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List of Abbreviation

PP- Polypropylene

MP- Microplastic

NP- Nanoplastic

DMEM- Dulbecco´s Modified Eagle´s Medium

LB - Lysogeny broth

MB- Marine broth

EtOH- Ethanol

DMSO- Dimethyl sulfoxide

FBS- Fetal bovine serum

Table of contents

1 ABSTRACT 8

2 ZUSAMMENFASSUNG 9

3 INTRODUCTION 10

3.1. GLOBAL PLASTIC POLLUTION 10
3.2. EFFECTS ON MARINE LIFE 10
3.3. HARMFUL EFFECT ON HUMAN HEALTH 11
3.4. THE NEED FOR REFERENCE MATERIALS TO STUDY EFFECTS OF MPS AND NPS 12
3.5. AIM OF THESIS 12

4 MATERIALS AND METHODS 13

4.1. ACCURATE PIPETTING OF GLYCEROL 13
4.2. PRODUCTION OF MPS (<38µM) 13
4.2.2. MEDIUM CHANGE FROM ETHANOL TO GLYCEROL 14
4.3. EFFECT OF PP CONCENTRATION IN GLYCEROL ON SIZE MEASUREMENTS 14
4.4. DISPERSION OF MPS INTO BIORELEVANT MEDIA 14
4.4.1. PP IN GLYCEROL WITH LYSOGENY BROTH (LB) + 0.5MOLAR (M) SODIUM CHLORIDE (NACL) SAMPLE PREPARATION 14
4.4.2. PP IN GLYCEROL WITH MARINE BROTH (MB) SAMPLE PREPARATION 15
4.4.3 PP IN GLYCEROL WITH DULBECCO'S MODIFIED EAGLE'S MEDIUM (DMEM) SAMPLE PREPARATION 15
4.4.4. PP IN GLYCEROL WITH DULBECCO'S MODIFIED EAGLE'S MEDIUM (DMEM) + 0.1, 1 AND 10% FETAL BOVINE SERUM (FBS) SAMPLE PREPARATION 16
4.5. SIZE CHARACTERISATION 17
4.5.1. MICROSCOPE (ECHO REBEL) 17
4.5.2. LASER DIFFRACTION (MASTERSIZER 3000, MALVERN PANALYTLCAL) 17
4.5.3. HEMOCYTOMETER (COUNTING CELL) 17
4.5.4. STABILITY CONTROL OF PP IN GLYCEROL 18
4.5.5. STATISTICAL ANALYSIS 18

5 RESULTS 19

5.1. GLYCEROL PIPETTING 19
5.2. EFFECT OF PP CONCENTRATION IN GLYCEROL ON PARTICLE SIZE MEASUREMENTS 20
5.3. DISPERSION OF MPS INTO BIORELEVANT MEDIA 21
5.3.1. PP PARTICLES IN GLYCEROL WITH LB + 0.5MOLAR (M) SODIUM CHLORIDE (NACL) 21
5.3.2. PP IN GLYCEROL WITH MARINE BROTH (MB) 22
5.3.3. PP IN GLYCEROL WITH DMEM + 0.1, 1 AND 10% FBS 23
5.4. PARTICLE SIZE CHARACTERISATION WITH THE MASTERSIZER3000 27
5.4.1. PP IN GLYCEROL AND ALL BIORELEVANT MEDIA 27
5.5. THE NUMBER OF PARTICLES/ML COUNTED WITH THE HEMOCYTOMETER 28

5.6. PP PARTICLE STORAGE STABILITY OVER 33 DAYS 29

6 DISCUSSION 30

6.1. THE IMPORTANCE OF MICROPLASTIC SIZE CHARACTERISATION 30

6.2. VARIOUS MEDIA IMPACTS ON THE SIZE DISTRIBUTION 30

7 CONCLUSION 32

8 REFERENCE 33

9 APPENDIX I 39

9.1. EFFECT OF PP CONCENTRATION IN GLYCEROL ON PARTICLE SIZE MEASUREMENTS 39
9.2. EFFECT OF PP CONCENTRATION IN GLYCEROL WITH LB + 0.5M NACL ON PARTICLE SIZE MEASUREMENTS 40
9.3. EFFECT OF PP CONCENTRATION IN GLYCEROL WITH MB ON PARTICLE SIZE MEASUREMENTS 41
9.4. EFFECT OF PP CONCENTRATION IN GLYCEROL WITH DMEM ON PARTICLE SIZE MEASUREMENTS 43
9.5. EFFECT OF PP CONCENTRATION IN GLYCEROL WITH DMEM + 0.1% FBS ON PARTICLE SIZE MEASUREMENTS 44
9.6. EFFECT OF PP CONCENTRATION IN GLYCEROL WITH DMEM + 1% FBS ON PARTICLE SIZE MEASUREMENTS 45
9.7. EFFECT OF PP CONCENTRATION IN GLYCEROL WITH DMEM + 10% FBS ON PARTICLE SIZE MEASUREMENTS 47
9.8. PARTICLE SIZE CHARACTERISATION WITH THE MASTERSIZER3000 48

1 Abstract

Polypropylene (PP) is recognized as one of the most dominant types of microplastic particles (MPs) in the environment, yet evidence about its toxic effect on human health (due to a lack of preparation methods) is lacking. MPs can be ingested by a wide range of organisms and therefore reference MPs and future research studies to evaluate the toxicity of MPs are needed. This thesis aims to characterise the stability of PP MPs in glycerol, LB, MB, DMEM, DMEM with 0.1%, 1%, 10% FBS and provide data for future studies that recognized the importance of understanding the potential of microplastic particles to induce adverse effects.

PP MPs (<38µm) were produced and seven serial dilutions of MPs in each media were prepared. The particle size distribution was characterized using three different methods. In addition to that, a glycerol pipetting experiment was done and the stability of PP particles in glycerol (that is used as a storage media) was investigated. Results revealed that the ability of these media to prevent aggregations is mostly concentration dependent. While the smallest particle size distribution, in the highest concentration of 5mg/g was achieved with DMEM with 1% FBS (90% are smaller than 26µm) and 10% FBS (90% are smaller than 25µm), the remaining media showed aggregations at the same concentration (MPs in glycerol are 90% smaller than 60µm, MPs in LB are 90% smaller than 48µm and MPs in MB are 90% smaller than 38,5µm). Furthermore, no aggregations were observed in any media from a conc. of 0,625 mg/g and below. This thesis indicates that the addition of serum protein (FBS) gives the preferably size distribution (MPs smaller than 38µm) and that glycerol is able to stabilize these particles over time, although the stability was investigated for a short period of time and long-term controls will be needed for confirmation.

2 Zusammenfassung

Polypropylen (PP) wurde als eines der dominierenden Arten von Mikroplastik Partikel (MP) in der Umwelt anerkannt, dennoch fehlen Beweise (aufgrund fehlender Zubereitungsmethoden) für ihre toxische Wirkung auf die menschliche Gesundheit. MP können von einer Vielzahl von Organismen aufgenommen werden und daher sind Referenz MP und zukünftige Forschungsstudien zur Bewertung der Toxizität von MP erforderlich. Diese Arbeit bestrebt die Stabilität von PP MP in Glycerol, LB, MB, DMEM, DMEM mit 0,1%, 1% und 10% FBS zu charakterisieren und Daten für zukünftige Studien bereitzustellen, die erkannt haben, wie wichtig es ist zu verstehen ob und in welchen Ausmaß PP Partikeln toxische Auswirkung auf den Menschen hervorrufen können.

PP MP (< 38 µm) wurden hergestellt und sieben serielle Verdünnungen von jedem Medium wurden zubereitet. Die Partikelgrößenverteilung wurde durch drei verschiedene Methoden charakterisiert. Darüber hinaus wurde ein Glycerol-Pipettiertechnik Experiment durgeführt und die Stabilität von PP MP in Glycerol (das als Speichermedium verwendet wird) untersucht. Die Ergebnisse zeigten, dass die Fähigkeit dieser Medien, Aggregationen zu verhindern, hauptsächlich konzentrationsabhängig ist. Während DMEM mit 1% (90% kleiner als 26µm) und 10% (90% kleiner als 25µm) FBS die kleinste Partikelgrößenverteilung ohne jegliche Aggregationen in der höchsten Konzentration von 5 mg/g erkennen lassen, zeigten die restlichen Medien Aggregationen in der gleichen PP-Konzentration (MP in Glycerol sind 90% kleiner als 60µm, MP in LB sind 90% kleiner als 48µm und MP in MB sind 90% kleiner als 38,5µm). Darüber hinaus wurden keine Aggregationen in den restlichen Medien (von einer konz. von 0,625 mg/g und darunter) detektiert. Diese Thesis weist darauf hin, dass die Zugabe von Serumprotein (FBS) die bevorzugte Größenverteilung ergibt (kleiner als 38µm) und das Glycerol in der Lage ist, diese Partikel im Laufe der Zeit zu stabilisieren, obwohl ein kurzer Zeitraum untersucht wurde und langfristige Kontrollen für die Bestätigung notwendig sind.

3 Introduction

3.1. Global plastic pollution

Microplastics are of great concern in terms of the harm they could produce on the environment, animal and human health, primarily due to their small size. Solid data to quantify the existence of the smallest microplastics in the environment and their potential to induce adverse effects on humans and marine life are limited (Barboza et al., 2018).

A study had a focus on the global challenge of the waste management system. They reported not having sufficient capacity at the global level for plastic recycling or deposition of plastic (Wilson et al., 2015). Yet, it is currently expected that the annual plastic rate production will be 1100 t by 2050 (Lear et al., 2021). Although plastic is known to be persistent in the environment, plastics for single use dominate (Lithner et al., 2011) and due to the non biodegradability accumulate in our environment (Geyer et al., 2017). Another study calculated that 275 million metric tons, in 192 coastal countries, was generated in 2010, from which 4.8 to 12.7 million metric tons entered the ocean (Jambeck et al., 2015). Since the start of the COVID 19 pandemic, the usage of plastic has increased even more due to the necessity of wearing face masks which are a potential source of microplastic since they are mostly disposed improperly (Kokalj et al., 2021).

Plastics fragment and disperse in the ocean (Barnes et al., 2009) and the majority of plastic marine debris is threatened by microplastic (particles smaller than 5 mm). Microplastic in all sizes have been detected in all oceans across the globe, including regions where coastal population density is much lower (Eriksen et al., 2014). Microplastic can be divided in primary and secondary and vary in size, shape, density, color and polymer composition (Leads et al. 2019). Primary microplastic are produced for a specific application like microbeads in cosmetics while secondary microplastic are created by breakdown of plastic good (Bonfanti et al., 2021) and can not be efficiently detected and collected for recycling (Andrady, 2017). These findings indicate how important it is to reduce plastic consumption by coordinating global action, which was stated by another study (Lau et al., 2020).

3.2. Effects on marine life

Microplastics are recognized as crucial contaminants in aquatic environments and there has been an increase in studies reporting about the toxicity of MPs (Adamovsky et al., 2021). One of them reported that 690 marine species were detected with MPs (White et al., 2018), while some others reported that MPs affect immune enzyme activity and gene expression of the Chinese mitten crab (Z. Liu et al., 2019). Studies indicate MPs can cause intestinal damage and oxidative stress in zebrafish, which is dependent on their size (Lei et al., 2018) and have adverse effects on the reproduction of marine fish (Wang et al., 2019). Fish can accidentally or intentionally ingest MPs which can cause various negative effects (e.g. cytotoxicity, physical damage, change in lipid metabolism and change in behavior) (Jovanović, 2017). MPs in the size range of 20-1000µm, have multiple effects on *Corallium rubrum* and can cause, in worse case scenario, coral death (Corinaldesi et al., 2021). Oysters have a 100% efficient particle size filter mechanism, which leads to ingestion of 6µm sized. MPs have been shown to negatively affect the reproduction of oysters (Sussarellu et al., 2016). Another study reported about the toxicity of MPs in zebrafish, stating that MPs cause inflammation and lipid accumulation, indicate oxidative stress and disturb the energy metabolism (Lu et al., 2016). While the toxicity of MPs on marine life has been reported in various studies, less has been reported about the size importance to cause these effects.

Therefore, a study reported about the negative effects of MPs on microalgae growth, that depended on the particle size. Their research indicated that the negative effects varied with particle size. While larger particles indicated adverse effects by blocking the light transport and affecting photosynthesis smaller particles adsorbed onto the algae surface and destroyed cells (G. Liu et al., 2020).

3.3. Harmful effect on human health

Studies reporting potential health risks for humans induced by MPs are lacking. However, it is known that the human population is exposed to MPs from a variety of sources (Sharma & Chatterjee, 2017). MPs could entry into the human body through ingestion, inhalation or through skin contact. Just a few weeks ago, a study was published showing evidence of plastics in the blood. MPs smaller than 20µm could penetrate organs while it is thinkable that MPs smaller than 10µm could cross cell membranes, the blood-brain barrier and accumulate in the liver, muscles and brain (Campanale et al., 2020). Moreover, there has been evidence that MPs smaller than 10µm could cause cell cytotoxicity in the form of oxidative stress, which was shown through *in vitro* studies (Schirinzi et al., 2017). Due to the lack of validation and standardisation methods suitable to assess human exposure to MPs through food consumption, a review study concluded that it is not possible, presently, to give an answer on the adverse effects of MPs as a result of food intake (Toussaint et al., 2019). Another study stated that the amount of MPs in seafood is very low, which indicates that the dietary exposure would be also low (Barboza et al., 2018).

Another important route of MP intake is via inhalation. A study reported about the theory that MPs could be aerosolised through wind actions and sea spray, and therefore transported to urban environments (Wright & Kelly, 2017). Just a few weeks ago, a study was published showing evidence of plastics in the lungs. They reported about evidence of MPs being released from the marine environment into the atmosphere (Allen et al., 2020). Another study evaluated the possibility of an toxic effect from MPs on the lung function of a group of polypropylene flocking plant workers and results showed an increase in respiratory symptoms when compared to the control (Atis et al., 2005).

It has been reported about the human exposure to MPs, where the skin route was considered as well. However, the size range was to big and therefore would the detected MPs not be able to penetrate through human skin (Abbasi & Turner, 2021).

There is an increase in evidence about MPs accumulating in the human body. A study reported the presence of MPs in human placenta, yet it is unknown how MPs reach the placenta (Ragusa et al., 2021). Another study provided evidence of MPs in human stool. Some of the participants used beauty products containing synthetic polymers, some drank fluids from plastic bottles daily, some consumed seafood and generally, their food was wrapped up in plastic. MPs were present in all stool samples, in a size range of 50-500µm. MPs bigger than 500µm were not detected, yet MPs smaller than 50µm could not be analysed due to technical limitations (Schwabl et al., 2019).

Although the number of MPs that reaches the brain is unknown, it is proven that MPs can reach the brain. Yet, only three (to that date) studies were published about MPs inducing neurotoxicity *in vitro* (Hoelting et al., 2013; Murali et al., 2015; Schirinzi et al., 2017). Human cerebral and epithelial cells showed, at high MP concentration, increased reactive oxygen species production. However, humans are exposed to lower levels of MPs during a long period of time, while these studies used high levels with short exposure time (Prüst et al., 2020). That the accumulated MPs in human body have potential to cause toxic effects, was stated in another study that investigated the cellular response of PP particles

in a size range of 25-200µm. They came to the conclusion that PP small sized, in high concentration, intensifies hypersensitivity and stimulates the immune system (Hwang et al., 2019).

It has been reported that MPs, in the presence of contaminants, can interact and lead to aggregation, adsorption and transformation and therefore lead to more potential toxic effects (Bhagat et al., 2021). Further research is therefore needed on the potential toxic effects of MPs on human health (Jiang et al., 2020).

3.4. The need for reference materials to study effects of MPs and NPs

In order to understand the potential of microplastic to induce adverse effects on humans and marine life, reference MPs needed to be generated for future research studies. Relevant data must be generated for a better understanding of the human exposure to MPs, their fate and toxicity/allergenicity. The aim should be understanding MP transmission to humans, establish methods for identification and quantification of MPs in foods, environmental media and tissues, establish analytical methods for MP detection, have detailed knowledge on microbial colonisation of MPs as vectors for potential pathogens, toxicology and fate in the gastro-intestinal or respiratory tracts and secondary organs (Enyoh et al., 2019). Furthermore, the effect dependence on the size of MPs needs to be considered which is another reason for reference MPs.

3.5. Aim of thesis

The aim of this thesis is to characterise the colloidal stability of PP MPs (<38µm) in various media using three different methods: microscopy, laser diffraction and a hemocytometer. It was reported that MPs are less stable than NPs due to their larger size and that the aggregation is concentration dependent. (Hü et al., 2017). Therefore, it was hypothesized that the stability of PP in various media is concentration dependent. A study reported that the addition of glycerol leads to a narrower particle size distribution (Saberi et al., 2013). It was hypothesized that glycerol is able to prevent aggregation and is a good storage media for MPs. Another study reported that nanocomposites aggregate in DMEM alone, whereas no aggregations were detected by adding 5% and 10% of FBS (Arora et al., 2017).Therefore, it was hypothesized that the addition of FBS will lead to MPs <38µm with a narrow size distribution.

4 Materials and Methods

- Polypropylene PP (Polypropylene, PP, isotactic, average Mw ~250,000, average Mn ~67,000). Catalog Number 427888-1KG (Sigma Aldrich).
- DMEM (without phenol red) was purchased from Thermo Scientific.
- NaCl was purchased from Roth.
- LB Broth, DMSO, FBS and all other reagents were purchased from Sigma Aldrich.

4.1. Accurate pipetting of glycerol

In this project, glycerol was used as a non-aqueous storage medium for MP suspensions because of the following reasons:

- glycerol is considered as generally safe (Saberi et al., 2013)
- glycerol is well-known in the pharmaceutical and cosmetic industries (Zhang & Grinstaff, 2014)
- A study indicated that glycerol has the ability to maintain the NP homogeneity over a long period of time and describe glycerol as a good NP stabilizer (Clergeaud et al., 2013)
- It has been reported that with glycerol the synthesis of highly homogeneous NPs was achieved (Genç et al., 2011)
- glycerol has a high density and viscosity (Ferreira et al., 2017)

Since glycerol has a high density and viscosity, it is hard to pipette accurately at room temperature. It has been reported that the viscosity of glycerol can be reduced at 40°C (Gulyaev & Solonenko, 2013). For this project a SOP was made for glycerol pipetting at room temperature (RT) and 40°C to see if there is a difference in the mean volume and volume uniformity. Glycerol was heated up in a water bath and the temperature was measured with a thermometer. Glycerol (1g) was transferred 10 times in 10 different vials and the mass of glycerol after cooling to RT was documented. The same process was performed for glycerol at room temperature. The mean value and arithmetic standard deviation was calculated for the 10 measurements and compared with each other.

