Influence of microplastic to bacterial antimicrobial susceptibility and aggregation

Bachelor thesis

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Mikroplasti mõju bakterite antibiootikumitundlikkusele ja agregatsioonile

Bakalaureusetöö

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not previously been presented for grading.

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(Digitally signed)

The paper conforms to requirements in force.

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(Digitally signed)

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Abbreviations

AMR – antimicrobial resistance

CFU – colony forming unit

csv – comma-separated values

ddPCR – droplet digital polymerase chain reaction

DIC – differential interference contrast

DNA – deoxyribonucleic acid

E. coli – *Escherichia coli*

EPS - extracellular polymeric substances

GFP – green fluorescence protein

LB broth – Luria-Bertani broth

MIC – minimum inhibitory concentration

MQ water – Milli-Q water; ultrapure water

OD_{600} – optical density at 600 nm

PBP – penicillin-binding protein

PDMS – poly-[dimethylsiloxane]

PTFE – polytetrafluoroethylene

qPCR – quantitative polymerase chain reaction

RNA – ribonucleic acid

TIF – Tagged Image Format

UV – ultraviolet

Introduction

Water-in-oil droplets are created by dispersing aqueous phase into oil phase. This allows to encapsulate microbial cell into microenvironments, either as single cells or small number of cells. Water-in-oil droplets can be created either by hand or with specialized equipment. In the first case varying sized droplets (polydisperse) are created. While they are easier and quicker to make, they are more difficult to analyse. Specialized equipment for droplet generation consists of microfluidic chips and pump systems. This method creates same-size droplets (monodisperse), which are easier to analyse.

Antibiotics are substances that have antimicrobial mechanisms. There are eight structure classes of antibiotics, which have different effects on bacterial cells. However, bacteria can develop resistance mechanisms to antibiotics, rendering them ineffective against them. Resistance to antibiotics makes bacterial infections harder to treat.

Microplastics are plastic pieces smaller than 5 mm which can enter the environment as primary or secondary microplastics. Primary microplastics are manufactured already as microscopic in size, while secondary microplastics fragment from bigger plastic pieces. Microplastics can offer bacteria a surface to attach to, mediating biofilm and plastisphere (plastic pieces colonized by microbial communities) formation. However, biofilm does not have to be attached to a surface and it can occur as non-surface attached aggregates. In such aggregates bacteria stick together and are therefore more resistant to environmental changes. Biofilm offers bacteria certain protection from antibiotics' harmful effect. While this is not a permanent resistance, it makes it difficult to get rid of bacteria. Plastisphere can harbour different bacterial species and antimicrobial resistance genes, enabling horizontal gene transfer of resistance genes.

The main aim of this thesis is to investigate the influence of microplastic to bacterial viability and autoaggregation in monodisperse droplets. The workflow of these experiments included droplet generation, droplet imaging with confocal microscope and image analysis with two different software. First part of this thesis is an overview of theoretical background of the important aspects discussed in the thesis. This includes droplets, their generation, and applications; antibiotics and resistance against antibiotics; microplastic pollution and effect on the environment; biofilm and plastisphere influence on antibiotic resistance. Second chapter is an explanation of the experiments conducted for this thesis. This includes bulk mixture and antibiotic aliquots preparation, droplet generation and imaging and the analysis with two software. The pipelines necessary for this thesis have been developed by Merili Saar (IAHM 2024) and Karoline Lindpere (LAAB 2024). The last chapter includes the experimental results as well as discussion and conclusions.

1. Literature overview

1.1. Water-in-oil droplets

Water-in-oil droplets are used to encapsulate small number or even single cells into micro- or nanodroplets. This has the advantage of separating microbial cells (for example bacteria, fungi, and algae) into individual microenvironments, which reduces interspecies competition and allows fast accumulation of metabolites. (He et al., 2022)

Water-in-oil droplets offer many advantages over other cell encapsulation techniques. Droplets can be generated at high volumes in a short amount of time making it a high throughput method of single-cell encapsulation. Droplets provide physical protection for the encapsulated cells, protecting the cells for unwanted damage during analysis. This also reduces the risk of cross-contamination. (Ling et al., 2020)

Droplets are created by dispersing aqueous phase into oil phase. Often an emulsifying agent such as surfactant is added. Surfactant consists of amphiphilic molecules and is used to stabilize the water-in-oil droplets and to inhibit the coalescence of droplets as they age. Without surfactant the droplets would be unstable and homogenize. (Shang et al., 2017)

1.1.1. Poly- and monodisperse droplets

Droplets can be created to be different sizes or the same size in a sample. Bulk emulsion is a rather straightforward method creating droplets that vary in size also known as polydisperse droplets. Droplet microfluidics' methods create uniformly sized, monodisperse droplets. This requires a different droplet generation method than bulk mixture. (Kaminski et al., 2016)

