the level of contamination and is used to differentiate between sample content and contamination.

A closer look on the left part of figure 2.3 gives a more detailed picture of the correlation between LoB and LoD explained by **Example 1** and illustrated on figure 2.4

Example 1

Two environmental samples were considered and treated with two different methods which are running through a quality control by checking the rate of contamination. The result of Method 1 as well as the result of Method 2 showed a concentration of 50 particles MP/L sample. Thus the amount of particles found in the blank experiments which were running parallel decides the LoB. For each environmental sample three experiments with blanks has been accomplished and the average amount of particles found are 5 particles MP/L blank for Method 1 and 20 particles MP/L blank for Method 2. Consequently the LoB in the second sample is higher which automatically means that the percentage of false positive is increasing which has a negative influence on the validation of the method why Method 1 would be considered as the more effective one.

To visualize Example 1 figure 2.3 has been reduced to show only the left part including LoB and LoD. If now figure 2.3 represents Method 1 from Example 1 and figure 2.4 represents Method 2 can be seen that the curves moved. As mentioned is the LoB higher in Method 2 which is reflected in the α and β errors. So all in all is a percentage higher LoB synonymous with a poorer quality of the method.

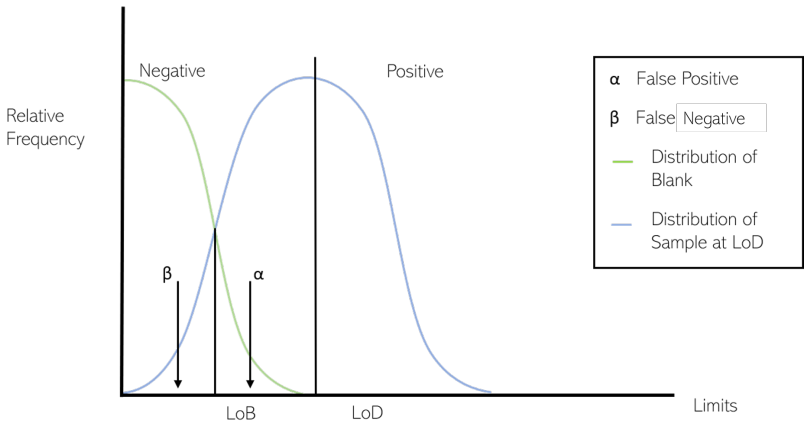

Figure 2.4.: The influence of a higher particle content in the blank, reflected in the size of errors.

Additional to the implementation of blank samples recovery studies are a classical technique for validating the performance of an analytical method in regard to be able to estimate the dimension of error [28].

On the contrary to blank samples which are used to estimate the extent of contamination, recovery experiments are designed to give an assessment of the extent of loss what to expect by using a method. Many studies, however, do not report on the exact procedures used, nor do they determine the recovery rate of microplastics from digestion methods, density separation, filtrations or the shift of laboratory equipment that have the potential to damage the structure, change the chemical composition or have an impact or physical characteristics on plastic polymers [2], [32]. Recovery experiments fulfill the purpose to test the method of its limits in terms of reality. Example 2 gives a short and easy impression of what recovery rate means.

Example 2

Again, two different methods have been tested but here to estimate the quality in regard to recovery rate. In a nutshell, a specific amount of particles are mixed with a sample which is running through the method and afterwards it is examined how many particles can be found again. Both methods have been running with a sediment sample which contained 100 particles. The experiments for each method have been repeated three times and the average result for Method 1 was a recovery of 98 particles while Method 2 showed an average of 78 particles. In this specific and easy case the amount of particles recovered are similar to the percentage rate which consequently means that Method 1 seems to show less loss of particles and thereby is considered to be better qualified than Method 2.

Thus, blank experiments are used to find the range of contamination caused by the treatment and recovery experiments used for the estimation of loss caused by the used method. So the next chapter will describe how far science is in regard to the discussed parameters in this chapter in the 'World of Microplastics'.

LOD and LOQ in the World of Microplastics

3.

The challenge of estimating LoD an LoQ in the world of microplastics refers to that these estimations until now have mainly been accomplished in regard to chemical concentrations. In particular regarding measurements of contamination in e.g. feed and food [33], in the validation process of analytical chemistry where these parameters especially are getting important when trace and ultra-trace quantities of an analyte must be detected [34], or in medicine aspects respecting drug detection [35]. In these cases the LoD is defined as the lowest concentration that with a specific certainty can be detected [28]. The question is how it is possible to project this parameter in 'the World of Microplastics' where LoD and LoQ are focused on sizes and amount of particles instead.

3.1. Understanding and Application of LoD and LoQ in Regard to Microplastics

Finding the LoD in regard to particle counting has been accomplished before by D.P. Fowler by looking on asbestos fibres or structures under a microscope and defining the level of decision and limit of detection [36] and Koelmans et. al. in regard to microplastics in fresh waters and drinking waters [37]. It is explained how 'the LoD can be involved in terms of number, mass concentrations and minimum and maximum detectable particles sizes inherent to the applied methodology' [37]. Based on microscopic counting these limits can be defined and estimated.

The detection limit is closely connected to the limit of blank as described in chapter 2.2 on page 11. In general it is useful to appropriate a LoD as soon as a background contamination of e.g. airborne particles is considered [30], [36]. Thus, **the LoD gives a statement of the amount of particles which minimum has to be observed in a sample to indicate the presence of the material with a reasonable certainty** [36].

The challenges for estimating LoD and LoQ regarding particles are explained as followed. The LoD is as described before in chapter 2 defined by itself depending on the result of the blank which in turn is depending on the contamination degree of the method. So the first step is to make some decisions to create a realistic basis of what limits can be reached. By defining these values a

limit of quantification is set. As example are in case of Aalborg University which is mentioned more detailed in the following part, the particles analyzed by FT-IR analysis after running through the experiments. The FT-IR microscope is able to detect particles down to ~5 μm which corresponds to the pixel size (5 μm x 5 μm) but the limit of quantification has been set regarding to the definitions for LoB, LoD and LoQ in section 2.1 on page 10, to 10 μm (x2 instead of 1.645) to ensure not only the presence of particles but also identify the material and composition with a certain percentage certainty. The three parameters are connected to estimate a reasonable limit of detection which in this case means the amount of particles that minimum has to be found in a sample after treatment to prove the presence of particles in the original environment. This connection has been outlined for illustration which can be seen on figure 3.1.

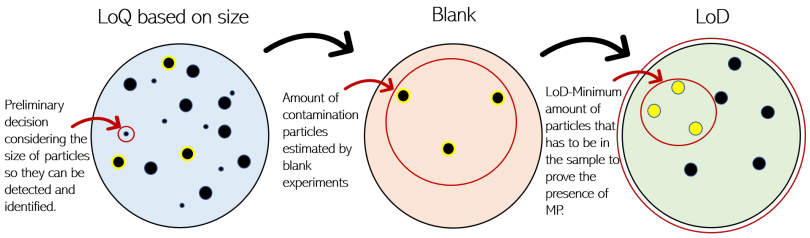

Figure 3.1.: Illustration of the connection of LoQ, the realisation of blank experiments and LoD.

LoQ The decision of only considering particles bigger than 10 μm means consequently that smaller particles are automatically ignored which on the contrary creates another error for false negative. The LoQ is based on the particle size on this illustration but there is another LoQ which is based on the amount of particles. This is going to be discussed in the further course of this part.

Blank The blank experiment is meant to eliminate the part of false positive particles which fit in the decided size range.

LoD The LoD defines the amount of particles which minimum has to be in the sample after running through the procedure.