4.2. Production of MPs (<38µm)

4.2.1. Milling and size fractionation of PP

Before the actual procedure to obtain microplastic, PP discs were prepared. PP (4g) was added in baking cups and melted in an oven at 180°C for one hour. After cooling down, the discs were collected in a stainless-steel box and stored at -70°C. Frozen plastic discs (20-25g) were smashed with a hammer and added to a blender (KOENIC Standmixer KBL 713 CONFORT EDELSTAHL) together with 40mL ethanol (96%) and milled for 1 min. A total of 7 cycles was performed and ethanol (5mL) was used to rinse all the plastic attached to the blender walls after every cycle. The milled plastic mixture was transferred to the 38µm sieve at top of the sieve tower, (RETSCH; woven wire mesh sieves – 200MM / 203MM, 60.101.000038) and rinsed twice with 10mL ethanol (to increase the efficiency of the sieving process). The sieve tower was operated for 5 minutes at level 7 with intensity 7. The PP fraction <38µm in ethanol was transferred to a glass bottle and stored for 24h to allow the MP fraction to sediment. The ethanol supernatant, which contained NP, was removed, the mass of the ethanol + plastic was

weighed and the concentration determined by using a rotary evaporator. The suspension was sonicated to homogenously disperse the particles and 1g was transferred to a pre-weighed vial. Ethanol was evaporated (50°C, 10mbar), the mass of the dry sample was documented and the concentration calculated. Batch numbers used for this project are B021121002, B171121003, C120122001, C260122001, C010222001 and C010222002.

4.2.2. Medium change from ethanol to glycerol

Reasons why glycerol was chosen as a storage medium for MPs are listed above. Therefore, it was needed to change the medium from PP in EtOH to PP in glycerol. Knowing the concentration of PP in ethanol made it possible to calculate the mass of glycerol to be added in order to get the desired concentration (20mg/g) of MPs in glycerol. MPs in ethanol were vortexed and sonicated for at least 1 min. The glycerol pipetting experiment showed that glycerol should be heated up to 40°C for easier pipetting. Glycerol was added to the suspension and evaporated to the pressure needed to extract ethanol. The mass was checked every 5 min and the extraction was completed when the difference in the last 3 measurements was < 10%.

4.3. Effect of PP concentration in glycerol on size measurements

To understand whether PP concentration in glycerol suspension affects the results of the size measurements, different dilutions were prepared and analysed. The suspension was vortexed and sonicated at 40°C before each dilution step. A stock solution (10mg/g) was prepared and diluted for a total of seven dilutions (5 mg/g, 2.5 mg/g, 1.25 mg/g, 0.625 mg/g, 0.3125 mg/g, 0.15625 mg/g and 0.078125 mg/g) in glycerol. Particle size distributions were then characterised as described in sections 4.5.1., 4.5.2. and 4.5.3.

4.4. Dispersion of MPs into biorelevant media

The sample preparation for PP in glycerol with all biorelevant media took place in the laminar flow.

4.4.1. PP in glycerol with lysogeny broth (LB) + 0.5molar (M) sodium chloride (NaCl) sample preparation

LB contains peptones, peptides, vitamins, trace elements (eg. magnesium) and minerals. PP in glycerol suspension (20mg/g) was mixed with LB + 0.5M NaCl. The suspension was vortexed and sonicated at 40°C before each dilution step. A stock solution (10mg/g) was prepared and diluted for a total of seven dilutions (5 mg/g, 2.5 mg/g, 1.25 mg/g, 0.625 mg/g, 0.3125 mg/g, 0.15625 mg/g and 0.078125 mg/g). Particle size distributions were then characterised as described in sections 4.5.1., 4.5.2. and 4.5.3.

4.4.2. PP in glycerol with Marine broth (MB) sample preparation

Media composition:

Components	Concentration(g/L)
Peptone	5.0
Yeast Extract	1.0
Ferric Citrate	0.1
Sodium Chloride	19.45
Magnesium Chloride	5.9
Magnesium Sulfate	3.24
Calcium Chloride	1.8
Potassium Chloride	0.55
Sodium Bicarbonate	0.16
Potassium Bromide	0.08
Strontium Chloride	0.034
Boric Acid	0.022
Sodium Silicate	0.004
Sodium Fluoride	0.0024
Ammonium Nitrate	0.0016
Disodium Phosphate	0.008

Table 1

Salts that were not available were replaced with NaCl. In order to replace the salts with NaCl, it was necessary to calculate the amount of ions (mols) the salts should give to the solution.
PP in glycerol suspension (20mg/g) was mixed with MB. The suspension was vortexed and sonicated at 40°C before each dilution step. A stock solution (10mg/g) was prepared and diluted for a total of seven dilutions (5 mg/g, 2.5 mg/g, 1.25 mg/g, 0.625 mg/g, 0.3125 mg/g, 0.15625 mg/g and 0.078125 mg/g). Particle size distributions were then characterised as described in sections 4.5.1., 4.5.2. and 4.5.3.

4.4.3 PP in glycerol with Dulbecco's Modified Eagle's Medium (DMEM) sample preparation

Media composition:

Components	Concentration (mg/L)
Amino Acids	
Glycine	30.0
L-Arginine hydrochloride	84.0
L-Cystine 2HCl	63.0
L-Glutamine	584.0
L-Histidine hydrochloride-H2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine hydrochloride	146.0
L-Methionine	30.0
L-Phenylalanine	66.0

L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine disodium salt dihydrate	104.0
L-Valine	94.0
Vitamins	
Choline chloride	4.0
D-Calcium pantothenate	4.0
Folic Acid	4.0
Niacinamide	4.0
Pyridoxine hydrochloride	4.0
Riboflavin	0.4
Thiamine hydrochloride	4.0
i-Inositol	7.2
Inorganic Salts	
Calcium Chloride (CaCl2) (anhyd.)	200.0
Ferric Nitrate (Fe(NO3)3"9H2O)	0.1
Magnesium Sulfate (MgSO4) (anhyd.)	97.67
Sodium Bicarbonate (NaHCO3)	3700.0
Potassium Chloride (KCl)	400.0
Sodium Chloride (NaCl)	6400.0
Sodium Phosphate monobasic (NaH2PO4-H2O)	125.0
Other Components	
Glucose	4500.0

Table 2

PP in glycerol suspension (20mg/g) was mixed with DMEM solution (not supplemented with serum). The suspension was vortexed and sonicated at 40°C before each dilution step. A stock solution (10mg/g) was prepared and diluted for a total of seven dilutions (5 mg/g, 2.5 mg/g, 1.25 mg/g, 0.625 mg/g, 0.3125 mg/g, 0.15625 mg/g and 0.078125 mg/g). Particle size distributions were then characterised as described in sections 4.5.1., 4.5.2. and 4.5.3.

4.4.4. PP in glycerol with Dulbecco's Modified Eagle's Medium (DMEM) + 0.1, 1 and 10% fetal bovine serum (FBS) sample preparation

FBS contains growth factors, antibodies, proteins, lipids, electrolytes, carbohydrates, hormones, enzymes and other undefined constituents.

DMEM was supplemented with either 0.1, 1 or 10% FBS prior to mixture with PP suspensions. PP in glycerol suspension(20mg/g) was then mixed with DMEM containing either 0.1, 1 or 10% FBS. The suspension was vortexed and sonicated at 40°C before each dilution step. Stock solutions were prepared and diluted for a total of seven dilutions (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Particle size distributions were then characterised as described in sections 4.5.1., 4.5.2. and 4.5.3.

4.5. Size characterisation

The size characterisation for PP in glycerol suspension in all media described above was done with three different methods: microscopy, laser diffraction and a hemocytometer.

4.5.1. Microscope (Echo Rebel)

All seven dilutions (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g) were vortexed and sonicated at 40°C for 1min and 20µl was pipetted onto a slide. A cover glass was added, 10 images were taken and the raw data was organized in form of histograms and cumulative distribution curves. The Echo Rebel software allows to add measurement annotations, length, area, cell count and scale bar on the iPad Pro with touch screen that is attached to the microscope. The measurement was repeated 3 times with 3 replicate batches.

4.5.2. Laser diffraction (Mastersizer 3000, Malvern Panalytlcal)

The measurement cell of the Mastersizer can be filled with any aqueous media which lead to the usage of particle free water for samples that are not aqueous (eg. glycerol), and usage of aqueous media without water to prevent possible additional aggregations (eg. DMEM). Before adding the sample (100µL) to the cell filled with pure media (5mL), it was needed to measure the background. A good measurement requires a clean, stable background. After the background signal was subtracted, the sample was added to the cell.

The highest concentration (5mg/g) was vortexed and sonicated for 1 min and 100µl were added to the cell. Not enough sample would lead to not representative data and the signal to noise ratio could be poor, while too much sample could lead to multiple scattering that could affect the reported size distribution. The obscuration level indicates the amount of laser light blocked by the sample and is a guide to know if the added sample amount is adequate. The obscuration level for wet measurements with particles from 1 to 100µm should be, according to the manufacturer, between 5-10%. Therefore, the Mastersizer could not be used for all dilutions since the obscuration was either too high or too low. For every media, one dilution sample was found with the obscuration level needed and measured. The measurement was repeated 3 times with 3 replicate batches.

4.5.3. Hemocytometer (counting cell)

The hemocytometer could not be used for all dilutions since too high or too low concentrated samples would not give accurate results. All serial dilution samples (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g) were observed and the concentration of 0.625mg/g showed the best results.

The coverslip was placed over the counting surface and the particle suspension (0.625mg/g, 10µl) was (after vortexing and sonicating) introduced to the edge of the coverslip with a fine tip transfer pipette. The capillary force would drag the liquid to fill the area under the coverslip. Enough liquid needs to be introduced so that the counting surface is just covered. Particles were counted in 5 different squares using a microscope focused on the grid lines of the counting area with a 10x objective. A counting rule was set and particles were counted on the top and left lines of a square, but not on the bottom and right lines. The counting was repeated on the other side of the hemocytometer, a total of 10 squares

were counted and an average number was calculated. The counting was repeated 3 times with 3 replicate batches. **Figure 1** shows an example of counted PP particles in one square. The equation used to calculate the number / ml is:

Concentration= Number of particles x 10.000 / Number of squares x dilution

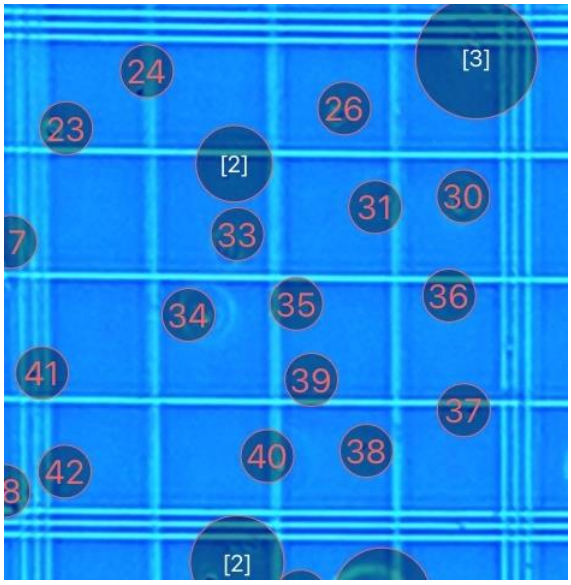


***Figure 1**. Number of PP particles in DMEM counted in one square at a conc. of 0.625mg/g.*

4.5.4. Stability control of PP in glycerol

Since glycerol has been used as the storage media, stability of PP particles in glycerol has been analyzed. Batch numbers UNIVIE008, UNIVIE009 and PP_IOA_032 were used for this experiment. Due to the small amount of samples of PP in glycerol, one batch could only be measured twice. The particle size distribution of UNIVIE008 was measured on day 1 and day 7, of UNIVIE009 on day 1 and day 14 and of PP_IOA_032 on day 1 and day 33. The Mastersizer 3000 was used for this experiment and the approach was explained in section 4.5.2.

4.5.5. Statistical analysis

To determine whether there is a significant difference between pippeting glycerol at 40°C and RT, a paired samples Student´s t-test was performed in Excel. A box was added to the Excel spreadsheet to insert the T-test results. The t-test was chosen from the statistical menu, cells which contain the replicates were highlighted and the P value was generated. $P< 0.05$ indicates that the difference is significant, while if $P> 0.05$ there is no significant difference between the two data sets.

5 Results

The aim of this project was to observe potential changes in measured particle size of PP in seven different media. Therefore, seven serial dilutions of PP particles were in every media prepared and analysed with three different methods. In addition to that, the highest dose of PP solution stabilised by each media should be found. The aim was also to have another way of knowing the concentration and therefore the hemocytometer was used. Furthermore, a glycerol pipetting experiment was done to observe potential ease in the pipetting technique.

5.1. Glycerol pipetting

A glycerol pipetting experiment was done to provide more data about the accuracy of pipetting glycerol for further experiments. **Figure 2** shows the difference of the mean in mass (± standard deviation) of glycerol pipetted at 40°C and RT (n= 20 measurements). A control (water) was pipetted as well to confirm that liquids with low viscosity can be pipetted accurately and the theoretical value of glycerol, based on a study from Volk & Kähler, 2018, was added to Figure 2. As referenced in section 4.1. glycerol at 40°C is less viscous. Therefore, it was hypothesized that glycerol at 40°C will be easier and more accurate for pipetting. As shown in Figure 2 glycerol pipetted at 40°C and cooled down at RT is closer to the theoretical value than glycerol pipetted at RT and pipetting glycerol at 40°C and RT showed a significant difference ($P<0.01$). Therefore, glycerol was warmed up to 40°C for all experiments.

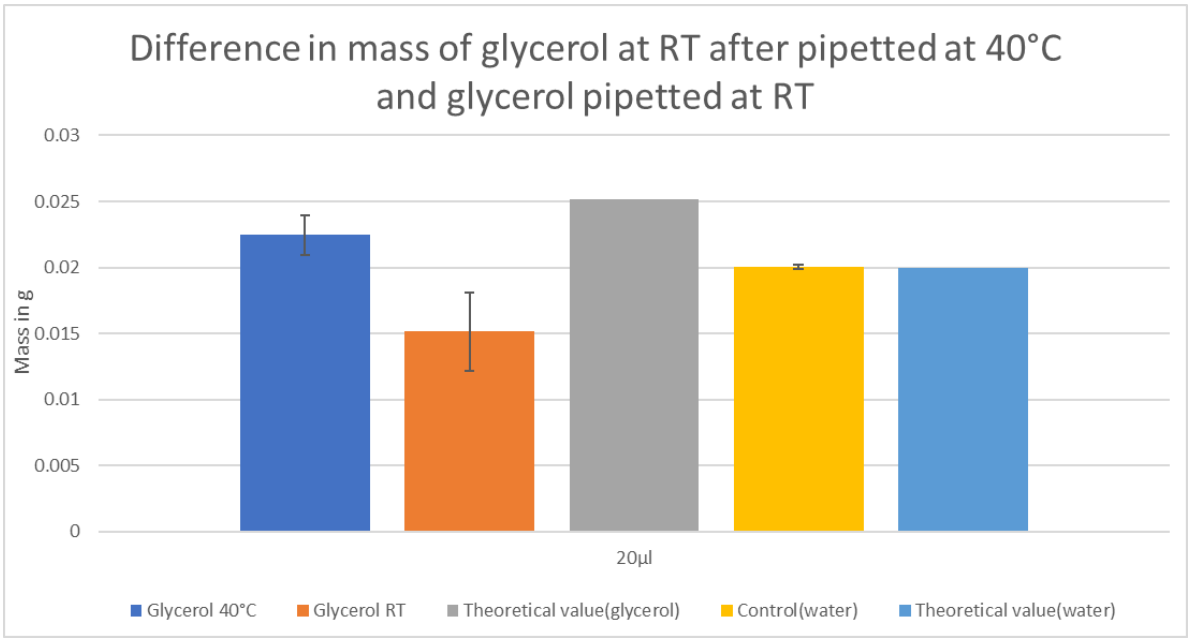


Figure 2. *Difference in mass of glycerol at RT after pipetted at 40°C and glycerol pipetted at RT. The theoretical value of glycerol was added as well as a control (water). Values represent the mean ± standard deviation of 20 measurements.*

5.2. Effect of PP concentration in glycerol on particle size measurements

Using a microscope, it was possible to analyse all seven concentrations of PP in glycerol and biorelevant media. Images were taken and the raw data was organized in form of histograms and cumulative distribution curves (Appendix I). The histograms showed a unimodal distribution and were used on the one hand to represent the particle size with the highest frequency directly from the peak, and on the other hand to indicate how far the remaining particle diameters scatter around this mean. The cumulative distribution curve shows what percentage of the PP particles are smaller or larger than a selected particle size. The median value (D50) indicates the particle size at which 50% of the particles are smaller and 50% larger, while the scattering parameters (D90/10) represent the ratio between the particle sizes at D90 and D10.

The images were compared to **Figure 3** (Picture of aggregated PP particles in glycerol) and **Figure 4** (Picture of PP particles in glycerol showing no aggregation). The measurement was repeated 3 times with 3 replicate batches.

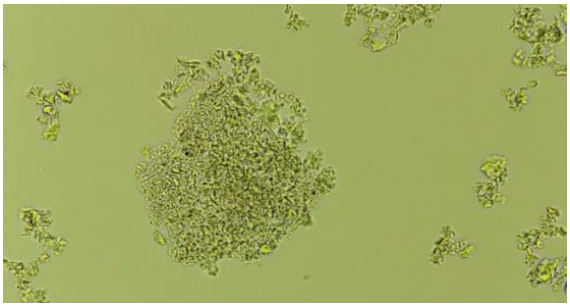


Figure 3. *Example of aggregated PP particles in glycerol.*

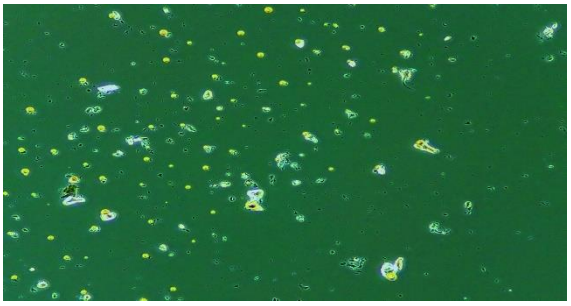


Figure 4*. Example of PP particles in glycerol without aggregation.*

Due to the high density and viscosity of glycerol, it was hypothesized that the PP particles will stay stable and therefore not aggregate. **Figure 5** shows the concentration dependence of the mean in particle size of three replicate batches. **Figure 6-7** show the D50 values and D90/10 ratio of PP in glycerol in all seven dilutions. Several important observations can be made from the graphs. First, in higher concentrations, such as 5mg/g and 2,5mg/g, PP particles are not stable and aggregate. Secondly, PP particles from a conc. of 1,25mg/g show that 90% of the particles are smaller than 32µm, which indicates that the aggregation is very low. PP particles at a conc. of 0,625mg/g and below are stable and show no aggregations. The particle-particle interactions decrease with lower particle concentration(Mangesana et al., 2008) and therefore the D50 values decrease with lower PP concentration. Another reason for the decrease of the D50 value is the ability of glycerol to reduce the particle size, as referenced in the discussion section. Hence, the stability of PP particles in glycerol depends on the concentration and the hypothesis was only partly right. Separate histograms and cumulative distribution curves of all dilutions are listed in Appendix I.