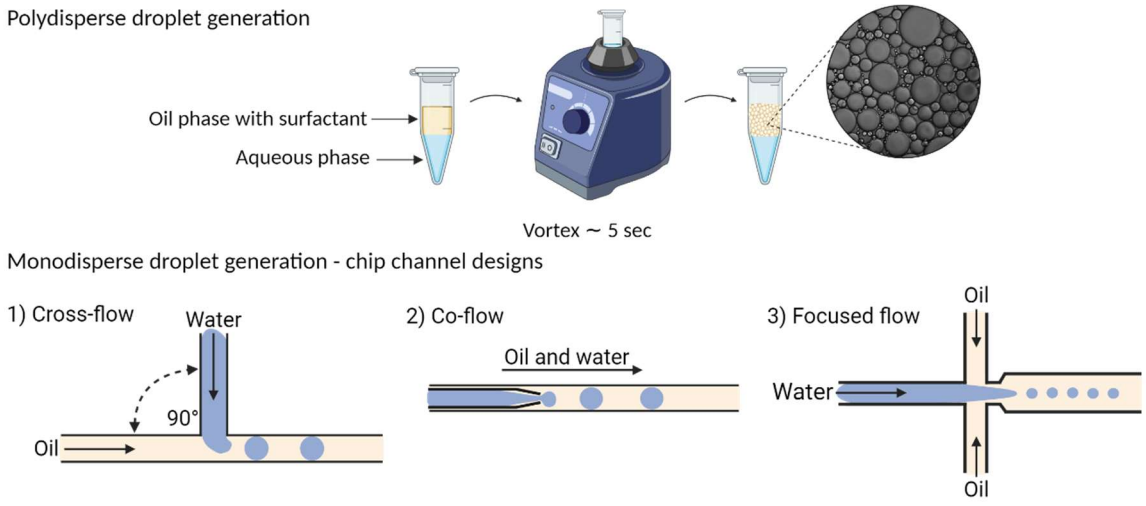

Figure 1 *Droplet generation methods. Upper part of the figure shows polydisperse droplet generation with vortex: oil and water phase are pipetted together into the same tube and vortexed for about 5 seconds which generates droplets that vary in size. Lower part of the figure shows common microfluidic chip channel designs for monodisperse droplet generation: 1) Cross-flow channel type: water and oil channels are usually at a 90˚ angle; however the exact degree can vary by specific channel design. 2) Co-flow channel type: water phase moves in the same direction as oil phase. 3) Focused-flow channel type: water phase flows horizontally, while the oil phase carrying channels flow vertically towards the water's flow. Created with BioRender.com*

Bulk emulsions are created by mixing the two phases (water and oil) together and applying force to break the aqueous phase into micelles and smaller droplets as shown in **Figure 1**. The force can come from shaking by hand or homogenizer device. This method is easy and affordable since it does not require any specialized equipment. However, it creates droplets that vary in size – polydisperse droplets. This lack of uniformity between droplets and certainty of equal distribution of reagents, makes analysing difficult. (Byrnes et al., 2018)

Uniformly sized droplets – monodispersed droplets are created by using microfluidic chips. In the chips there are microchannels where the two immiscible phases meet, and the aqueous phase gets broken up into same-sized droplets. (Kaminski et al., 2016)

Commonly used channel designs include (**Figure 1**):

1) Cross-flow-type (commonly known as T-type): water and oil phases meet at an angle and due to their immiscibility one phase is shaped into droplets inside the other phase (B. Li et al., 2023). This is a simple channel design that is widely used due to its ability to create highly monodisperse droplets up to certain throughput speeds (Ling et al., 2020).
2) Co-flow-type: water and oil phases get aligned parallelly and due to the oil phase's resistance, the water phase is cut off into droplets (B. Li et al., 2023).
3) Focused flow-type: both phases are forced through a small hole where the oil phase elongates and focuses the water phase's flow. This design creates droplets smaller than the microchannel's diameter. (Ling et al., 2020)

Contrary to polydisperse droplet generation, monodisperse droplet generation techniques require specialized equipment (chips, pump systems) and trained laboratory staff, limiting its use in all laboratories (Kaminski et al., 2016). In addition, monodisperse droplet generation is a lot more time consuming. Preparations for the droplet generation include more steps than just pipetting two phases together and the generation time itself depends on the specific flow rates assigned to pumps. However, the generated droplets are easier to analyse due to their uniform size (Byrnes et al., 2018).

1.1.2. Applications of droplets in microbiology

Droplets have several advantages in microbiology over classical methods. The advantages come from isolating the cells into their own microcompartment, ability for high throughput analysis and possibility to automate otherwise complex protocols. These are the main reasons droplet platforms are attractive and used in various fields in microbiology (**Figure 2**). (Kaminski et al., 2016; He et al., 2022)