The problem by considering the LoD based on the amount of contamination particles is the ignoring of properties like shape, size and polymer type. To analyze and interpret the particle content in a sample only based on the amount is considered as a starting point in bringing LoD into the world of microplastics but it is obvious that the situation is simplified.

Example 3

A sample has been analyzed and there have been found:

- 4 PP particles (~11 μm)
- 3 PS particles
- 5 PET particles (which by spectroscopic analysis can be assigned as polyester)
- 2 PE particles

While in the blank that was running in parallel have been found:

- 5 Polyester fibres
- 10 PP particles (~50 μm)

Now if the content would be estimated as described based on the amount, the result would show that the environmental sample contains no plastic particles because the blank showed 15 particles and the sample 14. If now the polymer type would be examined, the final result would be a content of 3 PS particles and 2 PE particles. Additional considering the size would result in that the 4 PP particles will be counted to the sample content and by looking on the shape the PET particles would as well be part of the sample. Suddenly the result will show a completely different value.

This problem provides discussion. The question is if it makes sense to include the LoD in the world of microplastics and transfer the term from concentration to particles and how to use it. As mentioned before in chapter 2 quality control and assurance is an important part of method development. However, the estimation of 'false positive' and 'false negative' is again part of quality control so there has to be found a solution before a standard method can be implemented.

The following chapters will describe the accomplishment of a recovery experiment, the statistical calculation of the LoD and the defined LoQ. Additional the described problem from 'Example 3' will come up once more and shown by an environmental sample.

Laboratory Experiments and Statistical Approaches

Method of Extracting Microplastics from a Matrix developed at AAU

4.

As described in chapter 3 there are a couple of different methods applied to extract microplastic particles from environmental matrices. The method used at Aalborg University is in principle including the following steps.

Digestion to extract the bio-organic matter.
Density Separation to remove inorganic matter.
Evaporation to create a reference amount of sample.
Scanning analysing the sample by FT-IR microscopy.

The number of digestion steps and the extent of density separation is depending on the considered matrix e.g. biota, sediment or water and size of the environmental sample. A high content of organic matter, as for example in biota samples, means consequently a more distinctive and detailed worked out digestion part while a sediment sample with a high content of inorganic matter is synonymous with a focus on the density separation. However all these steps include transfer from glassware over filtration to new glassware which are considered as sources of possible loss of particles and thereby creating an unknown part of 'false negative'. Additional the transferring procedures are also sources for airborne contamination which are synonymous to the creation of an unknown part of 'false positives'. Thus, to accomplish this method as standard method a quality control in form of recovery and blank experiments has to be done in respect to loss and contamination. The following sections will describe how a recovery experiment can be accomplished.

4.1. Practical Quality Control and Assurance

The relevancy and accomplishment of realising control experiments has been described in 2.2 on page 11. To make a blank sample is easy to realize in case of the described method from Aalborg University. Depending on the amount of environmental sample a reference quantity of filtered Milli-Q water is handled exactly the same way as the environmental sample and treated with the same procedures. The amount of particles analyzed in the blank sample is considered as the part of contamination occuring in the environmental sample. It is considered as advantageous to make more than one blank to refer to the average value. These particles are designated as 'false positive' as shown on figure 2.3.

The realization of blank experiments running parallel with the sample is the most common method to find the part of false positive particles. However, the realisation of recovery experiments to estimate the 'false negative' part of particles depends on the method and the considered material, concentration etc.. In case of the method used at Aalborg University a recovery experiment as part of this thesis has been planned and partly accomplished as described. [1]

1: The only partially carried out experiments are due to the outbreak of Covid19 in spring 2020 which caused a lock down of all laboratories in the institute.

In theory the recovery experiment of the considered method is about counting particles, adding them to a sample of cleaned sediment to avoid contamination in advance and perform the described method above or at least the most critical part for loss of particles, the density separation.

The 'Ideal' Recovery Experiment - Preliminary Considerations and Situation Dependent Changes

To design a recovery experiment as close to reality as possible more than one type of polymer should be considered with different sizes and shapes. Most sensible is to chose the ones that are typically found in environmental samples. These are mentioned in table 1.1 in chapter 1 on page 3, PE, PP, PS, PVC and PET in form of beads or granulate. In terms of the current situation described in the 'Preface' some necessary changes had to be made which made a restructuring of this thesis including the laboratory experiments was inevitable. Thus, in the further course of this thesis only the polymer that has been used (PS beads with a size of $\sim 106 \mu m$) will be considered. A closer insight to the original plan and the 'ideal' recovery experiment will be given in chapter 7 on page 47 including suggestions for further research based on this thesis. However, some preliminary conditions were made in the first couple of weeks that have to be noted. At the beginning of the laboratory work, all polymers were brought into solution with Milli-Q water. The different polymer types showed automatically various behaviour depending on their densities. Therefore, to bring every polymer listed in table 1.1 on page 4 in a more homogeneous distribution they were added to different solutions. This is illustrated on figure 4.1 based on the considered PS-beads. For the polymers that used to swim on top of Milli-Q water which included e.g. the PE beads a mixture of Milli-Q and some drops of 0.02% Tween 20 have been used while for the particles that used to sink in Milli-Q water as PVC granulate and PS-beads a solution of NaCL with a density of $\sim 1.2\ g/cm^3$ has been used.

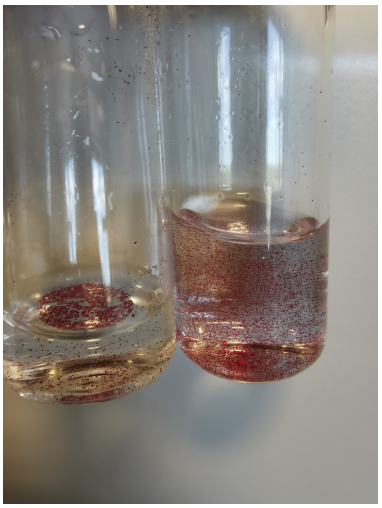

Figure 4.1.: Solution as solution. PS beads of 106 μm diameter in MilliQ water at the left and a NaCl solution with a density of 1.2 g/cm^3 to the right.

Furthermore in general it is considered as precondition to rinse every tool and glassware three times with Milli-Q water before use. However, this is no certainty for excluding contamination of

airborne particles or loss of material during performance. Additionally, people were wearing protective clothes as lab coats and gloves to avoid as much contamination as possible.

The next section will describe the relevancy of quality control followed by the implementation of the recovery experiment and the presentation of results, a discussion regarding the method and a final conclusion.

Counting with FlowCam

The FlowCam® 8000 Series Dynamic Imaging Particle Analyzer, shown on figure 4.2, is one of the new acquisitions of laboratory equipment at Aalborg University. It is commonly used to count and identify algae by digital imaging and fluorescence detection. Thus, for this thesis the FlowCam is used for a recovery experiment to count a specific amount of particles instead. It has to be mentioned that since there are no previously established methods available, a part of this thesis was to adjust the FlowCam and make it run.