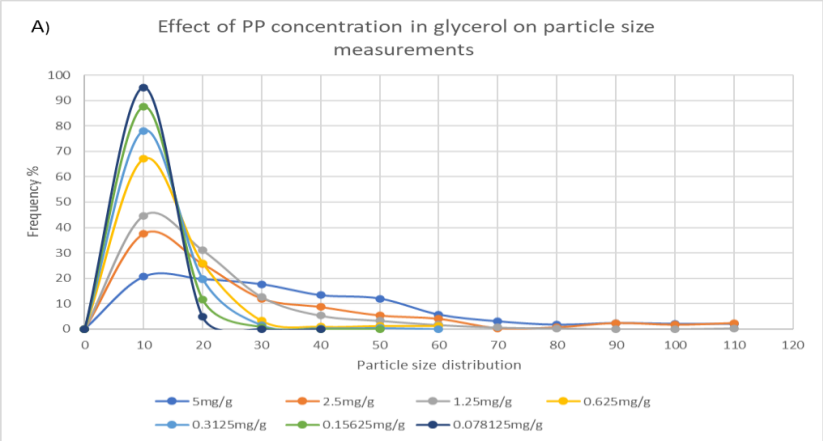


***Figure 5**. Particle size distribution of PP in glycerol in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

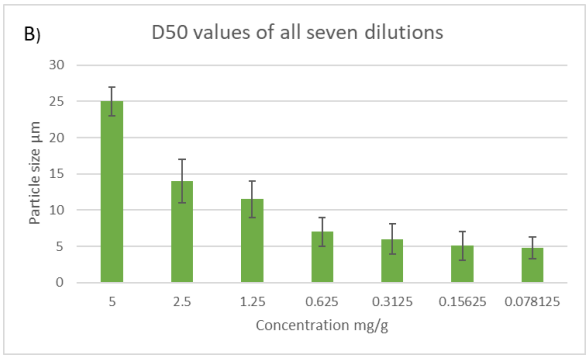


***Figure 6.** The D50 values of PP in glycerol in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

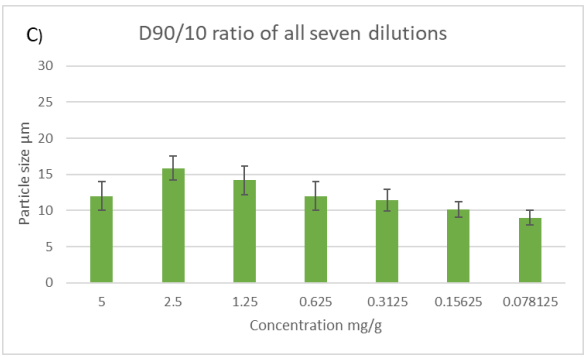


***Figure 7.** The D90/10 ratio of PP in glycerol in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

5.3. Dispersion of MPs into biorelevant media

5.3.1. PP particles in glycerol with LB + 0.5molar (M) sodium chloride (NaCl)

LB is primarily used for the growth of bacteria. Due to the composition of sodium chloride, peptone, and yeast extract, it was hypothesized that the media will stabilize PP particles in glycerol.

Figure 8 shows the concentration dependence of the mean in particle size of three replicate batches. **Figure 9-10** show the D50 values and D90/10 ratio of PP in glycerol with LB + 0.5M NaCl in all seven dilutions. In comparison with only glycerol as a media, PP particles in glycerol with LB show at the highest conc. of 5mg/g better results although there are still aggregates. The conc. of 2,5mg/g has 90% of particles smaller than 33µm and therefore low aggregation and from a conc. of 0,625mg/g and below particles are stable with no aggregation. It has been reported that a higher salt content can reduce particle size (Apte et al., 2003) which explains why the D50 value (apart from aggregation effects) decreases with decreasing concentration.

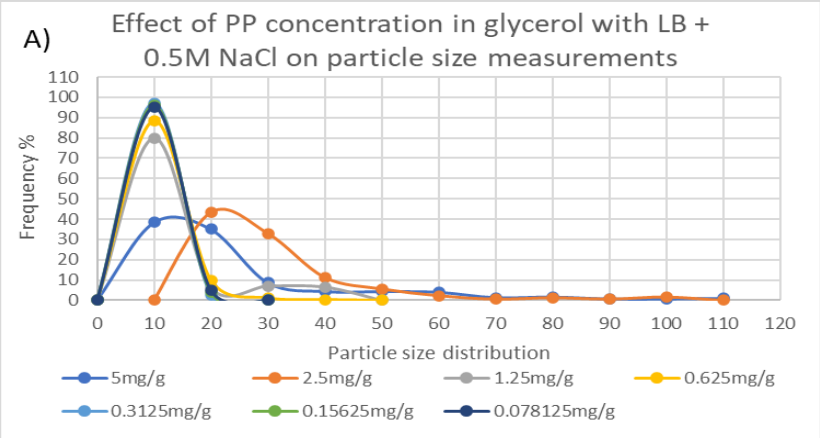


***Figure 8**. Particle size distribution of PP in glycerol with LB + 0.5M NaCl in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

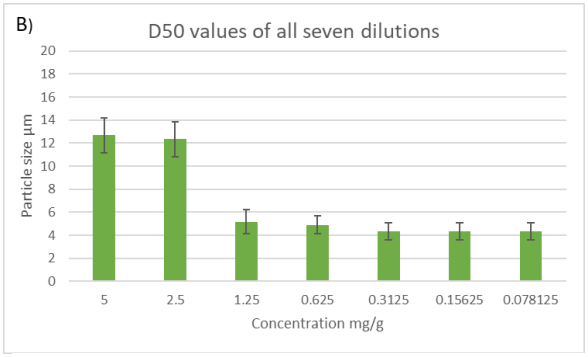


***Figure 9.** The D50 values of PP in glycerol with LB + 0.5M NaCl in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

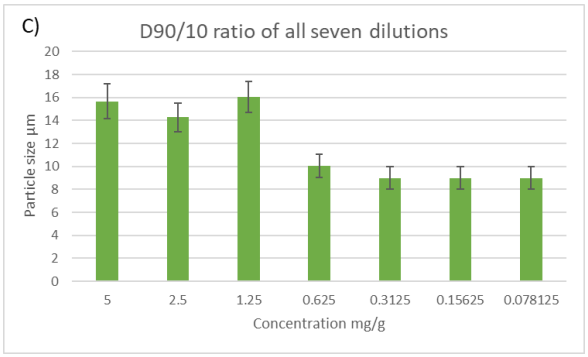


***Figure 10.** The D90/10 ratio of PP in glycerol with LB + 0.5M NaCl in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

5.3.2. PP in glycerol with Marine Broth (MB)

Marine broth is primarily used for the growth of marine bacteria. Due to the high salt concentration, peptone and yeast it was hypothesized that the media will have a positive impact on the stability of PP particles. **Figure 11** shows the concentration dependence of the mean in particle size of three replicate batches. **Figure 12-13** show the D50 values and D90/10 ratio of PP in glycerol with MB in all seven dilutions. Comparing with the results from above, PP in glycerol with MB at the highest conc. of 5mg/g show only low aggregations with 90% of the particles being smaller than 38,5µm. The D50 value, at the highest concentration, is 16µm and decreases with decreasing concentration. As referenced above, the high salt content could decrease the particle size.

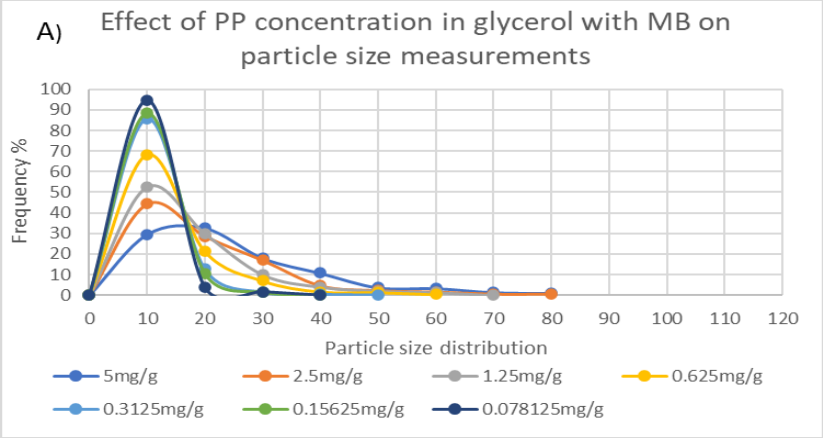


***Figure 11**. Particle size distribution of PP in glycerol with MB in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

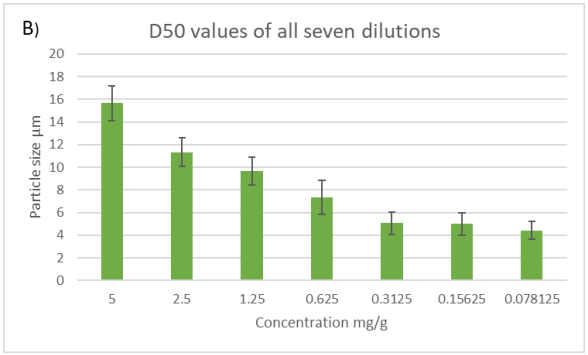


***Figure 12.** The D50 values of PP in glycerol with MB in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

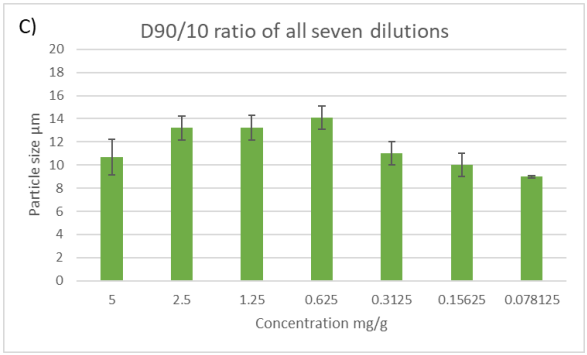


***Figure 13.** The D90/10 ratio of PP in glycerol with MB in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

5.3.3. PP in glycerol with DMEM + 0.1, 1 and 10% FBS

DMEM is primarily used to support the growth of mammalian cells. The media contains next to glucose salts, vitamins and amino acids. It was hypothesized that there will be a high protein serum (FBS) addition needed in order to stabilize PP particles. FBS contains lipids, proteins, electrolytes, carbohydrates, hormones, enzymes and is therefore used to support cell growth. The aim was to find the lowest dose of FBS that will stabilize PP particles in glycerol with DMEM. As referenced in the discussion section, FBS is able to reduce the average particle size.

Figure 14, 17, 20 and 23 shows the concentration dependence of the mean in particle size of three replicate batches. **Figure 15, 16, 18, 19, 21, 22, 24 and 25** show the D50 values and D90/10 ratio of PP in glycerol with MB in all seven dilutions. Results show that PP in the highest concentration (5mg/g) is not stable in glycerol with DMEM, yet still show lower aggregation than with glycerol alone, 0.1% FBS is not enough to stabilize PP particles at the same concentration and therefore can be compared to the results with DMEM without FBS, 1% of FBS is sufficient to stabilize the particles (90% of the particles are smaller than 26µm) and 10% FBS indicate no aggregations as well (90% of the particles are smaller than 25µm). There are no particles bigger than 100µm in comparison with glycerol alone. PP particles in DMEM alone show better stability from a concentration of 2.5mg/g (90% of the particles smaller

than 27,2µm) and below. The D90 at the highest concentration (5mg/g) with 10% FBS is only 1µm smaller than D90 with 1% FBS while the D90 from 2.5mg/g and below with 1% FBS are smaller than with 10% FBS. Possible explanations for the results will be discussed in the discussion part.

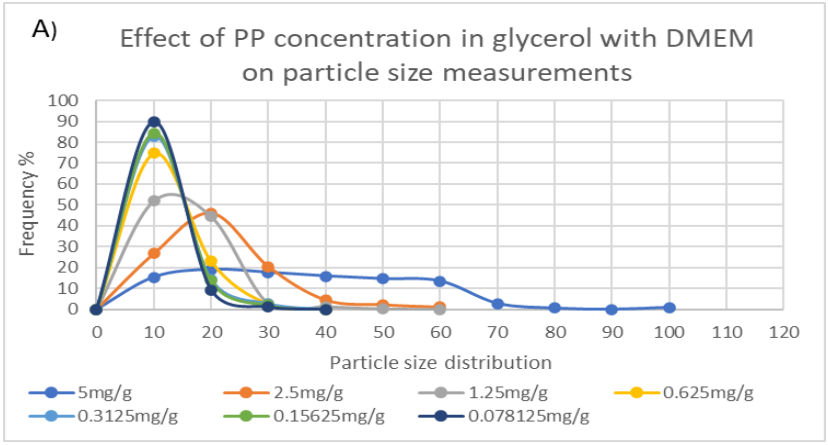


***Figure 14**. Particle size distribution of PP in glycerol with DMEM in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

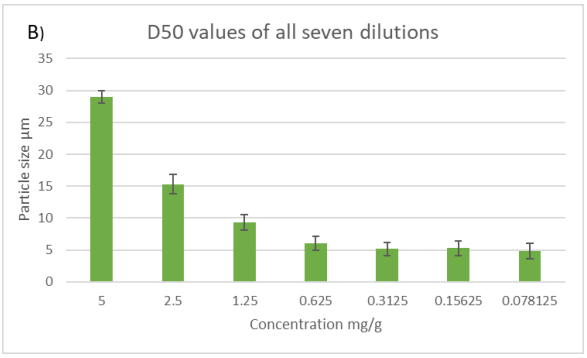


***Figure 15.** The D50 values of PP in glycerol with DMEM in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

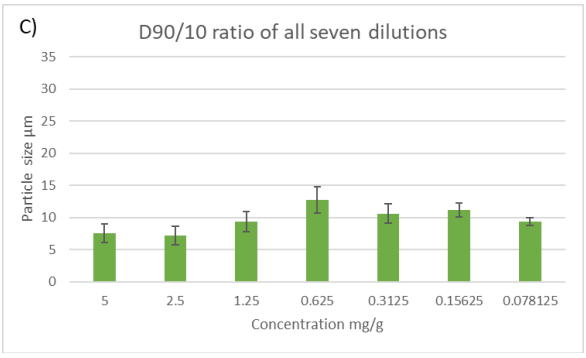


***Figure 16.** The D90/10 ratio of PP in glycerol with DMEM in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

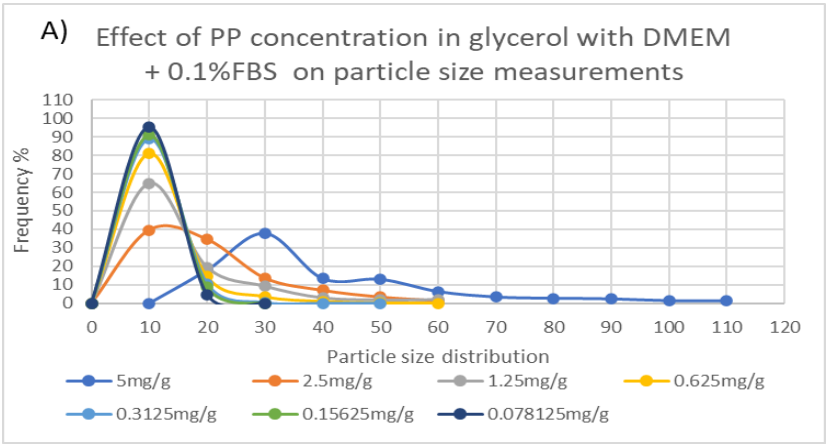


***Figure 17**. Particle size distribution of PP in glycerol with DMEM + 0.1% FBS in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

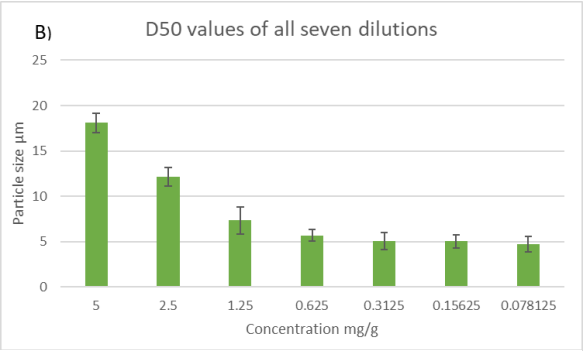


Figure 18. *The D50 values of PP in glycerol with DMEM + 0.1% FBS in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

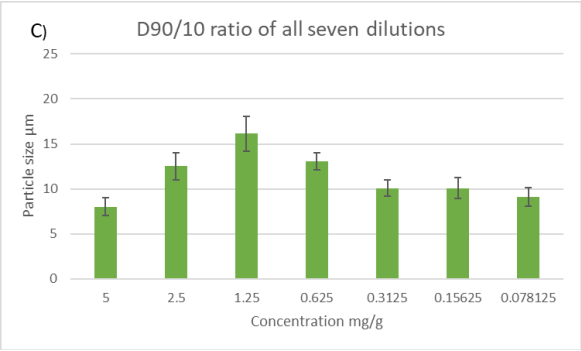


Figure 19. *The D90/10 ratio of PP in glycerol with DMEM + 0.1% FBS in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

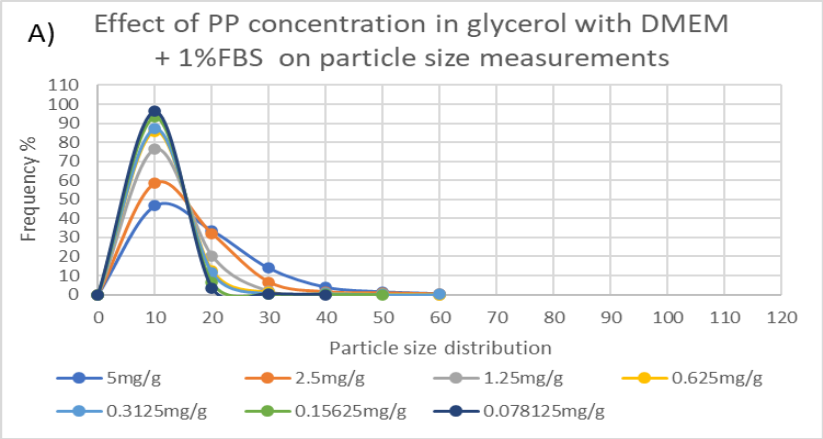


Figure 20*. Particle size distribution of PP in glycerol with DMEM + 1% FBS in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