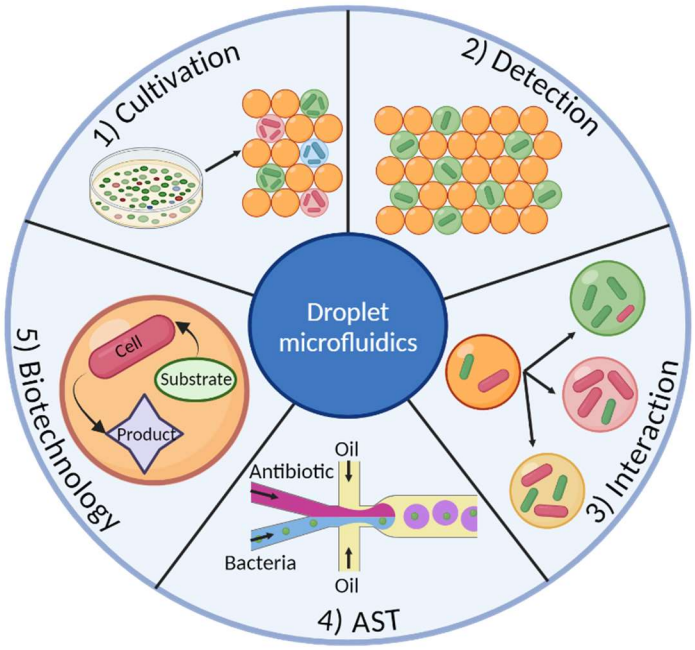

Figure 2 *Applications of droplet microfluidics in various fields of microbiology. 1) Droplet microfluidics is used to cultivate bacteria in their own microenvironments, eliminating interspecies competition. 2) Bacterial growth in droplets can be monitored with microscopes. 3) Droplets are used to study bacterial interactions by encapsulating different species into the same droplet and observing their influence on each other. 4) Chips can be designed to incorporate controlled concentrations of antibiotics to perform antibiotic susceptibility testing (AST) in droplets. 5) Droplets are useful in biotechnology since they require less reagents and have high-throughput applications.*

With traditional microbial culture methods (agar plates or broths), it takes time to see bacterial growth and slow growing microbes can even go undetected, because faster growing species take up all necessary nutrients. With droplet-based methods, interspecies competition is eliminated due to separating cells into their own microenvironments (**Figure 2**(1)). Bacterial growth in droplets can be monitored with brightfield or fluorescence microscopy (**Figure 2**(2)). (He et al., 2022)

Droplets provide a suitable platform to investigate interactions between bacteria (**Figure 2**(3)). Droplet microfluidics has been used to simplify complex microbial environments, to investigate interactions between many different bacterial strains (Jiang et al., 2022). Droplets have also been used to investigate bacteria and phage dynamics in different conditions (Nikolic et al., 2023). Droplet microfluidics has been proven effective for studying gene transfers between bacterial strains, giving an insight, and understanding of bacterial transformation mechanisms (Lam et al., 2019).

Droplets can be used for antibiotic resistance screening (**Figure 2**(4)). Determining the minimum inhibitory concentration (MIC) with traditional microdilution methods is a time-consuming task. Droplet-based systems can be designed to prepare droplets with various controlled concentrations of antibiotics and automated (Postek & Garstecki, 2022). In addition, droplets can be used to investigate antibiotic susceptibility at single cell level. This is useful for determining heteroresistance, where seemingly susceptible bacterial populations contain resistant sub-populations (Scheler et al., 2020).

Droplet microfluidics is an attractive method for not only microbiology but also biotechnology (**Figure 2**(5)). Microfluidic methods require small amounts of reagents, which is especially important when the reagents are expensive or difficult to produce. It combines aspects of flow cytometry and multiwell plate platform – the high-throughput applications of microfluidic methods are well suited for screening experiments and due to the small microenvironment, the cells are in, metabolites accumulate faster, making even small amounts of products detectable (Jain et al., 2024; Ortseifen et al., 2020). These properties have made droplet microfluidics platforms attractive for directed enzyme evolution. Cells can be separated from each other, and all further reactions take place inside the droplet, maintaining the linkage between cell and enzyme (Jain et al., 2024).

Microfluidic methods have a possible application in clinical labs as well. The most promising is droplet digital polymerase chain reaction (ddPCR). It has the advantage of providing absolute quantification and being more sensitive and specific than current methods (quantitative polymerase chain reaction (qPCR)). qPCR provides cycle threshold values where fluorescent signals are measured at certain timepoints, whereas ddPCR divides templates into separate droplets and results come from directly counting positive droplets. (H. Li et al., 2018)

1.2. Antibiotics and antimicrobial resistance in bacteria

Antibiotics are molecules that inhibit the growth of or kill bacteria. They can be produced by other microorganisms or be synthesised. Antibiotics are differentiated as antibacterials, antifungals and antivirals, depending on which microorganism they are effective to, but generally the term is used for antibacterial molecules (Etebu & Arikekpar, 2016).

Antibiotic resistance in bacteria occurs when bacteria have a mechanism which makes them immune to the drug being used against them. Infections by resistant bacteria are typically harder to treat and more prone to coming back. The widespread use and misuse of antibiotics has raised the antibiotic-resistant bacteria prevalence over the last decade (Christaki et al., 2020). Studies analysing antimicrobial resistance genes' abundance in sewage have highlighted low-income countries to be potential spots for new antibiotic resistance mechanisms emergence (**Figure 3**)(Hendriksen et al., 2019; Munk et al., 2022).