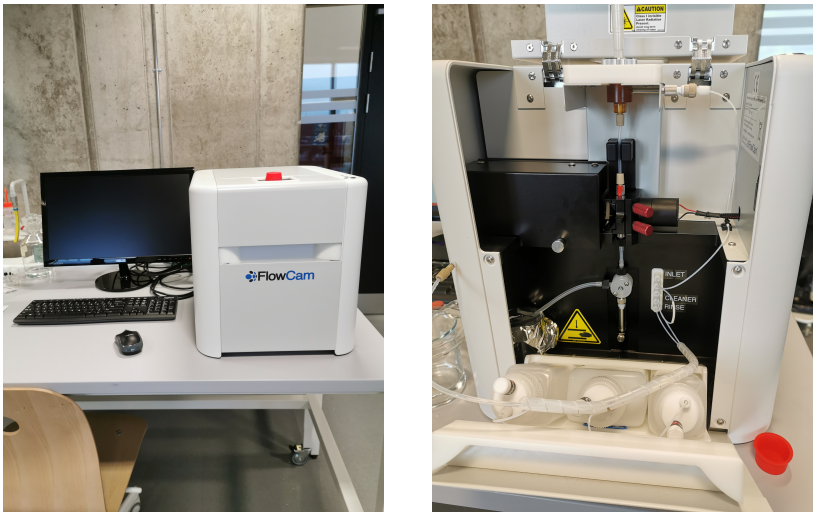

Figure 4.2.: Illustration of the FlowCam® 8000 Series Dynamic Imaging Particle Analyzer to count particles.

The recovery experiment itself was planned as listed on the following page while the detailed performance and design of the experiment will be described in the coming subsections.

1 Count a specific amount of particles.
2 Control manually by counting the particles under a microscope.
3 Add the known amount of beads to a sample of cleaned sediment.
4 Run the density separation experiment (explained in detail in the further progress of this thesis)
5 Count the particles under a microscope again.
6 Let the sample run though the FlowCam.

Based on figure 4.2 which just shows a picture of the FlowCam will the set-up and function for a better understanding be illustrated on figure 4.3 and described afterwards.

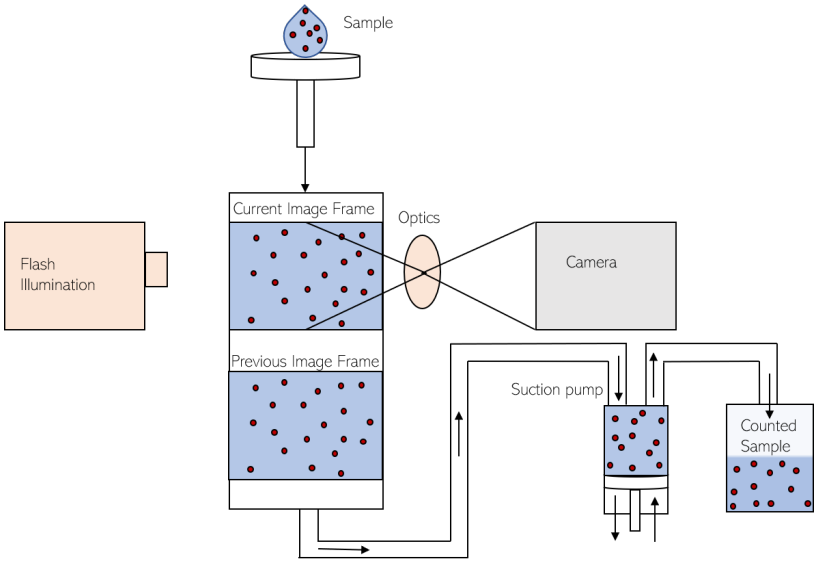

Figure 4.3.: Illustration of the function of the FlowCam.

The FlowCam is filled from above with the sample. Thereupon a suction pump is transporting the sample through a flow cell of specific dimension. In case of this thesis a flow cell with a depth of 300μm (instead of 100μm) and the dependent focus with a magnification of 4x has been selected since the considered PS beads have a size of ~106μm in diameter. The frequency of taken pictures and the flow velocity can be adjusted individually but carefully in consideration of that particles can be pictured more than once and consequently counted more than once which would automatically distort the final number. The FlowCam calculates a probability based on the flow velocity and imaging rate that all particles are getting photographed and counted. The highest percentage value to adjust was at approximately 72% with a flow velocity of 1mL and 9 pictures taken per second. In a nutshell, only 72% of all particles running through the FlowCam are counted. Consequently this has an impact on the accuracy of the final number of particles used for recovery. However, if the calculated certainty corresponds reality this would not represent a problem. Therefor, counting via

microscope was used as controlling and comparing factor which results are going to be discussed later. The settings of the FlowCam have to be chosen so no particles run through the flow cell before the counting starts or after it ends which means that the exact amount of mL has to be selected so the machine is running the right amount of time. After trials and errors it was found out that in this experiment the final amount of solution should be 10 mL whereof 2 mL are actual sample and additional the flow cell was filled with Milli-Q beforehand so no particles get sucked through the picturing part without counting. When the entire sample ran through the FlowCam, the remaining amount is used to flush out the rest of particles that got stuck in the beaker or somewhere before or after counting. The reason for this is shown up more detailed in the coming section. The FlowCam counts the particles and puts out images as shown on figure 4.4. The left picture shows the ideal sample while the right shows some fibres and airborne contamination.

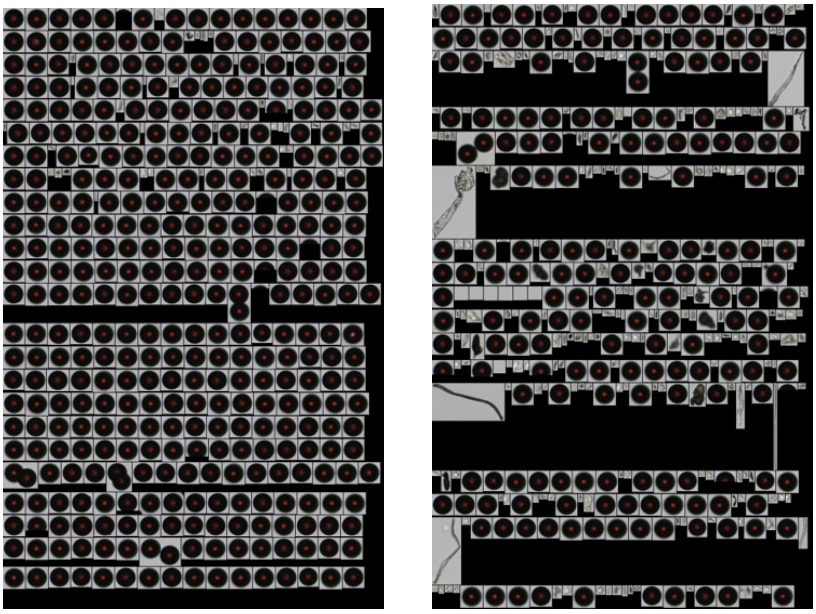

Figure 4.4.: Images of PS beads as output from the FlowCam. There is shown a clean sample on the left and a contaminated sample to the right.

To tell the FlowCam after the sample ran through which particles it has to take into account it is possible to create a library which describes the specific properties of the wanted material. Some examples of properties could be circularity, roughness, area, average blue, green or red, diameter or fiber curl. The tricky part is to find the right ranges of properties for one material so the FlowCam is just presenting the wanted ones. However, as visible on figure 4.4 some of the beads are connected which would make them fall out of the conditions of for example circularity or diameter. Thus the solution for this problem is simply to create two libraries, one for connected and one for the unconnected beads and add the counted particles of each library to the final number. So the

the preconditions of creating an optimal procedure for counting particles with the FlowCam can be summarized as followed:

Adjust Preconditions as flow velocity, imaging, flow cell size and magnification, amount of sample including flushing and pre-fill of the cell.

Input of the sample to the FlowCam in regard to volume and timing.

Select the wanted particles by creating and using a library with the specific properties.

Collect The counted particles to go on with the recovery experiment.

Counting with Microscope

The manual counting by microscope is meant as control for the amount the FlowCam came up with. Furthermore, the microscope counting is used as a common method in previous researches which makes it to a proven controlling approach. Literature examples for microplastics examination under a microscope are [8], [32], [38] and [39].