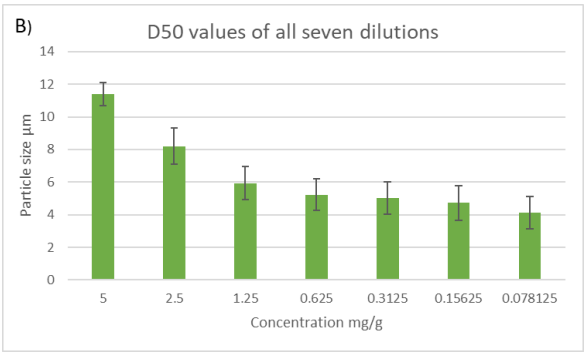


Figure 21. *The D50 values of PP in glycerol with DMEM + 1% FBS in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

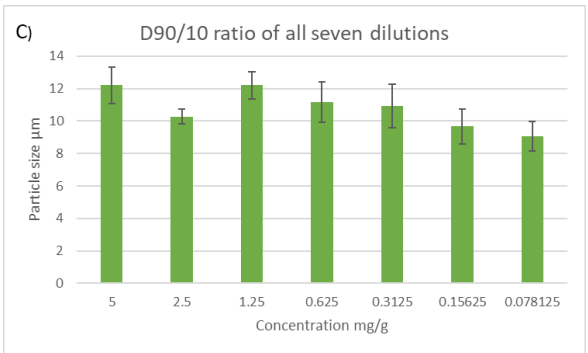


Figure 22. *The D90/10 ratio of PP in glycerol with DMEM + 1% FBS in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

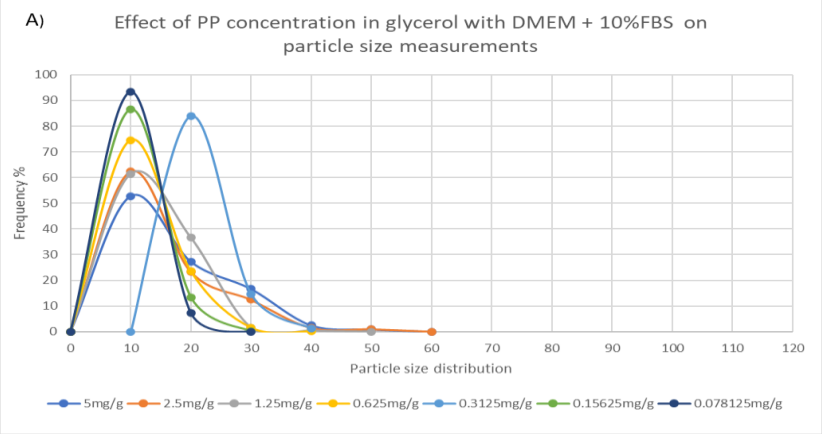


***Figure 23**. Particle size distribution of PP in glycerol with DMEM + 10% FBS in all seven concentrations (5mg/g, 2.5mg/g, 1.25mg/g, 0.625mg/g, 0.3125mg/g, 0.15625mg/g and 0.078125mg/g). Values represent the mean of n=3 batches.*

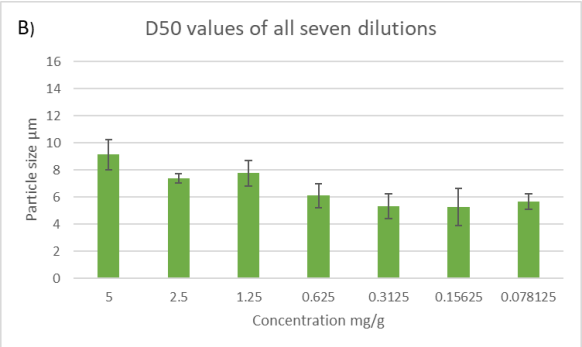


***Figure 24.** The D50 values of PP in glycerol with DMEM + 10% FBS in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

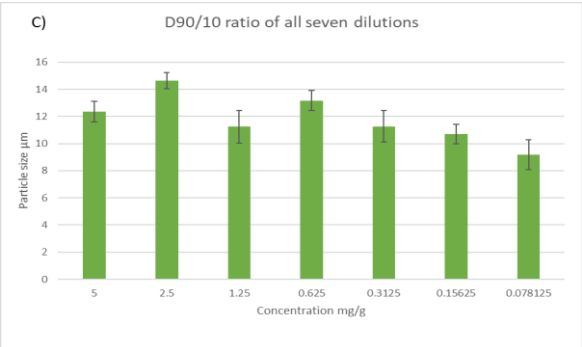


***Figure 25.** The D90/10 ratio of PP in glycerol with DMEM + 10% FBS in all seven dilutions. Values represent the mean ± standard deviation of n=3 batches.*

5.3.4. Data comparison

The data obtained with the microscope were explained above. **Figure 26** shows the D50 values and **Figure 27** shows the D90/10 ratio of PP particles in all media at a concentration of 1.25mg/g.

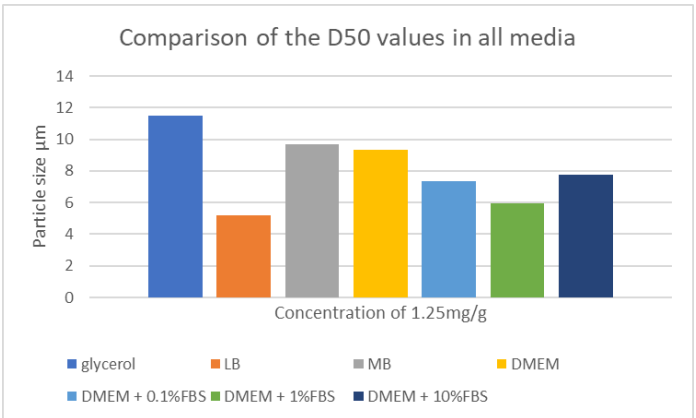


***Figure 26.** D50 values in all media. Values represent the mean of n=3 batches.*

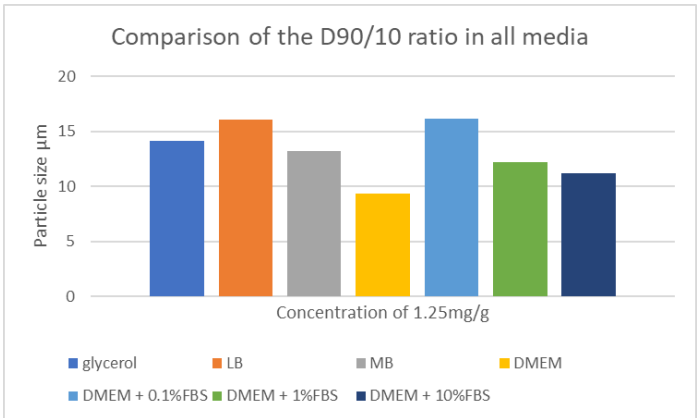


***Figure 27**. The D90/10 ratio in all media. Values represent the mean of n=3 batches.*

5.4. Particle size characterisation with the mastersizer3000

Since the obscuration level has a major impact on the quality of the results, it was not possible to measure all concentrations. For that reason, one concentration of every sample with an obscuration level between 5-10% was found and measured. The measurement was repeated with three replicate batches. The aim was to compare the results from the Mastersizer and microscope and to learn if the results are comparable and if not, which method is more accurate. The measurements were compared with table 3 (Measurement of PP suspension in glycerol with and without 5% Tween80 added). If the Dx would be double the size of the example 1 in Table 3, it was an indication that aggregation is likely to happen.

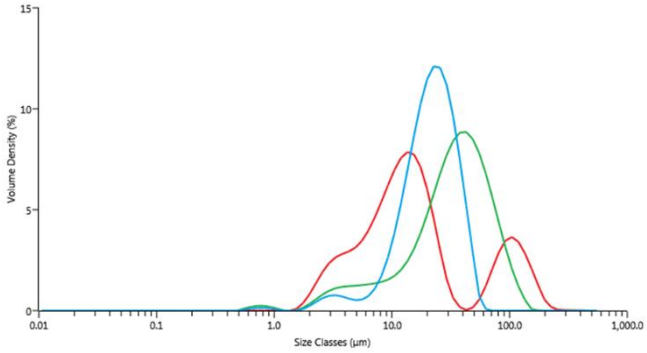
Sample name	Dx 10 (µm)	Dx 50 (µm)	Dx 90 (µm)	Obscuration level (%)
1) PP susp. in glycerol with 5% Tween80	3,48	9,65	28,4	17,13
2) PP susp. In glycerol without Tween80	12,4	26,2	48,5	8,22

Table3. Example of the size determination for a PP susp. in glycerol with and without 5% Tween80 added

5.4.1. PP in glycerol and all biorelevant media

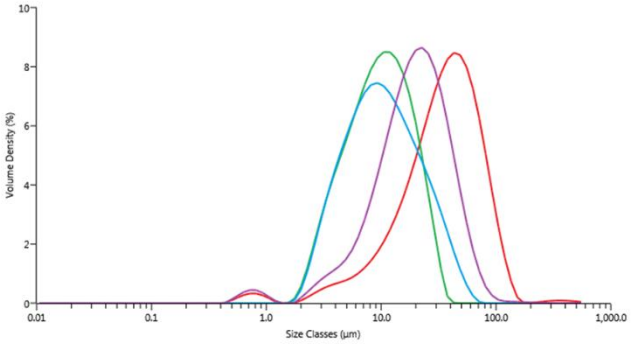
Figure 28. shows the mean in particle size of three replicate batches of PP in glycerol, PP in glycerol with LB + 0.5M NaCl and PP in glycerol with MB at the highest concentration (5mg/g). The particle distribution of PP in glycerol indicates a range from 1 to a bit over 100µm, which is comparable with the microscope results. The Mastersizer indicates that 90% of the particles are smaller than 74.9µm, while the microscope indicates that 90% are smaller than 60µm. Results of PP in glycerol with LB and NaCl indicate aggregation over time (according to the user manual of the Mastersizer 3000) due to the higher second peak at the size range around 100µm, the decrease of the obscuration level and increase of the D90 value. Results of this study were also compared to another study that used the same method and had similar results (Mayr et al., 2016). Data comparison from both methods indicates a bigger difference in the size range. The Mastersizer displays that 90% of the particles are smaller than 106µm, contrary, 90% of the particles with the microscope are smaller than 48µm. It is thinkable that with the microscope it is not possible to detect aggregations over time, which lead to a smaller particle size distribution. Result of PP in glycerol with MB indicates a narrow size distribution. The D90 from both methods are almost identical, D90 from the Mastersizer is 38µm and D90 from the microscope is 38.5µm, which indicates that PP particles are stable in MB and there are no aggregations over time.

Figure 29. shows the mean in particle size of three replicate batches of PP in glycerol with DMEM, 0.1, 1 and 10% FBS at the highest concentration (5mg/g). Both methods show that PP particles aggregate in glycerol with only DMEM. The Mastersizer displays that with the addition of 0.1% FBS 90% of the particles are smaller than 44µm, contrary, 90% of the particles with the microscope are smaller than 53µm. The particle distribution is yet comparable and both methods show aggregations. Therefore, 0.1% FBS is not sufficient to stabilize the PP particles but 1% FBS is, as result with both methods show. The D90 from both methods are close to each other. D90 from the Masterziser is 28.3µm while D90 from the microscope is 19.5µm. The Mastersizer indicates that 90% of the particles are smaller than 21.4µm with the addition of 10% FBS while 90% are smaller than 22.1µm measured with the microscope. The particle distribution is yet comparable and both methods show no aggregations.



	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	33	LB (red) GLYCEROL (green) MB (blue)	3,95	13,4	106
	22		7,20	34,2	74,9
	33		9,87	21,5	38,0
Mean			7,01	23,1	72,9
1xStd Dev			2,96	10,5	33,9
1xRSD (%)			42,3	45,5	46,6

Figure28. *Particle size distribution of PP in glycerol, LB and MB at the concentration of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*



	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	33		10,1	36,0	80,8
	34	DMEM (red) 10% FBS (green) 1% FBS (blue) 0.1% FBS (purple)	3,75	9,68	21,4
	47		3,81	9,96	28,3
	44		6,38	19,4	44,0
Mean			6,00	18,8	43,6
1xStd Dev			2,98	12,3	26,5
1xRSD (%)			49,7	65,8	60,8

Figure29. *Particle size distribution of PP in glycerol, DMEM, 0.1, 1 and 10% FBS at the concentration of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*

5.5. The number of particles/ml counted with the hemocytometer

Another way to determine the concentration can be achieved by using the hemocytometer. It allows to determine the number of particles in specified volume. Comparing the concentrations of PP in all media, it was possible to confirm the results from the microscope and Mastersizer. The highest concentration (highest number of particles/ml) has PP in glycerol with DMEM and 10% FBS followed by DMEM with 1%FBS which indicates that there are no aggregations, particles are small and individually distributed in the media.

Figure 30. shows the number of particles / ml in all seven media. Starting with the smallest number / ml (PP-glycerol) and ending with the highest number / ml (PP-glycerol-DMEM-10%FBS).

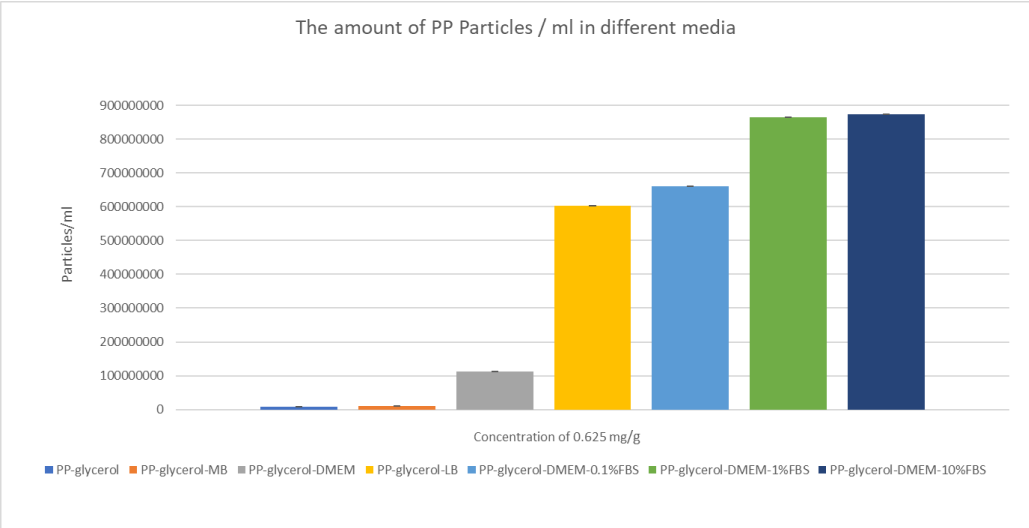


Figure 30. *The number of PP particles / ml counted with the hemocytometer in all seven media. The numbers represent counted particles in 10 squares in each media and calculated as described in the method section.*

5.6. PP particle storage stability over 33 days

Since glycerol has been used as the storage media, stability of PP particles in glycerol with a laser diffraction method (Mastersizer) over the time has been analysed. Three different samples containing PP in glycerol have been used for this experiment. The graphs do not overlap 100%, yet according to the manufacturers, this could be due to different vortex and sonication time. Long term storage stability controls will be needed to confirm that PP particles stay stable in glycerol.

Figure 31 indicate the stability of PP particles in glycerol after 7, 14 and 33days.

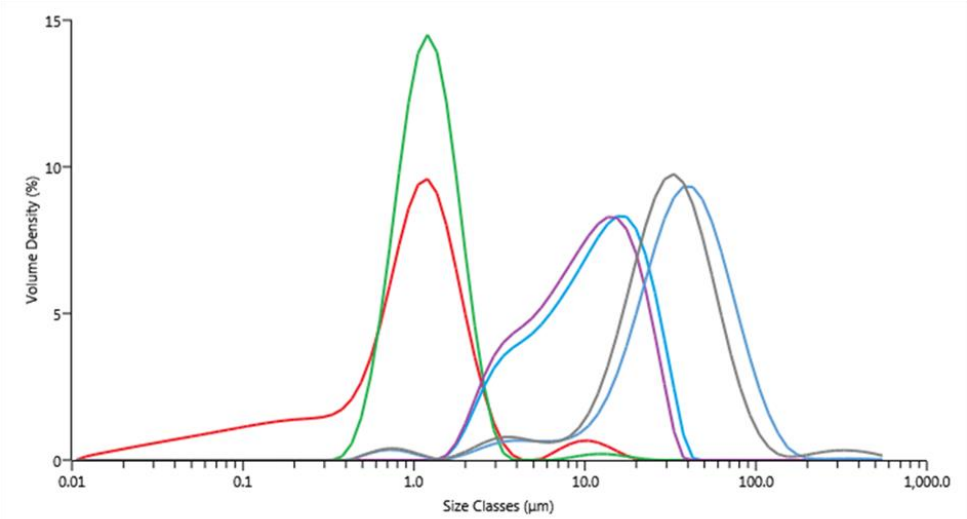


Figure 31. *Particle size distribution data of PP in glycerol over 33days. The values represent the mean in particle size of PP in glycerol.*

6 Discussion

6.1. The importance of microplastic size characterisation

This study investigated how the media selection affects the particle size distribution of PP particles and the data will be used to characterize PP plastic behavior (uptake and toxicity) in cell culture studies performed elsewhere. A major question in many studies is how MNPs affect the immune system, their allergenic potential in humans, the effect on the immune system, their allergenic potential in humans, the effect on the human response to respiratory and food allergens and the capability of MNPs to attach to molecules that could do harm to the human body (Enyoh et al., 2019). Previous studies stated that MNPs can cause reproductive toxicity in oysters (Sussarellu et al., n.d.) and accumulation of 5µm diameter MPs in fish gill, gut, liver and 20µm diameter accumulation in fish gill and gut, which caused toxic liver effects (Lu et al., 2016). The accumulation and distribution of MPs in aquatic organisms has been reported among many researchers, yet studies reporting potential health risks for humans are lacking. Therefore, a study focused on the accumulation of MPs in mice tissue and reported that the tissue accumulation and distribution strongly depend on the particle size. They used microplastic particles with a 5µm and 20µm diameter and detected accumulations in liver, kidney and gut. Results showed potential toxicity from MPs exposure (Deng et al., 2017). A review study summarized reports that indicated health impacts of micro- and nanoplastic contamination (Jiang et al., 2020). A table was made showing at what size range MPs were detected in aquatic organism but also in humans (up to 500µm). Based on their findings they assume that microplastic can affect human health and pointed out how necessary further studies are to address this topic (Jiang et al., 2020).