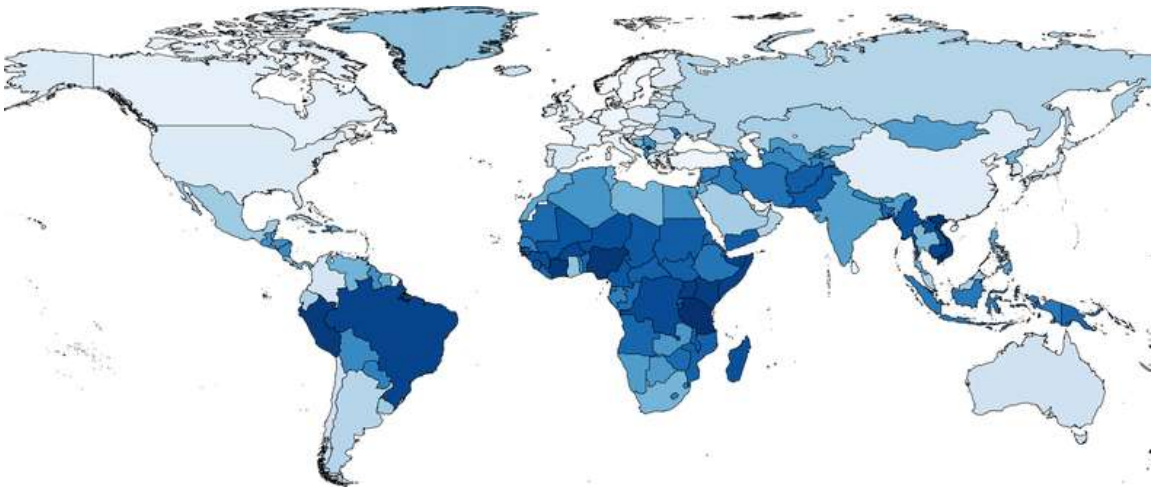

Figure 3 *Global predictions of antimicrobial resistance (AMR) abundance in all countries and territories in the world. Map coloured according to predicted abundance of AMR from light blue*

(low AMR abundance) to dark blue (high AMR abundance). From "Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage", by R. S. Hendriksen et al., 2019, Nature Communications 10(1), p. 6, (https://doi.org/10.1038/s41467-019-08853-3). CC BY 4.0.

There are over 670 000 infections caused by antibiotic-resistant bacteria in the European Union/European Economic Area each year. Out of those, about 33 000 people die because of the infection. Major issue is the resistance to last-line antibiotics like vancomycin and some carbapenems because there are limited treatment options to bacteria that have acquired those resistances. Often infections from such bacteria have fatal outcomes. (WHO Regional Office for Europe/European Centre for Disease Prevention and Control, 2022)

1.2.1. Antibiotics and their mechanisms of action

Antibiotics can be classified according to their molecular structures and mode of action. Molecular structures of antibiotic are the reason their targets or mode of action differ from each other. However, antibiotics in the same structural class usually behave similarly (Etebu & Arikekpar, 2016). Structurally there are eight antibiotics' classes which are divided between three mechanism groups (**Figure 4**)(Halawa et al., 2023):

1) Cell wall synthesis inhibitors:
    a. *β-lactams*: the antimicrobial activity comes from a reactive β-lactam ring. This class has four subgroups based on their side chains or rings: penicillins, cephalosporins, carbapenems and monobactams. β-lactam antibiotics interfere with peptidoglycan layer synthesis that is essential for the bacterial cell wall. (Kim et al., 2023)
    b. *Glycopeptides*: molecules are made of glycosylated cyclic non-ribosomal peptides. Glycopeptides inhibit the synthesis of bacterial cell wall by blocking the cross-linking of peptidoglycan. (Pootoolal et al., 2001)
2) Nucleic acid synthesis inhibitors:
    a. *Sulphonamides*: the centre of sulphonamide antibiotic is a sulphur atom bonded with double bonded oxygens, substituted amine group and an aniline group. This group of antibiotics inhibit deoxyribonucleic acid (DNA) replication by disrupting the synthesis of folic acid or tetrahydrofolate. (Ovung & Bhattacharyya, 2021)
    b. *Quinolones*: molecules have a bicyclic core structure. Almost all quinolone antibiotics are fluoroquinolones, where there is a fluorine atom in C6 position. Quinolones are distributed into generations based on their antibacterial spectrums. Quinolones interfere with DNA replication by targeting enzymes essential for this process. (Aldred et al., 2014)
3) Protein synthesis inhibitors:
    a. *Macrolides*: molecules have a 12- to 16-membered macrocyclic lactone ring as a core. Macrolides target bacterial ribosomes inhibiting protein synthesis. (Vázquez-Laslop & Mankin, 2018)
    b. *Oxazolidinones*: molecule includes a 2-oxazolidine in their structure. They inhibit protein synthesis at the ribosomal 50S subunit. (Pandit et al., 2012)
    c. *Aminoglycosides*: molecule is made of 3 aminocyclitol 2-deoxystreptamine rings connected to an amino sugar with amino and hydroxyl substitutions and a

streptidine ring. These antibiotics bind to ribosomal 30S subunits and inhibit the protein synthesis. (Serio et al., 2018)

d. *Tetracyclines*: molecules have a naphthacene core made of four aromatic rings. Tetracyclines are subgrouped into generations based on the method of synthesis. This class of antibiotics target the bacterial ribosome and disrupt protein synthesis by interfering with transfer ribonucleic acid (RNA). (Nguyen et al., 2014)