After the FlowCam procedure the particles were catched in a beaker and transferred over to a petri dish with a grid on the bottom to facilitate counting. The next paragraph will describe the preparation for the density separation and the experiment itself which is the next step after microscopic counting.

Density Separation

To prepare for the density separation, the cleaned sediment was running through a 'pre density separation' with SPT solution (Sodium Poly Tungstate) with a density of ~ 2.00 g/mL. At first, 200 g of sediment was weighted and added into a 1L beaker. The beaker was filled up to approximately 400 mL with SPT, mixed and left for sedimentation for 24 hrs for a 'pre-density separation'. This was meant to avoid introducing contamination to the recovery experiment. After 24 hrs the known amounts of PS beads were added to all three samples and mixed in. The sample was transferred into a separation funnel, aerated from the bottom for 30 minutes and left for sedimentation for 24 hrs again. This procedure is visualized on figure 4.5.

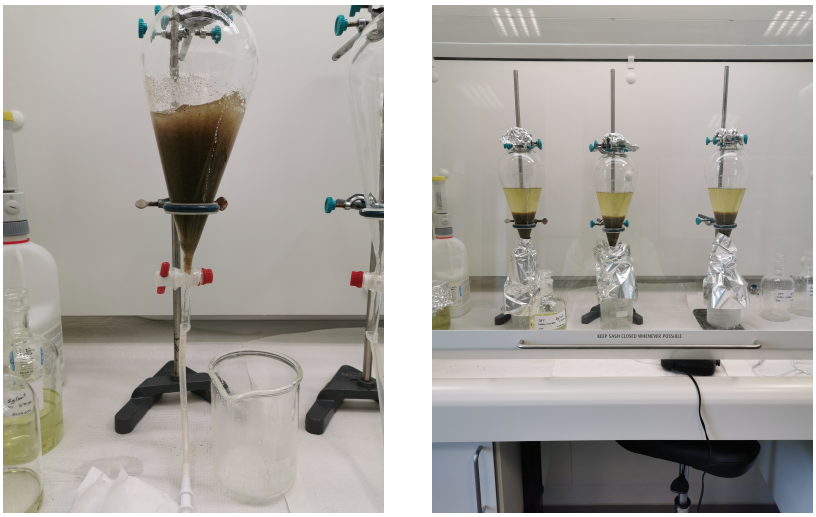

Figure 4.5.: Set up for the density separation.

In regard to the different densities shown up in table 1.1 on page 4 and the chosen density of the SPT at $\sim 2 g/cm^3$ it is assumed that after 24hrs the sample is separated in two layers. The upper part is where the particles are floating and the lower part is where the inorganic part of the sample has settled. So to split these two layers from each other the inorganic part is drained into a beaker. The upper 2-3 cm are drained into another beaker, the inner walls of the separation funnel are flushed with SPT to make sure everything stuck gets out and is kept. This procedure is repeated three times in total with the same sample which means after draining the first time the inorganic part is going through the same operation again and the upper part is added to the former one to have a final sample with all three upper parts in SPT. However, in this thesis this method is tested on its limits and one important lack of information is if it is anyhow necessary to repeat this step three times for which reason here every upper part is treated as separate sub samples and counted every one for itself.

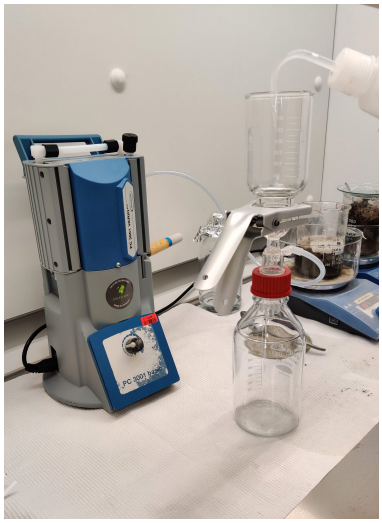

Figure 4.6.: Setup for the filtration procedure.

After all three operations have been accomplished the sample is going to be transferred from SPT to a 50% ethanol solution to afterwards evaporate the fluid and have the particles left in a petri dish ready for counting them under the microscope and FlowCam. This step is accomplished by vacuum filtration which will be explained and discussed more detailed in the following section but briefly summarized the sample of particles in SPT is transferred over to the filtration construction and filtrated over a stainless steel filter with a mesh size of 10 μm. The filter with particles is turned upside down into a beaker, covered with ethanol and ultra sonicated for five minutes to ensure the release of the attached particles and thereby minimize a risk of loss. Afterwards the filters are scratched and the ethanol is evaporated so the sample can be analyzed. The efficiency of the accomplished experiments and what is important to note during implementation is discussed

in the following section.

4.2. Results, Discussion and Conclusion

Issues and Advantages of Using the FlowCam and Microscope

Before consider the advantages and issues it is important to keep in mind that the FlowCam itself is not designed to count particles but algae. Therefore this experiment was meant as a trial to repurpose the function to unite counting and identification in one step and avoid unnecessary procedures during the method. Since the particles were counted as well by FlowCam and microscope they will be discussed and compared in the following paragraph.

The FlowCam as well as the microscope are user friendly instruments and easy to operate with. While the microscope includes manual counting and thereby the quality of the result is depending on the personal strategy of counting and the attitude to preciseness, the FlowCam works mechanically and the efficiency can be adjusted by finding the best possible ratio between flow velocity and pictures taken per second as described before. However, the best possible efficiency reached was ~ 72% which increases the error by counting with FlowCam. Thus, by only looking on the procedures both methods have issues. The FlowCam can save time but this is tied to optimal conditions as

The right size to make sure the flow cell is not gonna clogged so particles can go lost by disassembling and flushing.

Small sample size because 1mL takes approximately one minute to flow through the cell so at a certain size it more time saving to count by microscope.

The right adjustments so the FlowCam is running under optimal conditions so it is not gonna stop because of too low concentration or running dry.

These conditions are easily realized when the particles have to be counted before they are added to the sample because the size of the beads is known and the flow cell consequently is not going to be clogged. However, the FlowCam itself is creating a source of loss shown on figure 4.7 with red circles.

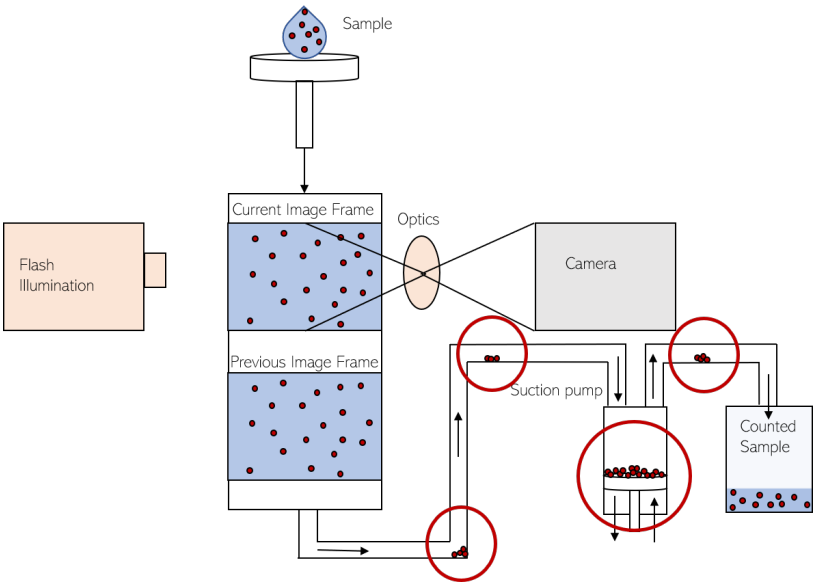

Figure 4.7.: Illustration of what happens in the FlowCam.