6.2. Various media impacts on the size distribution

Glycerol is well-known in the pharmaceutical and cosmetic industries and is listed on the FDA website (GRAS list) as generally safe and nontoxic (Zhang & Grinstaff, 2014). It has been reported that glycerol inhibited / decreased cell proliferation of BHK, CHO, HBL, MCF-7, depending on the glycerol concentration (Jung et al., 2010). Dumaswala et al., 1997 studied the effect of a glycerol-containing hypotonic media on erythrocyte phospholipid asymmetry and aminophospholipid transport during storage and believes that the protective effect is referable to glycerol. Another study used glycerol for the synthesis of palladium nanoparticles and reported that the addition of glycerol lead to the production of small palladium NPs stating that glycerol acts as a reducing agent. They also indicated that glycerol has the ability to maintain the NP homogeneity over a long period of time and describe glycerol as a good NP stabilizer (Clergeaud et al., 2013). A study that focused on the synthesis of gold nanoparticles, incorporated glycerol in the liposomal membrane. Results showed that with glycerol as a reducing agent, integrated into the membrane, the synthesis of highly homogeneous NPs was achieved (Genç et al., 2011). Glycerol can also be used for characterizing *in vitro* aerosol exposure, which was reported. The authors produced glycerol aerosols with a narrow size distribution and reported about their stability, robustness and reproducibility (Steiner et al., 2017). Results from this experiment show that glycerol alone at higher PP concentration is not able to prevent PP aggregation, however, low aggregation at a conc. of 1.25mg/g and no aggregation from 0.625mg/g and below were observed. The PP particle stability control experiment showed a decrease of relative standard deviation over time (which could be the result of different vortex and sonication time) in only glycerol and long-term stability controls will be needed to confirm if PP particles are stable in glycerol.

A review study indicated that MPs toxicity to humans and sea organisms could be affected trough bacterial growths on MPs surface, the additives they contain and their ability to adsorb contaminants (Hirt & Body-Malapel, 2020). Another study indicated that MP surface created a new environment for bacterial communities and that they adapted well to it (Chai et al., 2020). Therefore, in this study, LB + NaCl and MB were used to study whether PP particles are stable against aggregation. In microbiology, Lysogeny Broth (LB), is one of the most commonly used growth media (Ezraty et al., 2014). A study presented cultivation techniques for production of *R. marinus* DSM 16675 by using LB and MB broth with maltose. LB showed better results for obtaining high cell densities, though MB would give better results to produce EPSs (Ron et al., 2019). This study indicated that MB leads to a low aggregations of PP particles in high concentration, while LB showed more aggregates at the same concentration. LB is often used with 0.5M NaCl, as a study shows that had a focus on analysing the Endophytic *Bacillus safensis* strain ZY16 for improving phytoremediation of oil-contaminated saline soils. Due to the high salt tolerance of ZY16, ZY16 grew well in LB + 0.5M NaCl (Chen et al., 2019) while PP particles showed only low aggregation in LB + 0.5M NaCl in higher concentration.

DMEM can be used in cell culture studies and it has been reported that the usage of DMEM with 10% FBS for morphological characterization of adult mouse Leydig cells lead to fully elongated Leydig cells, whereas some grew aggregated and some grew individual (Wang & Cao, 2016). Another study had focused on analysing pectin-6-aminohexanoic-acid-magnetite nanoparticles for drug delivery and used DMEM alone, DMEM with 5% and 10% FBS to disperse the nanocomposites. The study reported aggregations of nanocomposites in DMEM alone, whereas no aggregations were detected by adding 5% and 10% of FBS (Arora et al., 2017). This statement corresponds to the results of the experiment of today. They also noticed a decrease in the average size of nanoparticles by increasing the FBS concentration. Furthermore, the study indicated that the actual serum coated MAP nanoparticles and the binding of the serum proteins on the nanoparticle surface prevented aggregation. Mao et al., 2010 stated that the surface of the particles changed depending on the addition of FBS since serum plasma proteins adsorbed on the particle surface. These findings could explain why the D90s from PP with DMEM + 1% FBS (from a conc. of 2.5mg/ and below) were smaller than the D90s with 10% FBS at the same concentrations. It is thinkable that the increased FBS concentration lead to increased binding on the microparticle surface, which was detected as a bigger particle. Further experiments will be needed to confirm this theory.

7 Conclusion

The fact that more than 3000 papers about micro- and nanoplastics were published from 2016 to 2020 (Bhagat et al., 2021) indicates how big of an issue plastic is for our environment, human and animal health. As referenced above, microplastic has been proven to accumulate not only in the nature, but also in humans and animals. Little is known about the possible toxic effects of MPs, and even less about the dependence of the particle size to induce these effects. Therefore, particle size characterisation of PP in various media provides data for future studies that will hopefully be able to analyse the toxicity of PP on human health.

Although glycerol did not prevent aggregation in high PP concentration, it has been used as a storage media for PP particles and results confirm the ability of glycerol to stabilize PP particles. Long term stability controls are needed for this experiment. Buford et al. 2007, reported that media containing proteins, lipids, protein/lipids produced CNPs with low aggregates which confirms the results from this study. The media with the smallest particle size distribution were DMEM with 1% and 10% FBS, concluding that the addition of protein serum and their ability to adsorb on the surface of particles leads to very small particle sizes. MB indicated a similar particle size distribution. The focus was given to the higher concentrated PP particle dilutions, whereas all media showed to lead to no aggregations in lower concentrations. There was no sign of aggregation in any media from a concentration of 0,625mg/g and below, which concludes that depending on the concentration all media described in the experiment of today could be used to characterise PP particle size distribution.

8 Reference

Abbasi, S., & Turner, A. (2021). Human exposure to microplastics: A study in Iran. *Journal of Hazardous Materials*, *403*, 123799. https://doi.org/10.1016/J.JHAZMAT.2020.123799

Adamovsky, O., Bisesi, J. H., & Martyniuk, C. J. (2021). Plastics in our water: Fish microbiomes at risk? *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *39*, 100834. https://doi.org/10.1016/J.CBD.2021.100834

AllenID, S., AllenID, D., Moss, K., le Roux, G., Phoenix, V. R., & Sonke, J. E. (2020). *Examination of the ocean as a source for atmospheric microplastics Missing plastic in the marine microplastic models*. https://doi.org/10.1371/journal.pone.0232746

Andrady, A. L. (2017). *The plastic in microplastics: A review*. https://doi.org/10.1016/j.marpolbul.2017.01.082

Atis, S., Tutluoglu, B., Levent, E., Ozturk, C., Tunaci, A., Sahin, K., Saral, A., Oktay, I., Kanik, A., & Nemery, B. (n.d.). *The respiratory effects of occupational polypropylene flock exposure*. https://doi.org/10.1183/09031936.04.00138403

Apte, S. P., Cagle, M., Bryant, R., Weidenbach, A., & Kulshreshtha, A. K. (2003). Use of sodium chloride to facilitate reduction of particle size of dexamethasone during ball milling. *Drug Development and Industrial Pharmacy*, *29*(3), 367–373. https://doi.org/10.1081/DDC-120018211

Arora, V., Sood, A., Shah, J., Kotnala, R. K., & Jain, T. K. (2017). Synthesis and characterization of pectin-6-aminohexanoic acid-magnetite nanoparticles for drug delivery. *Materials Science and Engineering: C*, *80*, 243–251. https://doi.org/10.1016/J.MSEC.2017.05.097

Barboza, L. G. A., Dick Vethaak, A., Lavorante, B. R. B. O., Lundebye, A. K., & Guilhermino, L. (2018). Marine microplastic debris: An emerging issue for food security, food safety and human health. In *Marine Pollution Bulletin* (Vol. 133, pp. 336–348). Elsevier Ltd. https://doi.org/10.1016/j.marpolbul.2018.05.047

Barnes, D. K. A., Galgani, F., Thompson, R. C., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 1985–1998. https://doi.org/10.1098/rstb.2008.0205

Bonfanti, P., Colombo, A., Saibene, M., Motta, G., Saliu, F., Catelani, T., Mehn, D., la Spina, R., Ponti, J., Cella, C., Floris, P., & Mantecca, P. (2021). Microplastics from miscellaneous plastic wastes: Physico-chemical characterization and impact on fish and amphibian development. *Ecotoxicology and Environmental Safety*, *225*. https://doi.org/10.1016/j.ecoenv.2021.112775

Buford, M. C., Hamilton, R. F., & Holian, A. (2007). *A comparison of dispersing media for various engineered carbon nanoparticles*. https://doi.org/10.1186/1743-8977-4-6

Bhagat, J., Nishimura, N., & Shimada, Y. (2021). Toxicological interactions of microplastics/nanoplastics and environmental contaminants: Current knowledge and future perspectives. *Journal of Hazardous Materials*, *405*, 123913. https://doi.org/10.1016/J.JHAZMAT.2020.123913

Campanale, C., Massarelli, C., Savino, I., Locaputo, V., & Uricchio, V. F. (n.d.). *A Detailed Review Study on Potential Effects of Microplastics and Additives of Concern on Human Health*. https://doi.org/10.3390/ijerph17041212

Corinaldesi, C., Canensi, S., Dell, A., Tangherlini, M., di Capua, I., Varrella, S., Willis, T. J., Cerrano, C., & Danovaro, R. (n.d.). *Multiple impacts of microplastics can threaten marine habitat-forming species*. https://doi.org/10.1038/s42003-021-01961-1

Chai, B., Li, X., Liu, H., Lu, G., Dang, Z., & Yin, H. (2020). Bacterial communities on soil microplastic at Guiyu, an E-Waste dismantling zone of China. *Ecotoxicology and Environmental Safety*, *195*, 110521. https://doi.org/10.1016/J.ECOENV.2020.110521

Chen, S., Lanza, G., Wu, T., Wang, R.-Q., W-h, G., X-b, L., J-b, X., W-j, X., Z-g, Y., Y-m, Z., R-q, W., Xu, J., Liu, J., Guo, W.-H., Li, X.-B., Xia, J.-B., Xie, W.-J., Yao, Z.-G., & Zhang, Y.-M. (2019). *Characterization and Initial Application of Endophytic Bacillus safensis Strain ZY16 for Improving Phytoremediation of Oil-Contaminated Saline Soils. Front Characterization and Initial Application of Endophytic Bacillus safensis Strain ZY16 for Improving Phytoremediation of Oil-Contaminated Saline Soils*. *10*, 991. https://doi.org/10.3389/fmicb.2019.00991

Clergeaud, G., Genç, R., Ortiz, M., & O'Sullivan, C. K. (2013). Liposomal nanoreactors for the synthesis of monodisperse palladium nanoparticles using glycerol. *Langmuir*, *29*(49), 15405–15413. https://doi.org/10.1021/la402892f

David C Wilson, Costas A Velis (2015): Waste management- still a global challenge in the 21st century: An evidence-based call for action

Deng, Y., Zhang, Y., Lemos, B., & Ren, H. (2017). Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure OPEN. *Nature Publishing Group*. https://doi.org/10.1038/srep46687

Dumaswala, U. J., Wilson, M. J., Jos6, T., & Daleke, D. L. (1997). BBN Effect of a glycerol-containing hypotonic medium on erythrocyte phospholipid asymmetry and aminophospholipid transport during storage. In *Biochimica et Biophysica Acta* (Vol. 1330).

Enyoh, C. E., Verla, A. W., Verla, E. N., Ibe, F. C., & Amaobi, C. E. (2019). Airborne microplastics: a review study on method for analysis, occurrence, movement and risks. In *Environmental Monitoring and Assessment* (Vol. 191, Issue 11). Springer International Publishing. https://doi.org/10.1007/s10661-019-7842-0

Eriksen, M., Lebreton, L. C. M., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., Galgani, F., Ryan, P. G., & Reisser, J. (2014). *Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea*. https://doi.org/10.1371/journal.pone.0111913

Ezraty, B., Henry, C., Hérisse, M., Denamur, E., & Barras, F. (2014). Commercial Lysogeny Broth culture media and oxidative stress: A cautious tale. *Free Radical Biology and Medicine*, *74*, 245–251. https://doi.org/10.1016/J.FREERADBIOMED.2014.07.010

Ferreira, A. G. M., Egas, A. P. V., Fonseca, I. M. A., Costa, A. C., Abreu, D. C., & Lobo, L. Q. (2017). The viscosity of glycerol. *The Journal of Chemical Thermodynamics*, *113*, 162–182. https://doi.org/10.1016/J.JCT.2017.05.042

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). *Production, use, and fate of all plastics ever made*. https://www.science.org

Genç, R., Clergeaud, G., Ortiz, M., & O'sullivan, C. K. (2011). Green synthesis of gold nanoparticles using glycerol-incorporated nanosized liposomes. *Langmuir*, *27*(17), 10894–10900. https://doi.org/10.1021/la201771s

Graz. (n.d.). *Conference Proceedings*. https://doi.org/10.3217/978-3-85125-456-3

Gulyaev, I. P., & Solonenko, O. P. (2013). Hollow droplets impacting onto a solid surface. *Experiments in Fluids*, *54*(1). https://doi.org/10.1007/s00348-012-1432-z

Hirt, N., & Body-Malapel, M. (n.d.). *Immunotoxicity and intestinal effects of nano-and microplastics: a review of the literature*. https://doi.org/10.1186/s12989-020-00387-7

Hoelting, L., Scheinhardt, B., Bondarenko, O., Schildknecht, S., Kapitza, M., Tanavde, V., Tan, B., Lee, Q. Y., Mecking, S., Leist, M., & Kadereit, S. (2013). A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles. *Archives of Toxicology*, *87*(4), 721–733. https://doi.org/10.1007/S00204-012-0984-2

Hü, T., Praetorius, A., Wagner, S., von der Kammer, F., & Hofmann, T. (2017). *Microplastic Exposure Assessment in Aquatic Environments: Learning from Similarities and Differences to Engineered Nanoparticles*. https://doi.org/10.1021/acs.est.6b04054

Hwang, J., Choi, D., Han, S., Choi, J., & Hong, J. (2019). An assessment of the toxicity of polypropylene microplastics in human derived cells. *Science of The Total Environment*, *684*, 657–669. https://doi.org/10.1016/J.SCITOTENV.2019.05.071

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan, R., & Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768–771. https://doi.org/10.1126/science.1260352

Jiang, B., Kauffman, A. E., Li, L., Mcfee, W., Cai, B., Weinstein, J., Lead, J. R., Chatterjee, S., Scott, G. I., & Xiao, S. (n.d.). *Health impacts of environmental contamination of micro-and nanoplastics: a review*. https://doi.org/10.1186/s12199-020-00870-9

Jovanović, B. (2017). Ingestion of microplastics by fish and its potential consequences from a physical perspective. In *Integrated Environmental Assessment and Management* (Vol. 13, Issue 3, pp. 510–515). Wiley-Blackwell. https://doi.org/10.1002/ieam.1913

Jung, H. il, Jeong, J. H., Lee, O. M., Park, G. T., Kim, K. K., Park, H. C., Lee, S. M., Kim, Y. G., & Son, H. J. (2010). Influence of glycerol on production and structural-physical properties of cellulose from Acetobacter sp. V6 cultured in shake flasks. *Bioresource Technology*, *101*(10), 3602–3608. https://doi.org/10.1016/j.biortech.2009.12.111

Kim, M.-S., Song, H.-S., Park, H. J., & Hwang, S.-J. (2012). Effect of Solvent Type on the Nanoparticle Formation of Atorvastatin Calcium by the Supercritical Antisolvent Process. In *Chem. Pharm. Bull* (Vol. 60, Issue 4).

Kokalj, A. J., Dolar, A., Drobne, D., Marinšek, M., Dolenec, M., Škrlep, L., Strmljan, G., Mušič, B., & Škapin, A. S. (n.d.). *Environmental hazard of polypropylene microplastics from disposable medical masks: acute toxicity towards Daphnia magna and current knowledge on other polypropylene microplastics*. https://doi.org/10.1186/s43591-021-00020-0

Lau, W. W. Y., Shiran, Y., Bailey, R. M., Cook, E., Stuchtey, M. R., Koskella, J., Velis, C. A., Godfrey, L., Boucher, J., Murphy, M. B., Thompson, R. C., Jankowska, E., Castillo, A. C., Pilditch, T. D., Dixon,

B., Koerselman, L., Kosior, E., Favoino, E., Gutberlet, J., … Palardy, J. E. (n.d.). *Evaluating scenarios toward zero plastic pollution*. https://www.science.org

Leads, R. R., & Weinstein, J. E. (2019). *Occurrence of tire wear particles and other microplastics within the tributaries of the Charleston Harbor Estuary, South Carolina, USA*. https://doi.org/10.1016/j.marpolbul.2019.06.061

Lear, G., Kingsbury, J. M., Franchini, S., Gambarini, V., Maday, S. D. M., Wallbank, J. A., Weaver, L., & Pantos, O. (n.d.). *Plastics and the microbiome: impacts and solutions Environmental Microbiome*. https://doi.org/10.1186/s40793-020-00371-w

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K. M., & He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. *Science of The Total Environment*, *619–620*, 1–8. https://doi.org/10.1016/J.SCITOTENV.2017.11.103

Lithner, D., Larsson, Å., & Dave, G. (2011). *Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition*. https://doi.org/10.1016/j.scitotenv.2011.04.038

Liu, G., Jiang, R., You, J., Muir, D. C. G., & Zeng, E. Y. (2020). Microplastic Impacts on Microalgae Growth: Effects of Size and Humic Acid. *Environmental Science and Technology*, *54*(3), 1782–1789. https://doi.org/10.1021/acs.est.9b06187

Liu, Z., Yu, P., Cai, M., Wu, D., Zhang, M., Chen, M., & Zhao, Y. (2019). Effects of microplastics on the innate immunity and intestinal microflora of juvenile Eriocheir sinensis. *Science of The Total Environment*, *685*, 836–846. https://doi.org/10.1016/J.SCITOTENV.2019.06.265

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., & Ren, H. (2016). Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (Danio rerio) and Toxic Effects in Liver. *Environmental Science and Technology*, *50*(7), 4054–4060. https://doi.org/10.1021/acs.est.6b00183

Mangesana, N., Chikuku, R.S., Mainza, A.N., Govender, I., van der Westhuizen, A.P., & Narashima, M.. (2008). The effect of particle sizes and solids concentration on the rheology of silica sand based suspensions. *Journal of the Southern African Institute of Mining and Metallurgy*, *108*(4), 237-243. Retrieved May 26, 2022, from http://www.scielo.org.za/scielo.php?script=sci_arttext&pid=S2225-62532008000400005&lng=en&tlng=en.