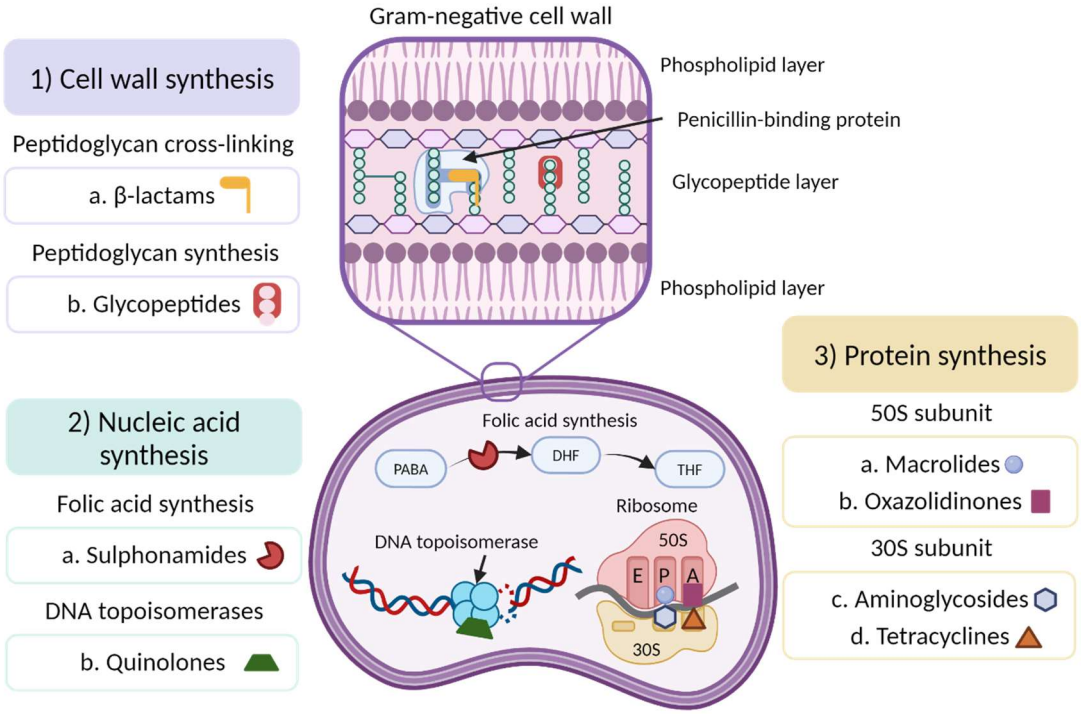

Figure 4 *Antibiotics and their mechanisms of action. Antibiotics are divided into three groups, based on their effect on the bacterial cell. Antibiotic classes inhibiting cell wall synthesis (1) are β-lactam and glycopeptide antibiotics; their exact binding sites inside the cell wall are marked yellow and red icons respectively. Nucleic acid synthesis inhibitors (2) are sulphonamides and quinolones; first disrupts the folic acid synthesis (red three-quarter circle), the other binds to DNA topoisomerase (green trapezoid). Protein synthesis (3) can be inhibited by binding to either 50S or 30S subunit. Antibiotics binding to 50S subunit are macrolides (light purple circle) and oxazolidinones (purple square). Antibiotics binding to 30S subunit are aminoglycosides (blue hexagon) and tetracyclines (orange triangle). Macrolides and aminoglycosides bind to ribosome's P-site, while oxazolidinones and tetracyclines bind to the A-site. The numbers and letters used in the figure correspond to the preceding list of antibiotic mechanisms and structures. Created with BioRender.com*

In this thesis, two different antibiotics were used: cefotaxime and kanamycin. Cefotaxime inhibits bacterial cell wall synthesis. It is a third-generation cephalosporin belonging into β-lactam antibiotics group. Third generation cephalosporins have a higher stability to degrading enzymes, making them effective against most Gram-negative bacteria. In their molecular structure, cephalosporins have a 6-membered sulphur containing ring fused to the β-lactam ring, which makes the molecule more resistant to β-lactam ring degradation. (Lima et al., 2020)

Kanamycin inhibits protein synthesis. It is an aminoglycoside antibiotic most effective on aerobic Gram-negative bacteria. It binds to the 30S ribosomal subunit causing misreading of messenger RNA during translation which produces nonsense proteins. Kanamycin possesses two amino sugars, which are glycosidically linked with central hexose. (Serio et al., 2018)

1.2.2. Antimicrobial resistance mechanisms in bacteria

Resistance to antibiotics in bacteria can be achieved by modifying either the antibiotic itself or the target in the cell or reducing antibiotic accumulation in the cell (**Figure 5**) (Christaki et al., 2020).