In reality it looks like seen on figure 4.8 which shows the pump head after a sample ran through. However, this can be circumvented by choosing a better solution so the particles won't sink that fast and get stuck, but this is only working for the pump, not for the inner side of the tubes which consequently have to be as short as possible.

The problem thereby is that the particles are counted and then get lost if the system will not be flushed carefully so it is assumed they are in the sample during the method but they aren't which means that the FlowCam during the recovery experiment creates 'false negative' particles whereas it is meant to find them instead. Additional it is calculating an error of $\sim 28\%$ (by giving a certainty that $\sim 72\%$ of the particles were counted) so already before the experiment starts, the number of particles is unknown.

Figure 4.8.: The head of the FlowCam pump which has to be cleaned after every counting experiment to ensure that all the particles counted are considered during the further procedure.

The advantage of the microscope is that the sample as well before and after running through the method just has to be evaporated on a petridish and can be counted since the size of particles doesn't matter. However, to create the optimal conditions for the FlowCam, the sample probably has to be filtered once more over a 150-180 μm steel filter which again creates a source of loss, discussed more detailed in the result part. Additional it is easier to compare the microscopic counting with other works which have been mentioned before since this method is used more commonly. So after a critical consideration of the density experiment the results will be demonstrated and discussed and a conclusion will give an subjective opinion of the methods and their efficiency.

Sources of Loss During Experiments

As mentioned the recovery experiment is part of validating the method and to show how much of the sample actually is lost and will not be considered in the final result. The goal is to recover as much as possible to reach the optimal method which reflects reality. One important information to counteract sources of loss is to figure out where the hot spots are located.

The PS beads showed a static behaviour which means they got stuck to all glassware and laboratory equipment no matter if they were in solution or not. The issues by using the FlowCam discussed before with figure 4.7 are partly attributed to this property. This could be circumvented by finding a fluid with a higher density that the FlowCam could handle and bring the particles in a more homogeneous solution which was shown on figure 4.1. However, the sample is transferred over to SPT solution after the microscopic counting. Based on the several transfers from glassware to glassware and used laboratory equipment it is important to flush everything carefully after usage. Since 'filtration' is a step that is repeated a couple of times during the method every time a switch between different solutions is necessary it is an important part to look on in regard to possible loss of particles.

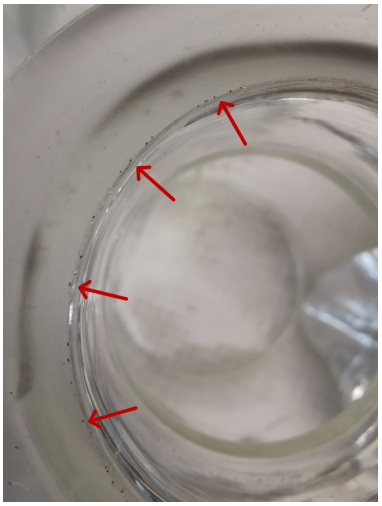

Figure 4.9.: Hotspot for loss of particles during filtration.

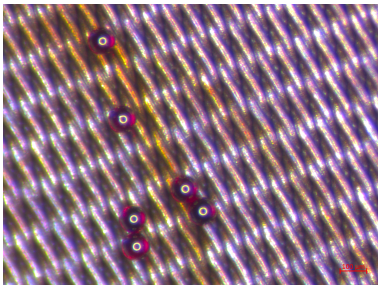

Figure 4.10.: PS-beads attached to the $10\mu m$ steel filter without ultrasonication.

Figure 4.9 shows the top part of the filtration setup where the sample is filled in and the particles hide below after removing it from the filter. Thus it is necessary to flush the glassware carefully after every filtration into a beaker to circumvent that these particles get lost. So the filtration steps between the experiments can be considered as the biggest source of loss for this method.

After the filtration the particles are sitting on a filter which has to be turned upside down in a new beaker, covered with the new solution, at this step 50% ethanol, and ultra sonicated for five minutes. Even if the ultra sonication is meant to 'shake' the particles off the filter it is no guarantee that no particles are stuck on the steel filter. Therefore it has to be scraped and flushed at least three times.

The main experiment the sample has been run through was the density separation. Even in SPT the particles showed a static behaviour which consequently presented some sources for loss. The main hot spots where the particles get stuck are shown on figure 4.11 and listed on the following page.

1 At the outer edge of the beaker.
2 At the outside of the glass funnel which is used to transfer the sample from beaker to separation funnel.
3 At the outside of the separation funnel plug caused from rotation by open and close the system to separate the upper and lower part.

Thus, to avoid losing particles all glassware has to be flushed minimum three times by considering the outer sides of the edges where the particles sit in consequence of their static behavior.

Figure 4.11.: Hotspots of loss during the density separation.

Final Results and Conclusion

The results of the recovery experiment are defined by the rate of loss that happened during the density separation with critical look at the two used counting methods.

Since counting with microscope is considered as a common counting method and used in different research works as [39] or [38] the results are considered as more 'correct' or closer to the real values while the results reached by FlowCam counting are used as an comparison for if there is an easier way of counting particles in the same or shorter amount of time. So the first counting before the particles were added to the sediment sample are shown in table 4.1 below.

Table 4.1.: Results of the Recovery experiment.

	FlowCam	Microscope	
Sample	Amount of Particles	Amount of Particles	Accordance [%]
1	2244	3103	**72.32**
2	439	611	**71.85**
3	1158	1174	**90.47**

It is shown which method counted how many particles in which sample and when result of the microscope is considered as more 'correct' how many the FlowCam found in contrast. Since the FlowCam calculated a certainty of ~ 72% the percentage improvement comes close to this value. However, sample three showed a result of 90.47% which consequently brings a higher variety in the assurance of the FlowCam calculation and has negative influence on the trust in the results. In comparison to the values from the beginning it is necessary to consult the results of counting after the density separation has been accomplished. These results are listed in table 4.2. Each sub sample represents one step of the density separation. As mentioned before would the density separation be repeated three times and all top parts concerted to one sample. However, in case of this thesis every sub sample has been collected independently

to test the necessity of realizing the density separation three times. So the sample has been counted as one before the experiment and divided into three sub samples afterwards. Additionally, sample 1 only shows results for sub samples 1.1 and 1.2. This is caused by an accident where the separation funnel broke which prevented continuation of the third round for this experiment.

Table 4.2.: Results of the Recovery experiment.

Sample 1						
	FlowCam			Microscope		
Sub Sample	Before	After	Recovery [%]	Before	After	Recovery [%]
1.1	|	1520	67.74	|	2404	77.74
1.2	2244	134	5.97	3103	193	6.22
1.3	|	-	-	|	-	-
Total	**2244**	**1654**	**73.71**	**3103**	**2597**	**83.69**
Sample 2						
	FlowCam			Microscope		
Sub Sample	Before	After	Recovery [%]	Before	After	Recovery [%]
2.1	|	170	38.72	|	552	90.34
2.2	439	37	8.43	611	54	8.84
2.3	|	-	-	|	1	0.16
Total	**439**	**207**	**47.15**	**611**	**607**	**99.35**
Sample 3						
	FlowCam			Microscope		
Sub Sample	Before	After	Recovery [%]	Before	After	Recovery [%]
3.1	|	647	55.87	|	1061	90.37
3.2	1158	109	9.41	1174	103	8.77
3.3	|	-		|	10	0.85
Total	**1158**	**756**	**65.28**	**1174**	**1174**	**100**

In this table the results of FlowCam counting and microscopic counting are directly confronted and can be compared. It has to be kept in mind that the first sample could only be repeated once because of an accident which made it impossible to repeat the density separation a third time. Therefor, the recovery rate of this experiment will not be considered for further discussions.