Mao, Z., Wan, L., Hu, L., Ma, L., & Gao, C. (2010). Tat peptide mediated cellular uptake of SiO2 submicron particles. *Colloids and Surfaces B: Biointerfaces*, *75*(2), 432–440. https://doi.org/10.1016/j.colsurfb.2009.09.017

Murali, K., Kenesei, K., Li, Y., Demeter, K., Környei, Z., & Madarász, E. (2015). Uptake and bio-reactivity of polystyrene nanoparticles is affected by surface modifications, ageing and LPS adsorption: In vitro studies on neural tissue cells. *Nanoscale*, *7*(9), 4199–4210. https://doi.org/10.1039/c4nr06849a

Murakami, H., Kobayashi, M., Takeuchi, H., & Kawashima, Y. (1999). Preparation of poly(dl-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method.

International Journal of Pharmaceutics, *187*(2), 143–152. https://doi.org/10.1016/S0378-5173(99)00187-8

Prüst, M., Meijer, J., & Westerink, R. H. S. (n.d.). *The plastic brain: neurotoxicity of micro-and nanoplastics*. https://doi.org/10.1186/s12989-020-00358-y

Ragusa, A., Svelato, A., Santacroce, C., Catalano, P., Notarstefano, V., Carnevali, O., Papa, F., Rongioletti, M. C. A., Baiocco, F., Draghi, S., D'Amore, E., Rinaldo, D., Matta, M., & Giorgini, E. (2021). Plasticenta: First evidence of microplastics in human placenta. In *Environment International* (Vol. 146). Elsevier Ltd. https://doi.org/10.1016/j.envint.2020.106274

Rohanová, D., Rohanová, R., Boccaccini, A. R., Horkavcová, D., Horkavcová, H., Ina Bozděchová, P., Bozděchov, B., Bozděchová, B., Bezdička, P., Bezdička, B., Mark´, M., Markétačastor, M. M., Markétačastorálov, M., & Markétačastorálová, M. (2014). *Is non-buffered DMEM solution a suitable medium for in vitro bioactivity tests?* https://doi.org/10.1039/c4tb00187g

Ron, E. Y. C., Sardari, R. R. R., Anthony, · Richard, van Niel, E. W. J., Gudmundur, ·, Hreggvidsson, O., & Nordberg-Karlsson, E. (2019). Cultivation technology development of Rhodothermus marinus DSM 16675. *Extremophiles*, *23*, 735–745. https://doi.org/10.1007/s00792-019-01129-0

Saberi, A. H., Fang, Y., & McClements, D. J. (2013). Effect of glycerol on formation, stability, and properties of vitamin-E enriched nanoemulsions produced using spontaneous emulsification. *Journal of Colloid and Interface Science*, *411*, 105–113. https://doi.org/10.1016/j.jcis.2013.08.041

Schirinzi, G. F., Pérez-Pomeda, I., Sanchís, J., Rossini, C., Farré, M., & Barceló, D. (2017). Cytotoxic effects of commonly used nanomaterials and microplastics on cerebral and epithelial human cells. *Environmental Research*, *159*, 579–587. https://doi.org/10.1016/j.envres.2017.08.043

Schwabl, P., Koppel, S., Konigshofer, P., Bucsics, T., Trauner, M., Reiberger, T., & Liebmann, B. (2019). Detection of various microplastics in human stool: A prospective case series. *Annals of Internal Medicine*, *171*(7), 453–457. https://doi.org/10.7326/M19-0618

Sharma, S., & Chatterjee, S. (2017). Microplastic pollution, a threat to marine ecosystem and human health: a short review. *Environmental Science and Pollution Research*, *24*(27), 21530–21547. https://doi.org/10.1007/s11356-017-9910-8

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Eve, M., Pernet, J., le Goïc, N., Quillien, V., Mingant, C., Epelboin, Y., Corporeau, C., Guyomarch, J., Robbens, J., Paul-Pont, I., Soudant, P., & Huvet, A. (n.d.). *Oyster reproduction is affected by exposure to polystyrene microplastics*. https://doi.org/10.1073/pnas.1519019113

Steiner, S., Majeed, S., Kratzer, G., Hoeng, J., & Frentzel, S. (2017). A new fluorescence-based method for characterizing in vitro aerosol exposure systems. *Toxicology in Vitro*, *38*, 150–158. https://doi.org/10.1016/j.tiv.2016.09.018

Toussaint, B., Raffael, B., Angers-Loustau, A., Gilliland, D., Kestens, V., Petrillo, M., Rio-Echevarria, I. M., & van den Eede, G. (2019). *Review of micro-and nanoplastic contamination in the food chain*. https://doi.org/10.1080/19440049.2019.1583381

Volk, A., & Kähler, C. J. (2018). Density model for aqueous glycerol solutions. *Experiments in Fluids*, *59*, 75. https://doi.org/10.1007/s00348-018-2527-y

Wang, J., Li, Y., Lu, L., Zheng, M., Zhang, X., Tian, H., Wang, W., & Ru, S. (2019). Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (Oryzias melastigma). *Environmental Pollution*, *254*, 113024. https://doi.org/10.1016/J.ENVPOL.2019.113024

Wang, J. Q., & Cao, W. G. (2016). Morphological characterization of adult mouse Leydig cells in culture. *Biochemical and Biophysical Research Communications*, *469*(4), 836–841. https://doi.org/10.1016/J.BBRC.2015.12.018

White, E. M., Clark, S., Manire, C. A., Crawford, B., Wang, S., Locklin, J., & Ritchie, B. W. (2018). Ingested Micronizing Plastic Particle Compositions and Size Distributions within Stranded Post-Hatchling Sea Turtles. *Environmental Science and Technology*, *52*(18), 10307–10316. https://doi.org/10.1021/acs.est.8b02776

Wright, S. L., & Kelly, F. J. (2017). Plastic and human health: a micro issue? In *Environ. Sci. Technol., Just Accepted Manuscript • Publication Date*. http://pubs.acs.org

Zhang, H., & Grinstaff, M. W. (2014). Recent advances in glycerol polymers: Chemistry and biomedical applications. *Macromolecular Rapid Communications*, *35*(22), 1906–1924. https://doi.org/10.1002/marc.201400389

9 Appendix I

9.1. Effect of PP concentration in glycerol on particle size measurements

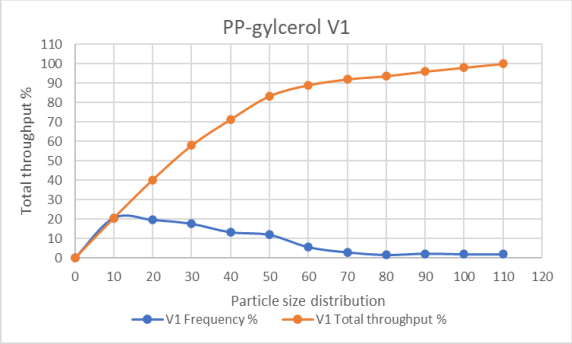


Figure 32. *Particle size distribution of PP in glycerol at a conc. of 5mg/g. D10= 5µm, D50= 25µm, D90= 60µm. The D90/10 ratio= 12µm. Values represent the mean of n=3 batches.*

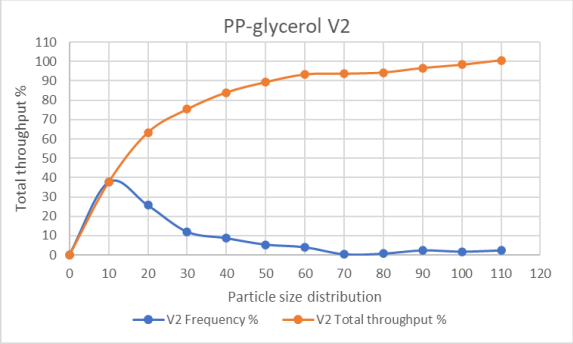


Figure 33*. Particle size distribution of PP in glycerol at a conc. of 2,5mg/g. D10= 3µm, D50= 14µm, D90= 50µm. The D90/10 ratio=16,6µm. Values represent the mean of n=3 batches.*

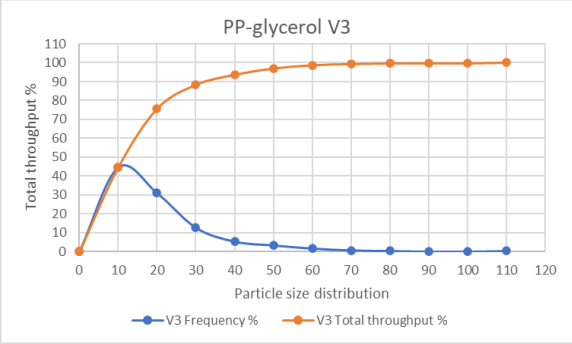


Figure 34. *Particle size distribution of PP in glycerol at a conc. of 1,25mg/g. D10= 2,2µm, D50= 11,5µm, D90= 32µm. The D90/10 ratio = 14,5µm. Values represent the mean of n=3 batches*

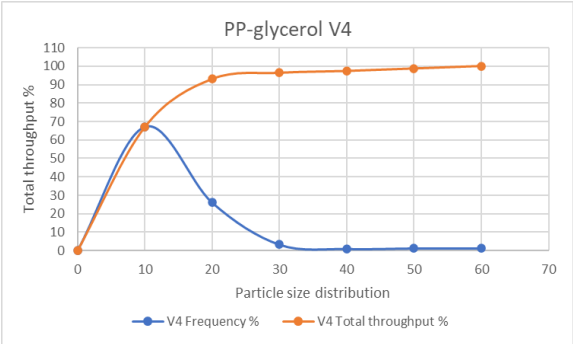


Figure 35. *Particle size distribution of PP in glycerol at a conc. of 0,625mg/g. D10= 1,5µm, D50= 7µm, D90= 18µm. The D90/10 ratio= 12µm. Values represent the mean of n=3 batches.*

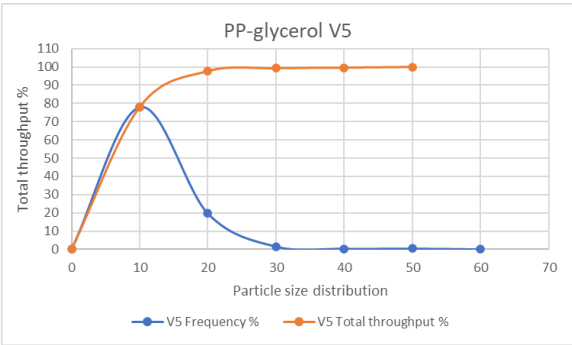


Figure 36. *Particle size distribution of PP in glycerol at a conc. of 0,3125mg/g. D10= 1,2µm, D50= 6µm, D90= 13,5µm. The D90/10 ratio= 11,25µm. Values represent the mean of n=3 batches.*

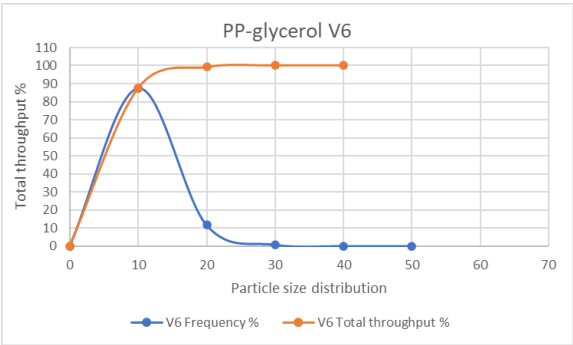


Figure 37*. Particle size distribution of PP in glycerol at a conc. of 0,15625mg/g. D10= 1µm, D50= 5,1µm, D90=10,5µm. The D90/10 ratio= 10,5µm. Values represent the mean of n=3 batches.*

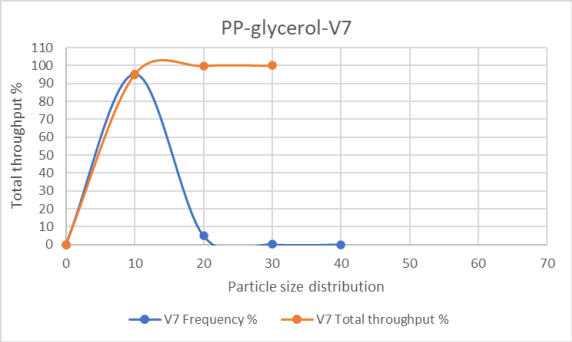


Figure 38. *Particle size distribution of PP in glycerol at a conc. of 0,0781mg/g. D10= 1µm, D50= 4,8µm, D90= 9µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

9.2. Effect of PP concentration in glycerol with LB + 0.5M NaCl on particle size measurements

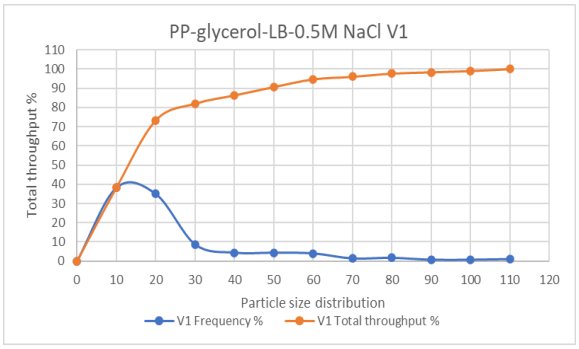


Figure 39. *Particle size distribution of PP-glycerol - LB + 0.5M NaCl at a conc. of 5mg/g. D10= 3µm, D50= 13µm, D90= 48µm. The D90/10 ratio= 16µm. Values represent the mean of n=3 batches.*

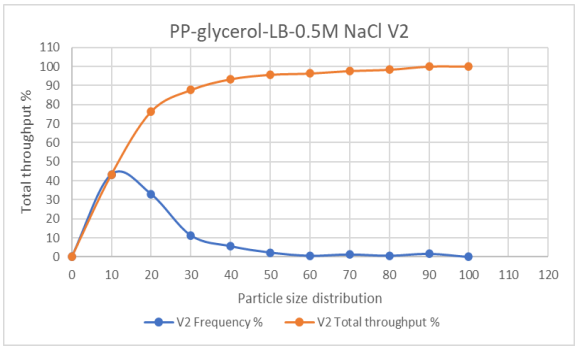


Figure 40. *Particle size distribution of PP-glycerol- LB + 0.5M NaCl at a conc. of 2,5mg/g. D10= 2,3µm, D50= 12µm, D90= 33µm. The D90/10 ratio= 14,3µm. Values represent the mean of n=3 batches.*

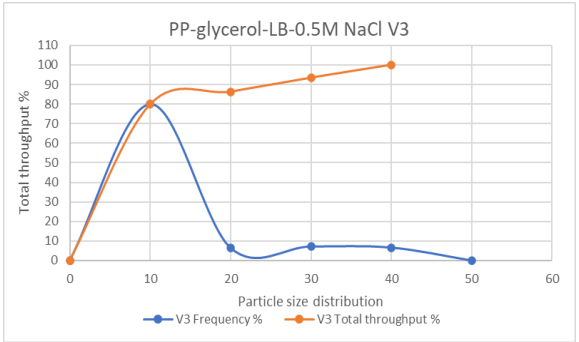


Figure 41*. Particle size distribution of PP-glycerol-LB + 0.5M NaCl at a conc. of 1,25mg/g. D10= 1,5µm, D50= 5,5µm, D90= 25µm. The D90/10 ratio= 16,6µm. Values represent the mean of n=3 batches.*

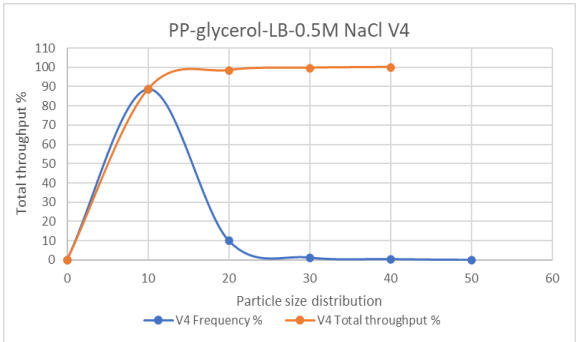


Figure 42. *Particle size distribution of PP-glycerol-LB + 0.5M NaCl at a conc. of 0,625mg/g. D10= 1µm, D50= 5,2µm, D90= 10,2µm. The D90/10 ratio= 10,2µm. Values represent the mean of n=3 batches.*

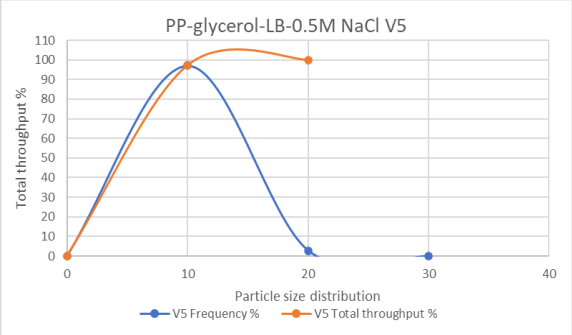


Figure 43. *Particle size distribution of PP-glycerol-LB + 0.5M NaCl at a conc. of 0,3125mg/g. D10= 1µm, D50= 4,5µm, D90= 9µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

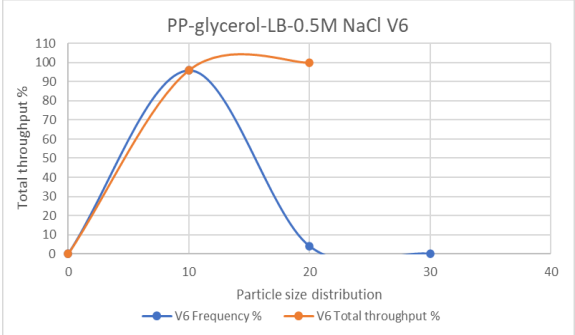


Figure 44. *Particle size distribution of PP-glycerol-LB + 0.5M NaCl at a conc. 0,15625mg/g. D10= 1µm, D50= 4,5µm, D90=9µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

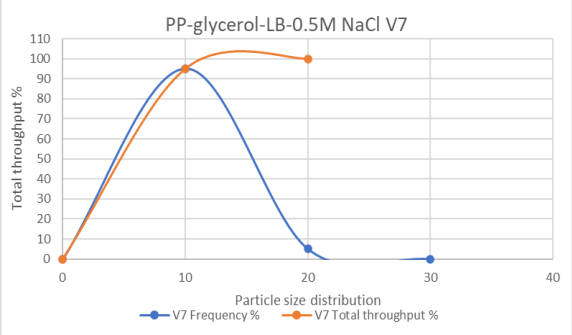


Figure 45. *Particle size distribution of PP-glycerol-LB + 0.5M NaCl at a conc. 0,0781mg/g. D10= 1µm, D50= 4,5µm, D90=9µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