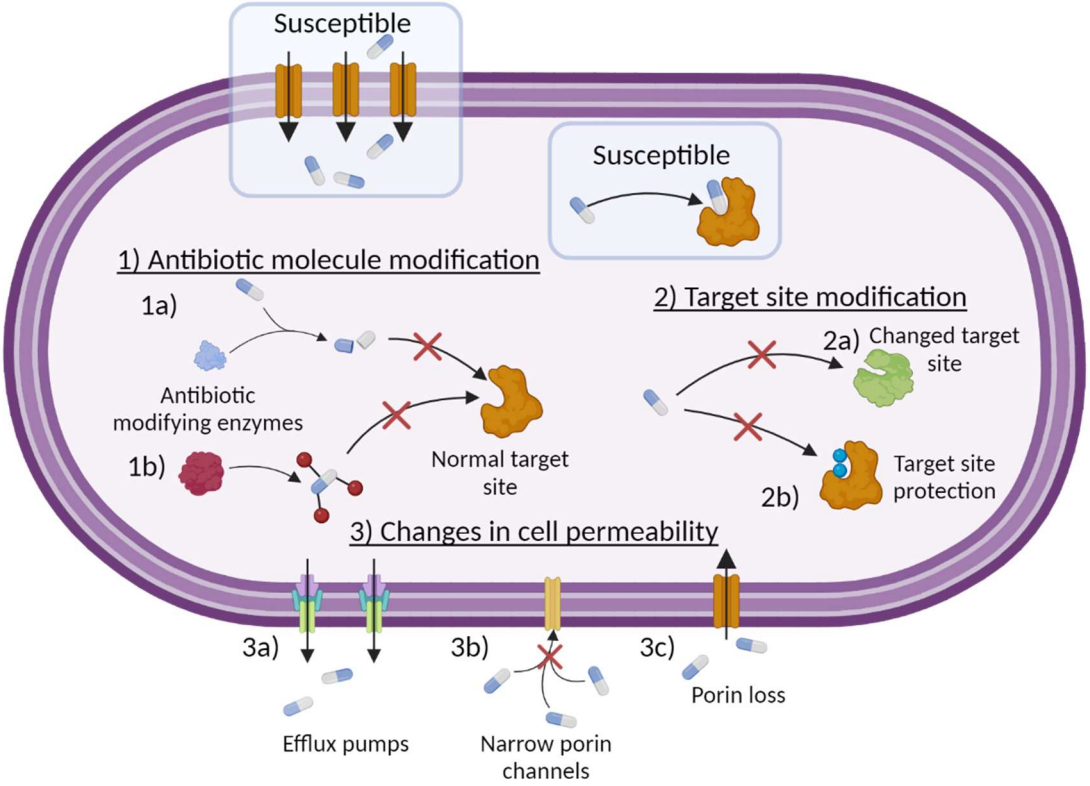

Figure 5 *Resistance mechanisms in bacteria. In light blue boxes are examples of susceptible bacteria's mechanisms: right box shows intact antibiotic molecule binding to a normal protein (orange); left shows three normal porin channels (orange) transporting antibiotic inside the cell. 1) Antibiotic molecule modification mechanisms: 1a) An extra bacterial enzyme (blue) breaks the antibiotic molecule down, preventing it for being able to bind to its target protein. 1b) An extra bacterial enzyme (red) modifies the antibiotic structure, so it is no longer able to bind to its target protein. 2) Target site modification mechanisms: 2a) The target protein has been modified (green), so the antibiotic can no longer bind to it. 2b) The target protein is protected by extra bacterial proteins (blue), that do not allow the antibiotic to bind. 3) Changes in cell permeability: 3a) Overactive efflux pumps pump the antibiotic out of the cell. 3b) Porin channel mutation caused it to become narrower (yellow), so the antibiotic molecule does not fit through. 3c) Bacterium has encountered porin loss, where there are less porin channel than normal, making the antibiotic concentration in the cell smaller. Created with BioRender.com*

The antibiotic molecule could get destroyed by specific enzymes (**Figure 5**(1)). This happens in β-lactam resistant bacteria, where the bacteria produce β-lactamase enzymes that destroy the amine bond in the β-lactam ring, making the antibiotic lose its antimicrobial activity (Christaki et al., 2020). Antibiotic molecules can also be subjected to different bacterial transferases, that modify the antibiotic molecule's structure, making them less active or less likely to bond to their targets due to the changed shape (G. D. Wright, 2005). This mechanism is seen in aminoglycoside resistance, where aminoglycoside molecule gets either acetylated, phosphorylated, or adenylated (Ramirez & Tolmasky, 2010).

Another way for bacterial cell to protect themselves from antibiotics, is to modify the antibiotic's targets (**Figure 5**(2)). This can mean replacing the target site with a different structure. For β-lactam antibiotics, the target in the bacterial cell is the penicillin-binding protein (PBP). Some bacteria replace the normal PBP with PBP2a, which does not bind β-lactam antibiotics well due to its changed structure. (Lambert, 2005). Target modification also includes target site alterations. Chromosomal mutations in bacterial gyrase and topoisomerase IV genes mediate quinolone resistance. There can also be no change to the target's structure itself and instead bacteria can encode proteins to block the target sites from the antibiotic binding there (Christaki et al., 2020).

Third way to counteract the antibiotics, is to not let the molecules even enter the cell. This is mediated by outer membrane permeability (**Figure 5**(3)). Gram-negative bacteria have the advantage of two membranes, making it difficult for hydrophilic antibiotics to permeate the cell (Kumar & Schweizer, 2005). Hydrophilic antibiotics enter the cells mainly by porin channels. Resistance by porin mutation is achieved by changes in the porin channel size or by porin loss. Some bacteria can pump antibiotic molecules out of the cell before they can do any damage. This is done by efflux pumps on the cytoplasmic membrane (Christaki et al., 2020).

1.2.3. Acquiring antimicrobial resistance

Antibiotic resistance in bacteria can be intrinsic, acquired, or adaptive (Christaki et al., 2020). Intrinsic resistance does not occur because of the selective pressure of the antibiotic but due to additional genes that contribute towards the resistance. Intrinsic resistance includes mechanisms that mediate outer membrane permeability discussed in the previous chapter. While low membrane permeability and porin mutations have a clear effect on the overall antimicrobial resistance, it is not substantial. However, efflux pumps have been shown to cause multi-drug resistance in Gram-negative bacteria and are the reason why *Pseudomonas aeruginosa* is resistant to multiple antibiotics (Cox & Wright, 2013).