Two different aspects have to be differentiated looking at the reached results. On the one hand these results are giving an estimation about the quality of the method. Since it is possible by working carefully and considering every hot spot and source of possible loss to reach a recovery of 100%, the density separation experiments and filtrations are considered as an efficient way to treat environmental samples for executing microplastics. And on the other hand it is obviously undisputed that the microscope

reaches the recovery rate up to 100% while the FlowCam only reaches a recovery rate up to 73.71 %. Based on this and in regard to the factors of time, adjustments and accuracy, the method by using the microscope does better. However, this does not mean that the FlowCam cannot be used for recovery experiments in 'the world of microplastics'. This thesis is just considered as the first try which now has to be expanded and improved. Chapter 7 on page 47 will give an introduction how this can be realized. However, before discussing the further practical procedure the next chapter describes and discusses the statistical approach of estimating the LoD and LoQ after the laboratory experiments have been finished.

Calculation of LoD and LoQ by Statistical Simulations

5.

The limit of detection in regard to this method is the amount of particles that with a particular percentage ensures the presence of microplastic particles in the sample which do not origin from contamination. As described in section 2.2 on page 11 is the limit of detection depending on the realization of blank experiments and their results. As reminder is figure 2.4 shown in the margin on figure 5.1 once more.

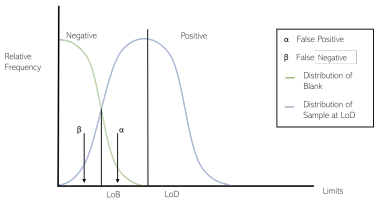

Figure 5.1.: Repetition of figure 2.4

The higher the amount of particles found in the blank compared to the number found in the environmental sample the higher is the limit of detection. To visualize the principle behind the theory the following example is meant to bring some light in the dark.

Example 4

An experiment for extracting microplastic particles from an environmental sediment sample and three parallel running blank experiments with Milli-Q water have been accomplished. Following amounts of particles has been found:

Environmental After analyzing the sample 16 particles have been found.

Blank After analyzing the blank samples an average value of 5 particles has been found.

Based on the ratio of blank and environmental sample the LoD and LoQ can be estimated. The LoD is synonymous with the amount of particles found in the blank because this is the minimum amount that has to be found to prove the presence of MP in the environmental sample. The LoQ is an individual set value which in this case is decided to be one particle more than the LoD. So basically would considering this example the LoD be 5 and the LoQ be 6. Consequently an amount of 11 particles will assumed to be in the sample.

This example is extremely simplified. The statistical model discussing in this chapter is calculating different quantiles which give an percentage range where the 'true' value is within. This is shown by some environmental samples in the following course. from three different lakes in Norway where the in- and the outlet have been sampled. The meaning by realizing statistical calculations is to find an answer to two questions.

1 Is the number of particles found in the sample corresponding the reality?

   - Calculating the range of where the real value must be with a specific certainty.

2 What is the correlation between the value found in the environmental sample, the amount found in the blank and the estimation of the LoD/LoQ.

The used program was written by Professor Jes Vollertsen from Aalborg University. The approach was to find an estimation or the real situation in three Norwegian lakes based on environmental sub samples taken in the in- and outlet. The calculations are based on the total volume (V_{total}) of the lakes, the volume of the sub sample (V_{sub}) and the amount of microplastic particles found in the sub sample (MP_{sub}). The V_{total} would be considered as indefinite size but to be able to give an estimation of the microplastic content in the system it has to be defined for the model. The chosen volume and the microplastics content of the three considered lakes numbered 1, 2 and 3 are shown in table 5.1

Table 5.1.: Results of the statistical calculations.

1		
	In	Out
V_{total} $[m^3]$	1000	1000
V_{sub} $[m^3]$	0.6	0.6
MP_{sub}	10	24
MP Content Air and Lab Blank	21	21
2		
	In	Out
V_{total} $[m^3]$	1000	1000
V_{sub} $[m^3]$	0.48	0.42
MP_{sub}	29	8
MP Content Air and Lab Blank	23	23
3		
	In	Out
V_{total} $[m^3]$	1000	1000
MP_{sub}	17	15
V_{sub} $[m^3]$	0.48	0.46
MP Content Air and Lab Blank	28	28

It has been found out that it makes no difference if the value was chosen to be 1000 m^3 as shown on table 5.1 or 10000 m^3 because it as soon as the V_{total} is more than 1000 times bigger than V_{sub} it is considered to run towards infinity. However, the size of the sub sample matters and gives different ranges of the real value. Therefor it is important to work with the highest possible amount

of sample. Briefly speaking, the higher the reference amount the lower will the uncertainty be. Consequently, these calculations are based on the results reached by the laboratory experiments finding (MP_{sub}). So the results of LoD and LoQ are depending on the contamination degree shown by the blank sample.

The following table illustrates the results of the statistical calculations from the Inlet of sample 2 as an explanatory example of the electronic appendix B.1. It shows the particle concentration found in the sample after treatment (dark green) and the associated range between the 5 percentile and the 95 percentile (light green). The true value is with a certainty of 90% within this range. The dark orange line represents the amount of particles found in the air and lab blank, in this case also considered as the LoD, while the light orange lines shows the range wherein the true value is located with a certainty of 90%. The blue marking line shows the chosen limit of quantification. Similar to the common use for the LoQ in the field of e.g. concentrations as described in chapter 2 on page 9 and 3 on page 15 where the LoQ is optional equivalent or much higher than the LoD is the LoQ value in this case equal to one particle more than then LoD and different from chapter 3 on page 15 not based on particle size but amount. Easier explained this does mean that the amount of particles in the blank are considered as contamination and therefor not part of the final result. However, if the sample content shows more than the blank it is considered as proof of an occurrence of particles in the examined environment, so one particle more in the sample than in the blank would in theory mean that the MP content in sample would be one. The matching distribution the program comes up with for this sample is shown on figure 5.2

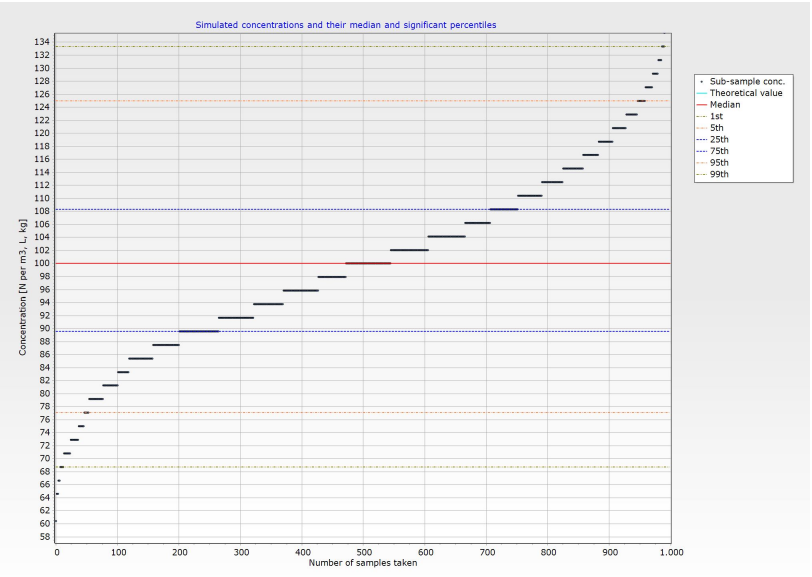

Figure 5.2.: Result of content distribution of sample 2 In .