9.3. Effect of PP concentration in glycerol with MB on particle size measurements

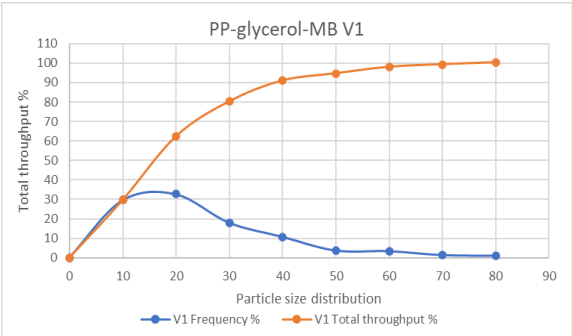


Figure 46. *Particle size distribution of PP-glycerol-MB at a conc. of 5mg/g. D10= 3,5µm. D50= 16µm, D90= 38,5µm. The D90/10 ratio= 11µm. Values represent the mean of n=3 batches.*

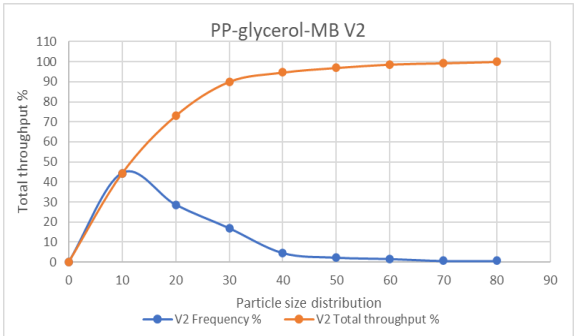


Figure 47. *Particle size distribution of PP-glycerol-MB at a conc. of 2,5mg/g. D10= 2,2µm, D50= 11,5µm, D90= 30µm. The D90/10 ratio= 13,6µm. Values represent the mean of n=3 batches.*

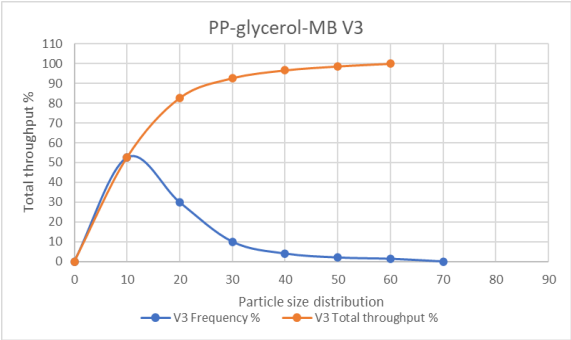


Figure 48. *Particle size distribution of PP-glycerol-MB at a conc. of 1,25mg/g. D10= 1,9µm, D50= 9,5µm, D90= 26µm. The D90/10 ratio= 13,7µm. Values represent the mean of n=3 batches.*

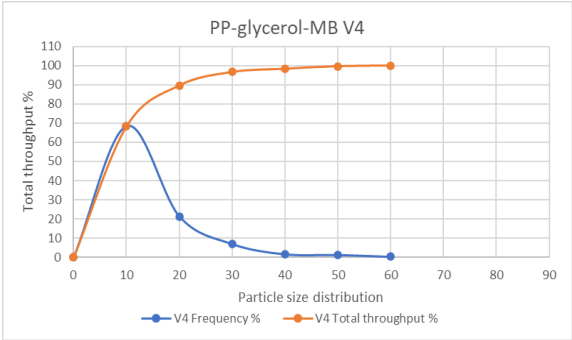


Figure 49*. Particle size distribution of PP-glycerol-MB at a conc. of 0,625mg/g. D10= 1,4µm, D50= 7µm, D90=20µm. The D90/10 ratio= 14,3µm. Values represent the mean of n=3 batches.*

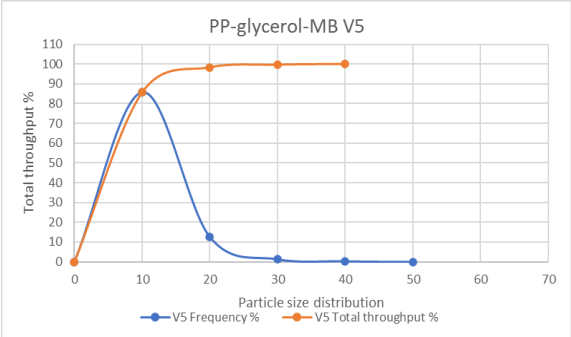


Figure 50. *Particle size distribution of PP-glycerol-MB at a conc. of 0,3125mg/g. D10= 1µm, D50= 5,2µm, D90= 11µm. The D90/10 ratio= 11mm. Values represent the mean of n=3 batches.*

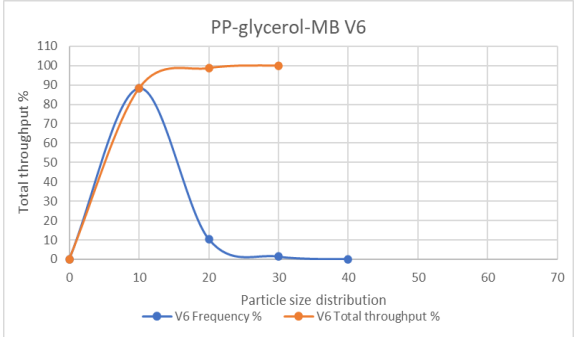


Figure 51. *Particle size distribution of PP-glycerol-MB at a conc. of 0,15625mg/g. D10= 1µm, D50= 5µm, D90= 10,1µm. The D90/10 ratio= 10,1µm. Values represent the mean of n=3 batches.*

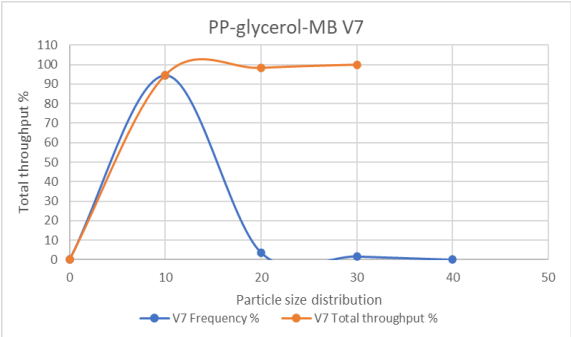


Figure 52*. Particle size distribution of PP-glycerol-MB at a conc. of 0,0781mg/g. D10= 1µm, D50= 4,8µm, D90= 9,1µm. The D90/10 ratio= 9,1µm. Values represent the mean of n=3 batches.*

9.4. Effect of PP concentration in glycerol with DMEM on particle size measurements

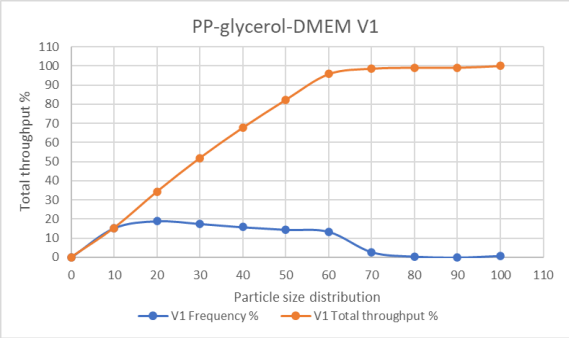


Figure 53. *Particle size distribution of PP-glycerol-DMEM at a conc. of 5mg/g. D10=7µm, D50= 29µm, D90= 55µm. The D90/10 ratio= 7,8µm. Values represent the mean of n=3 batches.*

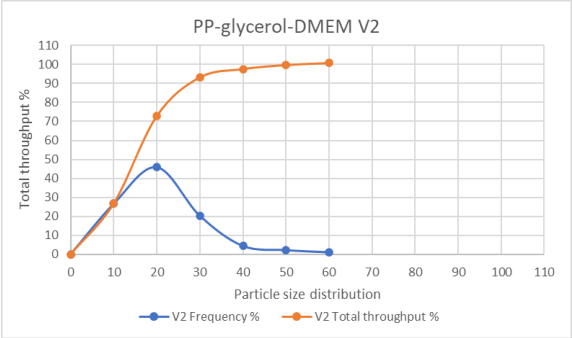


Figure 54. *Particle size distribution of PP-glycerol-DMEM at a conc. of 2,5mg/g. D10= 4µm, D50= 15µm, D90= 27,2µm. The D90/10 ratio= 6,8µm. Values represent the mean of n=3 batches.*

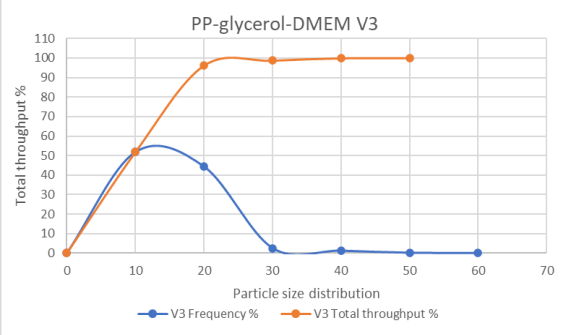


Figure 55. *Particle size distribution of PP-glycerol-DMEM at a conc. of 1,25mg/g. D10= 2µm, D50= 9,5µm, D90= 18µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

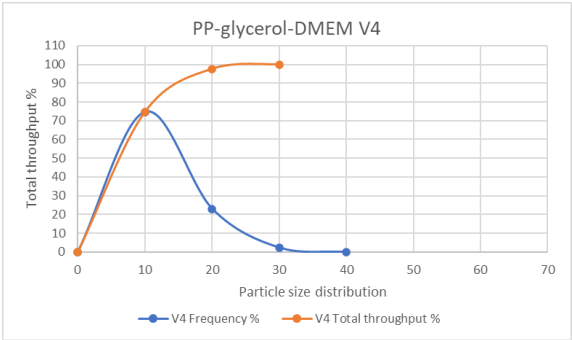


Figure 56. *Particle size distribution of PP-glycerol-DMEM at a conc. of 0,625mg/g. D10= 1,2µm, D50= 6,1µm, D90= 14,5µm. The D90/10 ratio= 12,1µm. Values represent the mean of n=3 batches.*

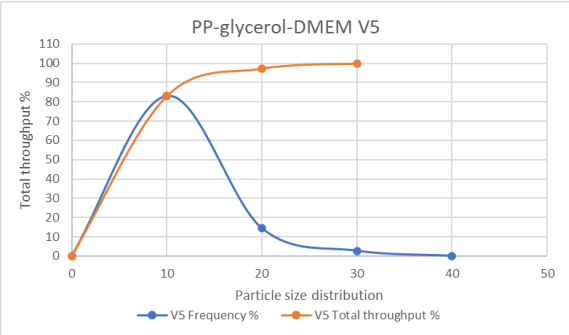


Figure 57. *Particle size distribution of PP-glycerol-DMEM at a conc. of 0,3125mg/g. D10= 1,1µm, D50= 5,5µm, D90= 11,9µm. The D90/10 ratio= 10,8µm. Values represent the mean of n=3 batches.*

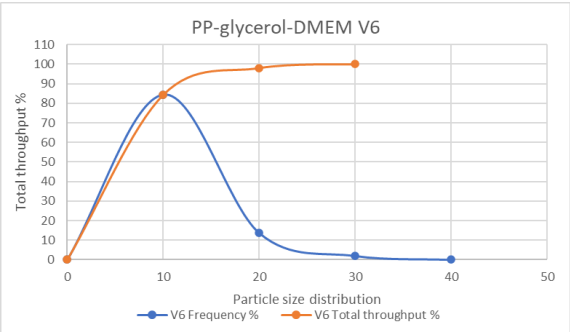


Figure 58. *Particle size distribution of PP-glycerol-DMEM at a conc. of 0,15625mg/g. D10= 1µm, D50= 5,4µm, D90= 11,5µm. The D90/10 ratio= 11,5µm. Values represent the mean of n=3 batches.*

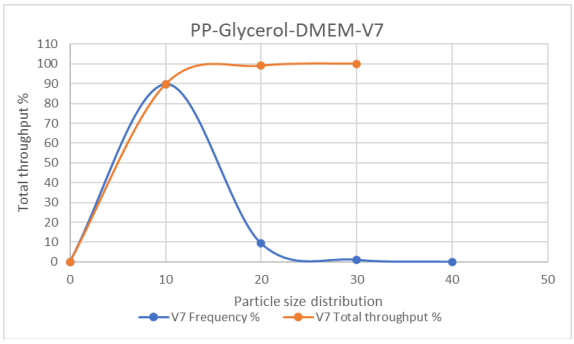


Figure 59. *Particle size distribution of PP-glycerol-DMEM at a conc. of 0,0781mg/g. D10= 1µm, D50= 5µm, D90= 10µm. The D90/10 ratio= 10µm. Values represent the mean of n=3 batches.*

9.5. Effect of PP concentration in glycerol with DMEM + 0.1% FBS on particle size measurements

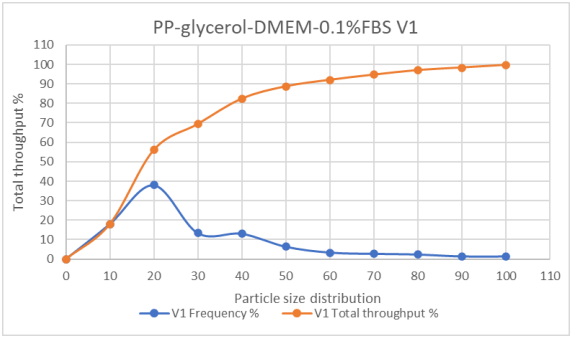


Figure 60*. Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 5mg/g. D10= 6,5µm, D50= 18µm, D90= 53µm. The D90/10 ratio= 8,1µm. Values represent the mean of n=3 batches.*

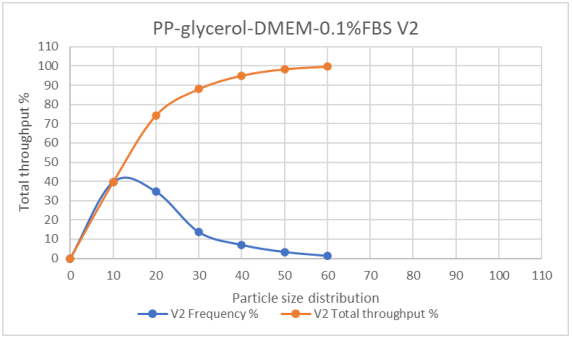


Figure 61. *Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 2,5mg/g. D10= 2,5µm, D50= 12,5µm, D90= 31,5µm. The D90/10 ratio= 12,6µm. Values represent the mean of n=3 batches.*

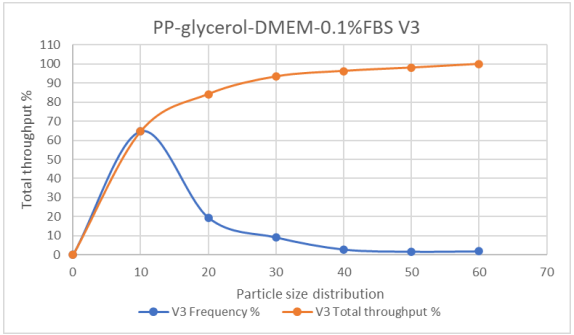


Figure 62. *Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 1,25mg/g. D10= 1,5µm, D50= 7µm, D90=25µm. The D90/10 ratio= 16,6µm. Values represent the mean of n=3 batches.*

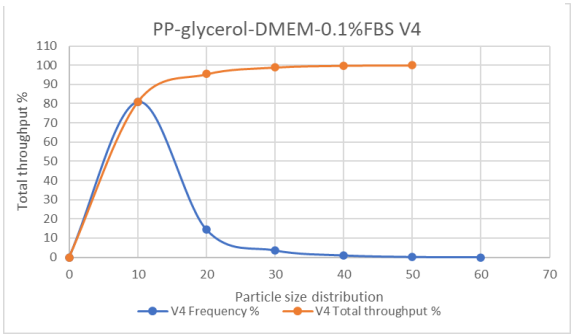


Figure 63. *Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 0,625mg/g. D10= 1µm, D50= 5,8µm, D90= 13µm. The D90/10 ratio= 13µm. Values represent the mean of n=3 batches.*

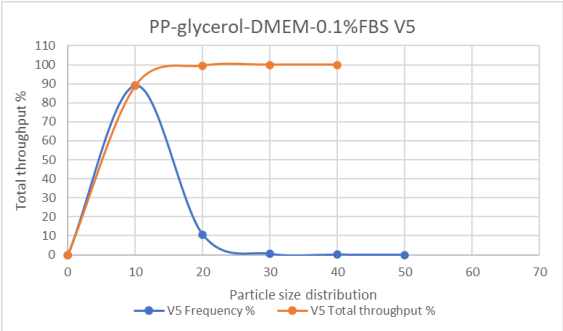


Figure 64. *Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 0,3125mg/g. D10= 1µm, D50= 5µm, D90= 10,1µm. The D90/10 ratio= 10,1µm. Values represent the mean of n=3 batches.*

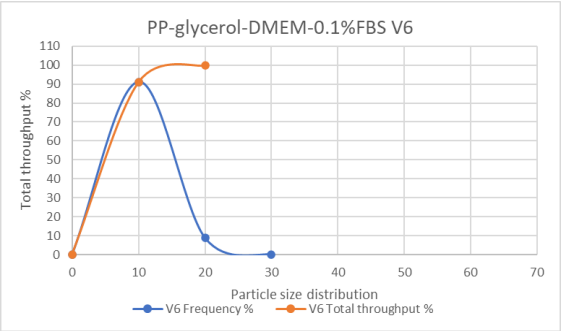


Figure 65. *Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 0,15625mg/g. D10= 1µm, D50= 5µm, D90= 10µm. The D90/10 ratio= 10µm. Values represent the mean of n=3 batches.*

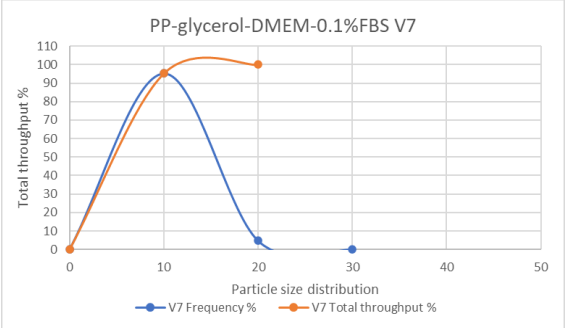


Figure 66*. Particle size distribution of PP-glycerol-DMEM-0.1%FBS at a conc. of 0,0781mg/g. D10= 1µm, D50= 4,8µm, D90= 9µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

9.6. Effect of PP concentration in glycerol with DMEM + 1% FBS on particle size measurements

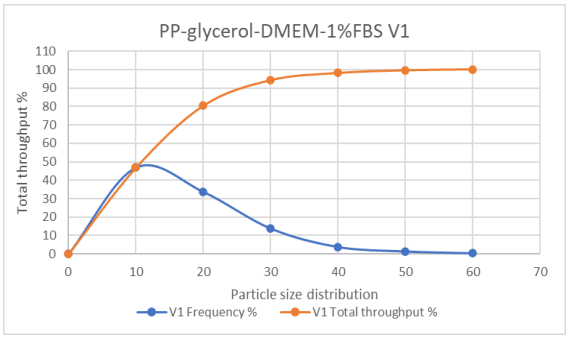


Figure 67. *Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 5mg/g. D10= 2,1µm, D50= 11µm, D90= 26µm. The D90/10 ratio= 12,4µm. Values represent the mean of n=3 batches.*