Adaptive resistance is a temporary survival mechanism caused by environmental stimuli. It is a result of gene expression modulation that causes phenotypic changes when in the hostile environment. Bacteria with adaptive resistance has been shown to become susceptible again after the antibiotic has been removed from the environment. Adaptive resistance is also exhibited in biofilms. (Salimiyan Rizi et al., 2018)

Acquired resistance occurs when previously susceptible bacteria become resistant by either a mutation or exchange of genetic material (Christaki et al., 2020). This is further influenced by antibiotics by applying a selective pressure to gain resistance. The exchange of genetic material occurs through horizontal gene transfer. Its mechanisms are transformation, transduction, and conjugation (**Figure 6**) (Holmes et al., 2016). Transformation happens when free DNA fragments from dead cells enter other bacteria and are incorporated into its chromosome. Transduction is a transfer of genetic material by bacteriophage. Conjugation happens between two bacterial cells during physical contact where a plasmid is transferred between the bacteria by sex pilus (Christaki et al., 2020).

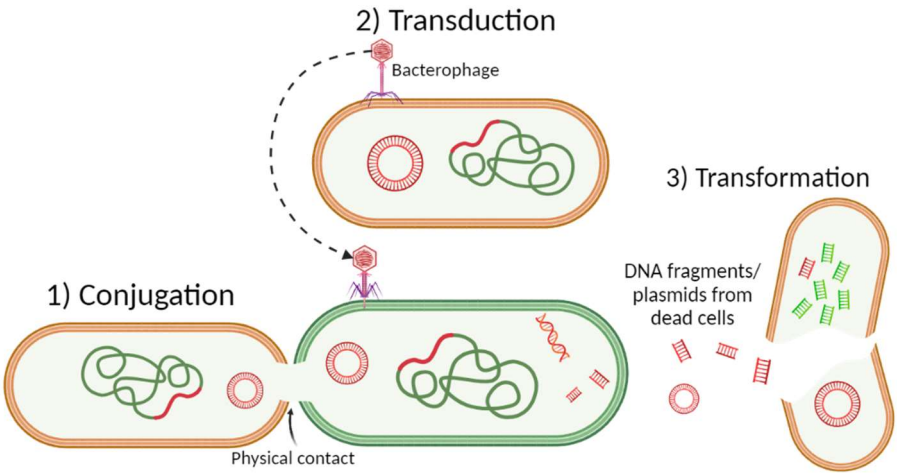

Figure 6 *Horizontal gene transfer mechanisms. Green marks susceptible bacteria and normal DNA. Orange marks resistant bacteria, red marks resistance genes. 1) Conjugation: resistance genes get transferred during a physical contact between two live bacteria. 2) Transduction: resistance genes get transferred between bacteria by bacteriophages. 3) Transformation: DNA fragments or plasmids from dead cells can get incorporated to a susceptible bacteria's chromosome. Created with BioRender.com*

1.3. Plastic pollution

Plastics are organic synthetic polymers, which are used widely due to their durability, inexpensiveness and lightweight. Approximately 9.2 billion tonnes of plastics have been produced since 1950s, when use of plastic started to grow rapidly, and more than half of it has been produced after 2004. (United Nations Environment Programme, 2021)

The main leakage of plastics to the environment usually occurs after the use of the plastics and during disposal. However, plastics escape into the environment in form of plastic pellets or fibres at every stage of their life cycle – production, transport, conversion (United Nations Environment Programme, 2021). It is estimated that 79% of plastic wastes so far is in landfills, 12% gets burned and only 9% recycled (Ramasamy & Harit, 2023).

Plastic waste can get fragmented into smaller pieces by ultraviolet (UV) radiation, biological or physical means – becoming microplastics when their size becomes less than 5 millimetres and nanoplastics when their size becomes less than 1 micrometre (Ramasamy & Harit, 2023).

1.3.1. Micro- and nanoplastic sources

Microplastics are small pieces of plastic that measure under 5 millimetres and nanoplastics measure under 1 micrometre (Ramasamy & Harit, 2023). The fragment sizes are shown in **Figure 7**. Microplastics in the environment are classified as primary and secondary (Ramasamy & Harit, 2023).

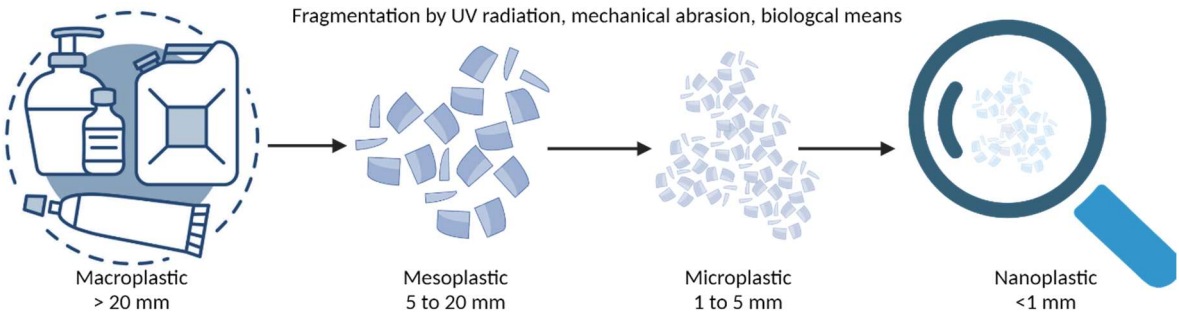

Figure 7 *Plastic fragmentation and the fragments' sizes. The biggest size of plastic fragments is called macroplastics. Due to UV radiation, mechanical abrasion or biological means plastic get fragmented down to meso-, micro- and nanoplastic sizes. Created with BioRender.com*

Primary microplastics are already manufactured as microscopic particles in shape of pellets or beads. Pellets are used as raw materials in the plastic industry. Beads are found in detergents and hygiene products as exfoliating materials like soaps, cleansers, and toothpaste (Ramasamy & Harit, 2023). Primary microplastics such as pellets usually find their way into the environment due to accidental spills or during handling and transportation. Microbeads however find their way into the sewage system after using products that contain them (United Nations Environment Programme, 2021). Despite the many applications, primary microplastics make up only a fraction of the global microplastic pollution. The main concern is secondary microplastics (Ramasamy & Harit, 2023).