Particle concentration	Sample replicates	1th percentile	5th percentile	25th percentile	Median concentration	75th percentile	95th percentile	99th percentile
0	1	0	0	0	0	0	0	0
1	1	0	0	0	0	2,1	4,2	6,3
2	1	0	0	0	2,1	2,1	6,3	8,3
3	1	0	0	2,1	2,1	4,2	8,3	10,4
4	1	0	0	2,1	4,2	6,3	10,4	12,5
5	1	0	0	2,1	4,2	6,3	10,4	14,6
6	1	0	0	4,2	6,3	8,3	12,5	14,6
7	1	0	2,1	4,2	6,3	8,3	14,6	18,8
8	1	0	2,1	4,2	8,3	10,4	14,6	20,8
9	1	0	2,1	6,3	8,3	12,5	16,7	20,8
10	1	2,1	4,2	6,3	10,4	12,5	18,8	22,9
11	1	2,1	4,2	8,3	10,4	14,6	18,8	25
12	1	2,1	4,2	8,3	12,5	14,6	20,8	25
13	1	2,1	4,2	10,4	12,5	16,7	22,9	27,1
14	1	4,2	6,3	10,4	14,6	16,7	25	27,1
15	1	4,2	6,3	10,4	14,6	18,8	25	29,2
16	1	4,2	6,3	12,5	14,6	18,8	27,1	31,3
17	1	4,2	8,3	12,5	16,7	20,8	27,1	31,3
18	1	6,3	8,3	14,6	18,8	22,9	29,2	33,3
19	1	6,3	8,3	14,6	18,8	22,9	29,2	33,3
20	1	6,3	10,4	14,6	18,8	25	31,3	37,5
21	1	6,3	10,4	16,7	20,8	25	33,3	37,5
22	1	8,3	10,4	16,7	20,8	27,1	33,3	39,6
23	1	8,3	12,5	16,7	22,9	27,1	35,4	41,7
24	1	8,3	12,5	18,8	22,9	29,2	35,4	41,7
25	1	8,3	14,6	20,8	25	31,3	37,5	43,8
26	1	10,4	14,6	20,8	25	31,3	39,6	45,8
27	1	12,5	14,6	20,8	27,1	31,3	39,6	45,8
28	1	10,4	16,7	22,9	27,1	33,3	41,7	47,9
29	1	12,5	16,7	22,9	29,2	33,3	43,8	47,9
30	1	12,5	16,7	22,9	29,2	35,4	43,8	50
31	1	14,6	18,8	25	29,2	35,4	45,8	50
32	1	14,6	18,8	27,1	31,3	37,5	45,8	52,1
33	1	14,6	18,8	27,1	33,3	39,6	47,9	54,2
34	1	16,7	20,8	27,1	33,3	39,6	50	56,3
35	1	18,8	22,9	29,2	35,4	39,6	50	56,3
36	1	14,6	20,8	29,2	35,4	41,7	50	56,3
37	1	16,7	22,9	31,3	35,4	43,8	52,1	58,3
38	1	18,8	22,9	31,3	37,5	43,8	52,1	60,4
39	1	18,8	25	33,3	39,6	43,8	54,2	60,4
40	1	18,8	25	33,3	39,6	45,8	56,3	60,4
41	1	20,8	25	33,3	39,6	47,9	56,3	62,5
42	1	20,8	27,1	35,4	41,7	47,9	58,3	66,7
43	1	22,9	27,1	37,5	41,7	50	58,3	66,7
44	1	20,8	27,1	37,5	43,8	50	60,4	66,7

Based on the laboratory results from table 5.1 every sample was running through the statistical program which results are listed below in table 5.2.

1		
	In	Out
MP Content Sample	10	24
95% range of reality	5-18	15-37
MP Content Air and Lab Blank	21	21
95% range of reality	13-33	13-33
Decision Value for LoQ	22	22
2		
	In	Out
MP Content Sample	29	8
95% range of reality	17-44	3-18
MP Content Air and Lab Blank	23	23
95% range of reality	13-39	12-29
Decision Value for LoQ	24	24
3		
	In	Out
MP Content Sample	17	15
95% range of reality	9-31	7-27
MP Content Air and Lab Blank	28	28
95% range of reality	18-45	18-43
Decision Value for LoQ	29	29

Table 5.2.: Results of the statistical calculations including the ranges from the electronic appendix.

At this point it is useful to remind the discussion from chapter 3 which pointed out one of the struggling problems that comes up considering LoD in regard to microplastics. Because in this example only the MP content in total has been taken into consideration as well as the entire content in the blank. No differences between particles have been considered. Consequently, if the focus is on sample 1, the inlet will not show MP content at all, while the outlet shows 2 particles based on the LoQ. But if the LoD would comply after the polymer type instead of the general amount the content would probably be higher. To illustrate this statement table 5.3 shows the real content of polymers in the in and outlet of sample 1 compared to the blank (taken from the electronic Appendix B). Basically, it is a blank correction used for a more recessed estimation of the sample content. The third sample has not been considered because the blank content is so high that the estimated content still is zero.

Table 5.3.: Results after the blank corrections.

1					
	Previous Values			After Correction	
Polymer Type	In	Out	Blank	In	Out
PE	1	1	8	0	0
PP	0	5	0	0	5
Polyester	9	18	9	0	9
PA	0	0	1	0	0
PVC	0	0	0	0	0
PS	0	0	2	0	0
PU	0	0	1	0	0
Total Content after Correction	0	3	21	0	14

2					
	Previous Values			After Correction	
Polymer Type	In	Out	Blank	In	Out
PE	0	0	9	0	0
PP	11	2	0	11	2
Polyester	16	4	10	6	0
PA	0	2	1	0	1
PVC	2	0	0	2	0
PS	0	0	2	0	0
PU	0	0	1	0	0
Total Content after Correction	8	0	21	19	3

If now each polymer type would be considered individually, the result would vary from the previous one. The inlet for sample 1 still shows less content than the blank, but the particle content in the outlet shows a result of 9 polyester particles and 5 PP particles, so 14 particles in total instead of 3. Looking at sample 2 is obvious that the content increased as well. Inlet shows a content of 19 particles instead of 8 and the outlet contains 3 particles instead of 0. Thus, is generally an increasing trend visible by considering the polymer types.

This dilemma is one of the lacks that has to be defined to include LoD and LoQ in quality management in future microplastic research. The following chapters will discuss and conclude the questions, definitions and methods that have been used during this thesis and give an outlook on how the next steps will look like with this thesis as possible basis.

Discussion and Conclusion

6. Discussion and Conclusion

The aim of this thesis was to realize recovery experiments and thereby consider the FlowCam8000 as possible opportunity for counting and characterizing polymer particles. The experiments have been accomplished in triplicates using PS beads with 106 μm diameter as described in Chapter 4 on page 21 before. The terms, methods, results and calculations have been shown, explained and discussed. Thus, a few summary questions have to be answered yet. Thereby it is to be kept in mind that this thesis has to be viewed from two angles. On one side is the method to extract microplastics from an environmental matrix which was subjected to a quality control and on the other hand the quality assurance itself in form of how to accomplish the recovery experiment to reach the best possible result. Basically, 4 statements finally need to be discussed and concluded.

1 Assessment of the method with specific focus on the density separation.
2 Estimation of the efficiency for using the FlowCam for recovery experiments.
3 The necessity of using statistical approaches for estimating the ranges of LoD and LoQ.
4 Evaluating the question of meaning regarding LoD and LoQ as parameters for microplastic research?