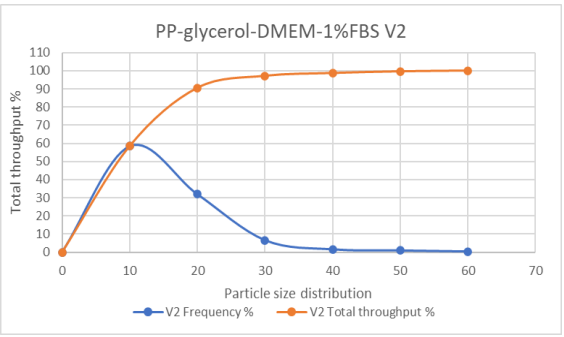


Figure 68*. Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 2,5mg/g. D10= 1,9µm, D50= 8,2µm, D90= 19,5µm. The D90/10 ratio= 10,3µm. Values represent the mean of n=3 batches.*

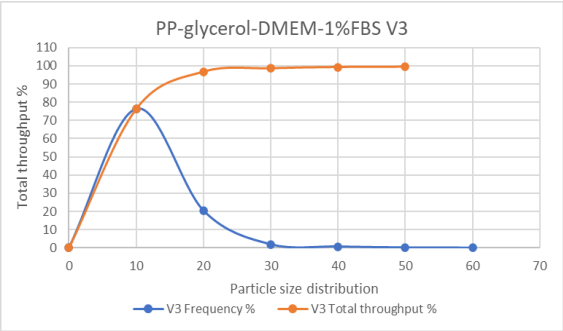


Figure 69. *Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 1,25mg/g. D10= 1,2µm, D50= 6µm, D90= 14,5µm. The D90/10 ratio= 12,1µm. Values represent the mean of n=3 batches.*

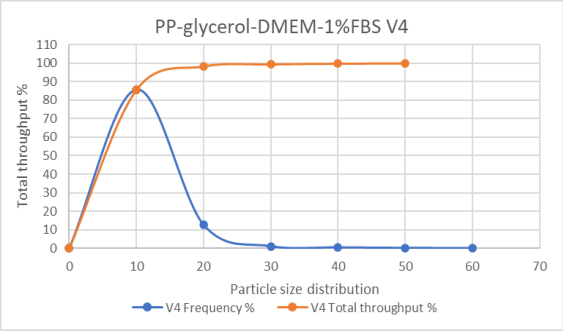


Figure 70. *Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 0,625mg/g. D10= 1µm, D50= 5,2µm, D90= 11µm. The D90/10 ratio= 11µm. Values represent the mean of n=3 batches.*

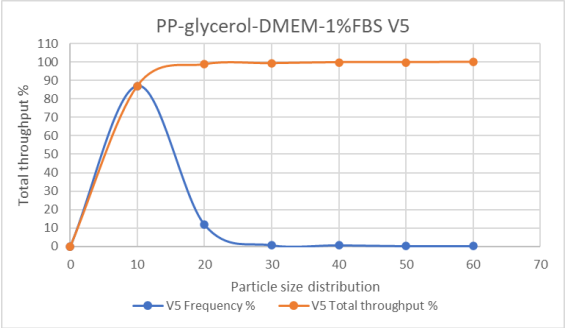


Figure 71. *Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 0,3125mg/g. D10= 1µm, D50= 5,1µm, D90= 10,9µm. The D90/10 ratio= 10,9µm. Values represent the mean of n=3 batches.*

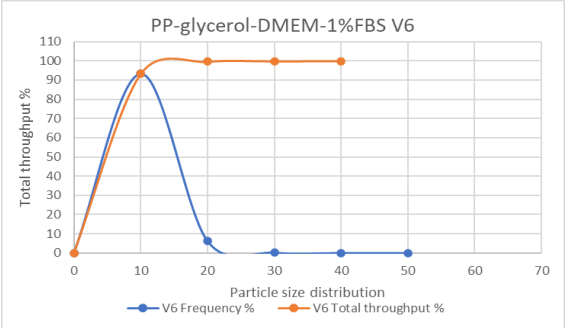


Figure 72*. Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 0,15625mg/g. D10= 1µm, D50= 4,9µm, D90= 9,5µm. The D90/10 ratio= 9,5µm. Values represent the mean of n=3 batches.*

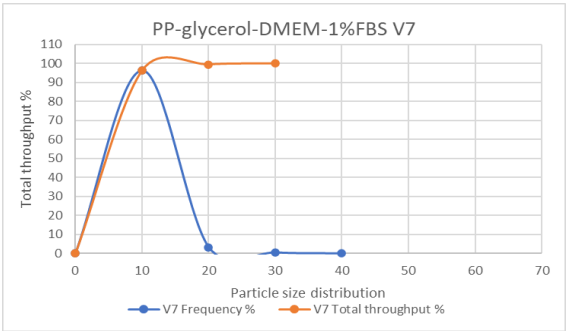


Figure 73. *Particle size distribution of PP-glycerol-DMEM-1%FBS at a conc. of 0,0781mg/g. D10= 1µm, D50= 4,5µm, D90= 9µm. The D90/10 ratio= 9µm. Values represent the mean of n=3 batches.*

9.7. Effect of PP concentration in glycerol with DMEM + 10% FBS on particle size measurements

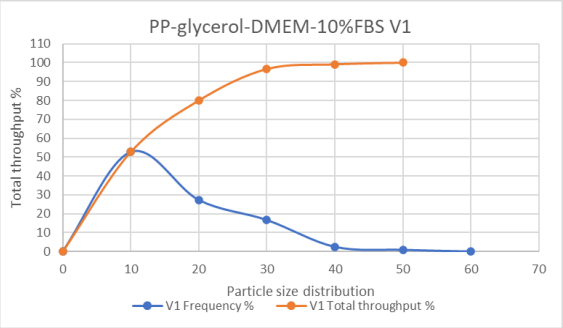


Figure 74. *Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 5mg/g. D10= 2µm, D50= 9,2µm, D90= 25µm. The D90/10 ratio= 12,5µm. Values represent the mean of n=3 batches.*

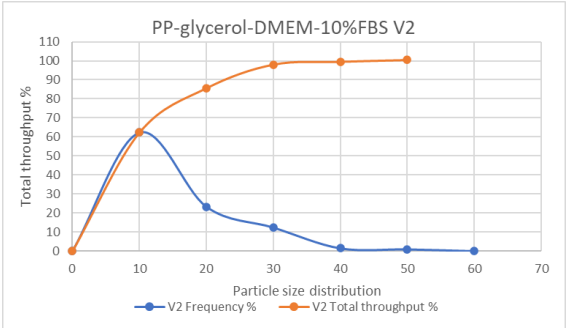


Figure 75. *Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 2,5mg/g. D10= 1,5µm, D50= 7,6µm, D90= 22,1µm. The D90/10 ratio= 14,7µm. Values represent the mean of n=3 batches.*

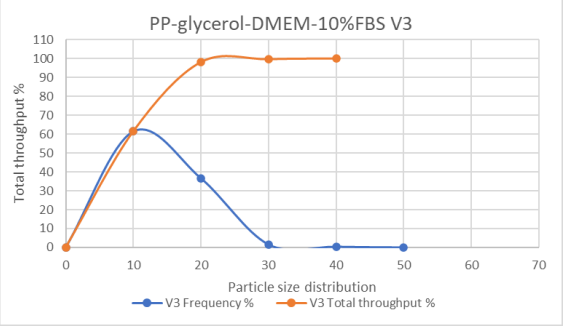


Figure 76. *Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 1,25mg/g. D10= 1,5µm, D50= 7,5µm, D90= 17µm. The D90/10 ratio= 11,3µm. Values represent the mean of n=3 batches.*

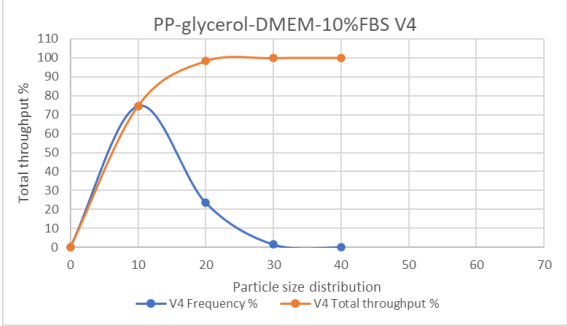


Figure 77. *Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 0,625mg/g. D10= 1,1µm, D50= 6,1µm, D90= 14,5µm. The D90/10 ratio= 13,2µm. Values represent the mean of n=3 batches.*

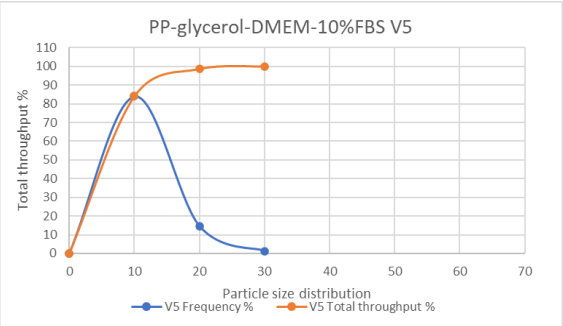


Figure 78. *Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 0,3125mg/g. D10= 1µm, D50= 5,4µm, D90= 11,5µm. The D90/10 ratio= 11,5µm. Values represent the mean of n=3 batches.*

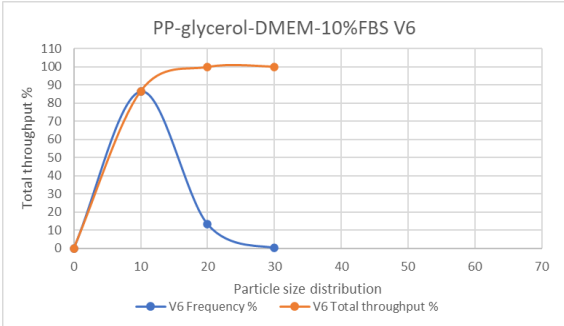


Figure 79*. Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 0,15625mg/g. D10= 1µm, D50= 5,1µm, D90= 10,9µm. The D90/10 ratio= 10,9µm. Values represent the mean of n=3 batches.*

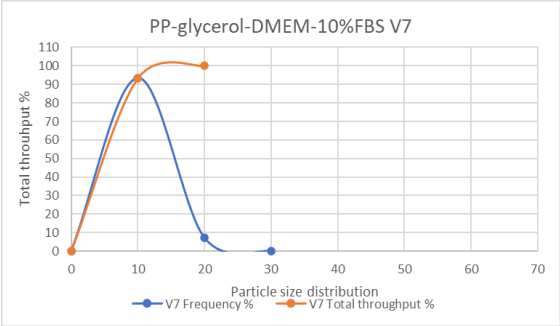
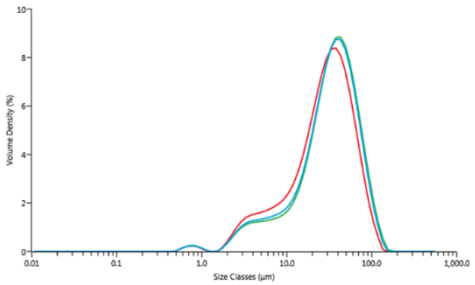


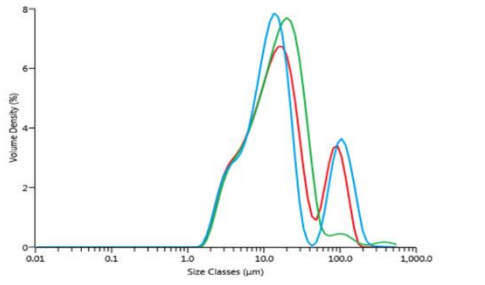
***Figure 80**. Particle size distribution of PP-glycerol-DMEM-10%FBS at a conc. of 0,0781mg/g. D10= 1µm, D50= 4,9µm, D90= 9,3µm. The D90/10 ratio= 9,3µm. Values represent the mean of n=3 batches.*

9.8. Particle size characterisation with the mastersizer3000



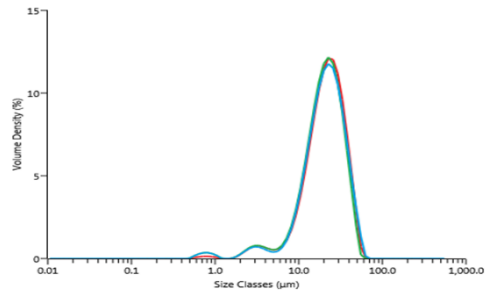
	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	22	Average of 'PP_GL_V1_SPL2'	7,20	34,2	74,9
	11	Average of 'PP_GL_V1_SPL1'	5,77	29,0	65,6
	33	Average of 'PP_GL_V1_SPL3'	6,77	33,1	72,8
Mean			**6,58**	**32,1**	**71,1**
1xStd Dev			**0,731**	**2,77**	**4,87**
1xRSD (%)			**11,1**	**8,62**	**6,86**

***Figure 81.** Particle size distribution of PP-glycerol at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches obtained with dynamic light scattering.*



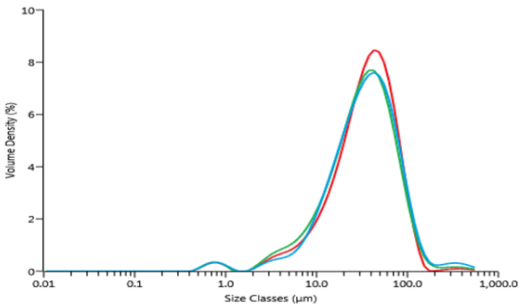
	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	11	Average of 'PP_GL_LBBr_NaCl_V1_SMPL1'	3,96	14,7	82,9
	22	Average of 'PP_GL_LBBr_NaCl_V1_SMPL2'	4,17	15,0	36,8
	33	Average of 'PP_GL_LBBr_NaCl_V1_SMPL3'	3,95	13,4	106
Mean			**4,03**	**14,4**	**75,1**
1xStd Dev			**0,124**	**0,860**	**35,1**
1xRSD (%)			**3,09**	**5,97**	**46,8**

***Figure 82**. Particle size distribution of PP-glycerol-LB-0.5M NaCl at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*



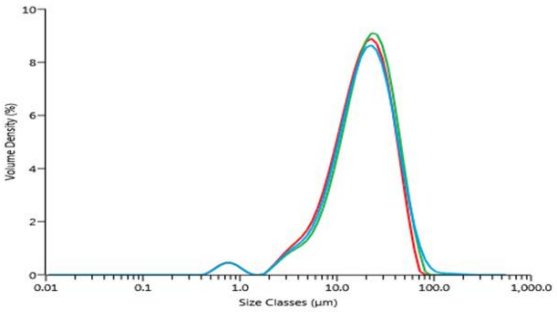
	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	33	Average of 'V1_smpl3'	9,87	21,5	38,0
	22	Average of 'V1_smpl2'	9,02	20,4	35,8
	11	Average of 'V1_smpl1'	9,58	21,1	38,3
Mean			**9,49**	**21,0**	**37,4**
1xStd Dev			**0,428**	**0,574**	**1,32**
1xRSD (%)			**4,52**	**2,73**	**3,55**

***Figure 83.** Particle size distribution of PP-Glycerol-MB at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*



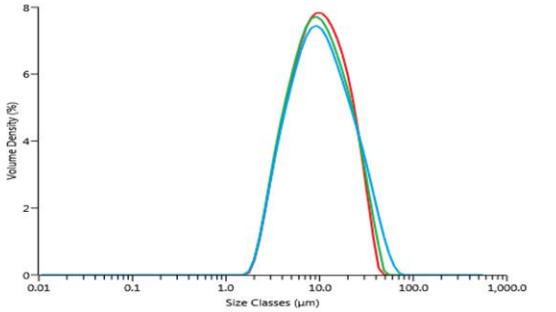
	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	33	Average of 'V1_smpl3'	10,1	36,0	80,8
	22	Average of 'V1_smpl2'	8,68	33,1	82,1
	11	Average of 'V1_smpl1'	10,5	35,3	88,7
Mean			**9,76**	**34,8**	**83,8**
1xStd Dev			**0,955**	**1,53**	**4,22**
1xRSD (%)			**9,79**	**4,41**	**5,04**

***Figure 84.** Particle size distribution of PP-glycerol-DMEM at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*



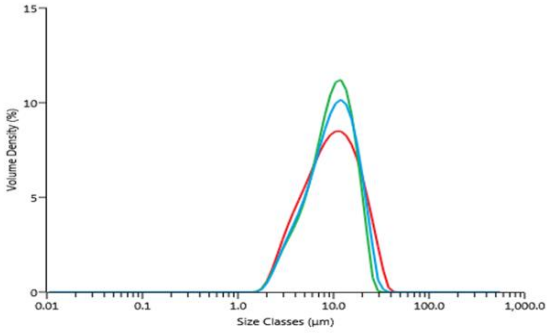
	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	22	Average of 'V1_smpl2'	5,97	18,5	39,5
	33	Average of 'V1_smpl3'	6,76	20,2	43,1
	44	Average of 'V1_smpl1_take2'	6,38	19,4	44,0
Mean			**6,37**	**19,4**	**42,2**
1xStd Dev			**0,394**	**0,886**	**2,38**
1xRSD (%)			**6,18**	**4,57**	**5,64**

Figure 85. *Particle size distribution of PP-glycerol-DMEM-0.1% FBS at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*



	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	25	Average of 'V2_smpl1'	3,80	9,68	23,5
	36	Average of 'V2_smpl2'	3,75	9,54	24,7
	47	Average of 'V2_smpl3'	3,81	9,96	28,3
Mean			**3,79**	**9,73**	**25,5**
1xStd Dev			**0,0326**	**0,216**	**2,50**
1xRSD (%)			**0,861**	**2,22**	**9,81**

Figure 86. *Particle size distribution of PP-glycerol-DMEM-1% FBS at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*



	Record Number	Sample Name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
	33	Average of 'V2_smpl3'	3,75	9,68	21,4
	22	Average of 'V2_smpl2'	4,06	9,74	17,7
	11	Average of 'V2_smpl1'	4,03	9,97	19,3
Mean			**3,95**	**9,80**	**19,5**
1xStd Dev			**0,170**	**0,154**	**1,87**
1xRSD (%)			**4,30**	**1,57**	**9,63**

Figure 87. *Particle size distribution of PP-glycerol-DMEM-10% FBS at a conc. of 5mg/g. The figure represents the mean in particle sizes of three replicate batches.*