To give a qualitative statement for the entire method it is necessary to realize a recovery experiment by running a known amount of particles though the entire procedure including digestion, all filtrations, the density separation followed by evaporation and the analysing part. Thus, an assessment for the recovery of this thesis is only regarding the density separation. Based on the discussion regarding the hotspots of loss during the experiment and the results presented in section 4.2 on page 28 it can be stated that even if there is a risk of loosing particles it is possible to reach a recovery of 100%. This has a positive influence on the method assessment and the final estimation of the quality assurance. Briefly speaking, the result reached for the recovery experiment is considered as the highest recovery rate that could be reached which clearly means that all sources and hot spots of loss can successfully circumvented. However, the goal is to reach the highest possible recovery rate for the **entire** method which will be discussed in the 'Outlook'.

By looking at the 100% of recovery it has to be kept in mind that this result has been reached by using the traditional method of microscopic counting. So by considering the recovery experiment out of the other view and give an estimation of efficiency the microscope showed a more exact and trustful result compared to the FlowCam. The operation of the FlowCam was aimed for counting particles and trying to characterize them based on properties so as shape, roughness, area, fibre curl and circularity. Since the current situation prevented a more detailed research in regard to other polymers than the PS beads, which showed an almost perfect circularity, it was easily realizable to create a library. By using the library as filter it is possible to only show the amount of beads containing in the sample. This makes the operation with the FlowCam to an easy and not time consuming method for particle counting and detection. Since it was part of this thesis to set up the machine it was not possible to run the recovery experiment and adjust the FlowCam planned. The 'Outlook' will get deeper into what future recovery studies have to consider. Thus at this point it is not possible to make a final statement about the efficiency of the FlowCam because the time was not sufficient and the thesis had to be restructured. So even if the results show a higher efficiency by using the microscope, this doesn't mean that it can't be used for counting. Basically, the application is considered as not finished calibrating and still is a possible option to count and characterize microplastics in the future.

Based on the results reached by laboratory experiments which show the particle content in blank and environmental sample some statistical approaches by calculating the LoD and LoQ can be accomplished. The calculations demonstrated their necessity by showing up the ranges in which the 'real' value is located. By statistical calculation it is possible to give a percentage certainty of the present particle amount in the environmental sample whereas laboratory experiments only give one value. Thus since it is not possible to sample and investigate the entire environmental system statistical approaches are indispensable. The problem thereby was shown in 'Example 3' and in Chapter 5. The more the calculations and considered terms are summarized and simplified the higher is the uncertainty of the result. Briefly speaking, to get an assessment of the entire system statistical approaches are necessary. How precisely the results will be depends on how detailed the differentiation of polymers is chosen.

All in all the difficulty of including LoD and LoQ into microplastic research has been discussed and shown several times during this thesis. Even if there still are many lacks of definitions, methods and adjustments the consideration of LoD is a common and proven way of quality control. Therefor, the implementation of LoD and LoQ

as a concept in regard to the amount of particles is considered as a first approach which needs expansion. The methods used in this thesis are not considered as final solutions but as a start in the right direction for accreditation of a standard method regarding the extraction of microplastic particles from environmental matrices.

7. Outlook

At this point the laboratory and statistical part have been completed, described and discussed as detailed as the current situation regarding the outbreak of Covid19 allowed. As indicated in section 4.1 on page 21 the focus of this thesis has been shifted from the limits of FlowCam by performing a recovery experiment for the considered method from AAU to a simplified version of laboratory work with a more precise focus on the theory behind quality control and assurance. This included definitions of the concepts of the 'Limit of Detection' and 'Limit of Quantification'. This final chapter has the task to give an outlook on possible future works where the knowledge achieved during this thesis can be used as starting point.

The original plan was to look on at least five different polymer types (PE, PP, PS, PVC and PET) to cover the most common types in environmental samples. Each polymer should have been characterized individually in regard to its properties, to create suitable libraries for the FlowCam and test it on its limits to detect the particles and differentiate them from others. The idea was to create a mixture of polymers as they occurrence in environmental samples and see if the FlowCam can differentiate them. Additionally, since the different polymer types showed diverse densities the best suitable solution should have been found which creates the best homogeneous distribution for the FlowCam. The polymers should have been tested compatibility for used chemicals and treatments so as $ZnCl_2$ (which can be used for density separation as well), SPT and ultra sonication to answer the question if these terms have an influence on the particles composition, shape or surface conditions.

It was planned to count the particles via FlowCam and microscope, transfer them over to a sediment sample and let them run through a density separation with $ZnCl_2$ in a bigger construction than the separation funnels. After counting the particles again with FlowCam and microscope the evaporated sample should as well be analyzed with FT-IR spectroscopy especially when mixing the polymers in one sample to control the result of particle content and polymer occurrence the FlowCam detected. So a task for future work will be to test the FlowCam on its limits and find out how to use it for recovery. For example is it interesting to find out if the calculation of 72% particles counted corresponds to reality by repeating the procedures more than three times. All in all a future

task will be to find out the exact borders of the FlowCam so its strengths can be used for the right things. Additionally it could be interesting for future projects to see how each of the polymer types behaves on the steel filters after filtration, if are they as easy to remove by ultra sonication, scratching and flushing like the PS beads.

Anyhow, if all these questions are answered and the best possible counting method has been discovered a recovery experiment has to be realized for the entire method. This starts with digestion and goes over to the density separation followed by evaporation and analysing including all filtrations and transfers from glassware to glassware.

This thesis is intended to create a basis from where future researches can start and at some point give a staunchly estimation of the recovery potential of the method from Aalborg University and maybe finally be able to create a standard.

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Appendix

A.

Used Values for Statistical Calculation

1						
	In			Out		
Environmental Volume [m^3]						
Sampled Matrix Volume [m^3]	1.006			1.075		
Concentrated Sample Volume [mL]	5	5	5	5	5	5
Analysed Subsample Volume [mL]	1	1	1	1	1	1
Total Number of MP per Sub Sample	2	3	5	6	4	14
Number of PE per Sub Sample	1	0	0	1	0	0
Total Number of PP per Sub Sample	0	0	0	2	1	2
Total Number of Polyester per Sub Sample	1	3	5	3	3	12
Total Number of PA per Sub Sample	0	0	0	0	0	0
Total Number of PVC per Sub Sample	0	0	0	0	0	0

Table A.1.: Values from Sampling Site 1.

Table A.2.: Values from Sampling Site 2.

2						
	In			Out		
Environmental Volume [m^3]						
Sampled Matrix Volume [m^3]	1.040			1.049		
Concentrated Sample Volume [mL]	5	5	5	5	5	5
Analysed Subsample Volume [mL]	0.8	0.8	-	0.7	0.7	-
Total Number of MP per Sub Sample	12	1	-	3	1	-
Number of PE per Sub Sample	0	0	-	0	0	-
Total Number of PP per Sub Sample	5	1	-	1	0	-
Total Number of Polyester per Sub Sample	7	0	-	1	0	-
Total Number of PA per Sub Sample	0	0	-	1	1	-
Total Number of PVC per Sub Sample	0	0	0	0	0	0

3						
	In			Out		
Environmental Volume [m^3]						
Sampled Matrix Volume [m^3]	1.020			1.012		
Concentrated Sample Volume [mL]	5	5	5	5	5	5
Analysed Subsample Volume [mL]	0.8	0.8	0.8	0.7	0.8	0.8
Total Number of MP per Sub Sample	1	8	8	0	8	7
Number of PE per Sub Sample	0	0	2	0	1	1
Total Number of PP per Sub Sample	1	4	3	0	1	2
Total Number of Polyester per Sub Sample	0	3	3	0	6	4
Total Number of PA per Sub Sample	0	0	0	0	0	0
Total Number of PVC per Sub Sample	0	1	0	0	0	0

Table A.3.: Values from Sampling Site 3.

B.

Electronic Appendix

B.1. Results of Statistical Calculations of the LoD and LoQ