Secondary microplastics originate from larger plastic pieces. Upon reaching the environment, the plastic begins to slowly degrade and fragment into smaller pieces – eventually reaching microscopic sizes. The breakdown takes place due to UV radiation (from sunlight) and mechanical action from wind or waves (Ramasamy & Harit, 2023).

Secondary microplastics enter the environment mainly after being discarded but also during use of the plastic (Ramasamy & Harit, 2023). During use, microplastics are released from tyres and road marking due to the mutual abrasion. Another source is synthetic clothing which releases microfibers into air during normal wear but also into the wastewater during washing (United Nations Environment Programme, 2021). After use, the largest secondary microplastic sources are landfills, where if plastic is not properly stored then the degrading plastic fragments can be carried away by wind (Ramasamy & Harit, 2023).

1.3.2. Microplastics in environment

Microplastics have been found all over the environment from bodies of water to air to even human placenta. The origin of microplastics vary, therefore there is a mix of several polymers in the environment. Particles can have different sizes, shapes, densities, and additives, which influence their effect in the environment (Koelmans et al., 2022). The different microplastic sources in the environment and their pathways are also shown in **Figure 8**.

Largest contributor to soil contamination is sewage sludge, collected from both domestic and industrial sources (Surendran et al., 2023). Even though wastewater gets thoroughly treated, a large volume of microplastics is still released into the environment (Ramasamy & Harit, 2023). The soil contamination can lead to surface and ground water contamination, which means microplastic particles can find their way into drinking water. Microplastics in the soil get spread horizontally and

vertically by soil biota, plant root activity, digging mammals but also by different agricultural practices (Surendran et al., 2023).

Around 80% of microplastics found in oceans are from land-based sources. This is largely due to transportation of microplastics by rainfall and wind (Ramasamy & Harit, 2023). Microplastics in the marine space get easily incorporated into the food chain: first being eaten by invertebrates and moving up the food chain to higher trophic organisms (S. L. Wright et al., 2013). Ingestion of microplastics can cause starvation and death or growth inhibition to smaller aquatic organisms (Xu et al., 2020).

Airborne microplastic particles originate from many different sources. Microplastic has been found in dust and snow, but air is also a good carrier of the particles (O'Brien et al., 2023). The small microplastics are easily inhaled by humans and other animals which can lead to chronic inflammation in the lungs (Prata et al., 2020).

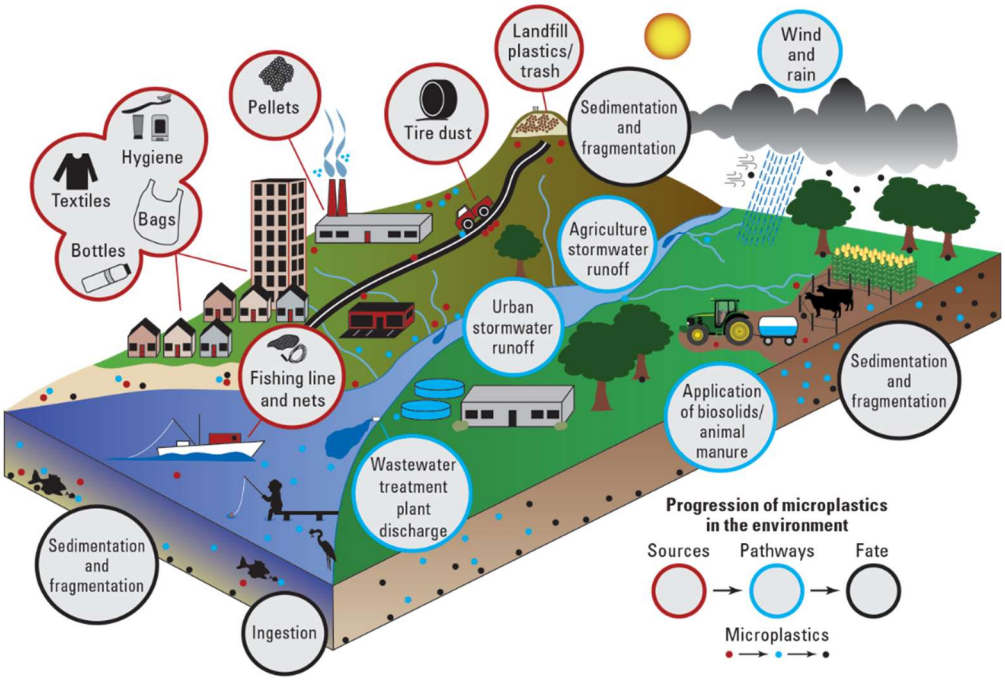

Figure 8 *Microplastic sources, pathways, and fate conceptual diagram. Microplastic sources are circled in red; microplastic pathways, which contribute to microplastic spread in environment are circled in blue; microplastic fates in the environment are circled in black. From Microplastic sources, pathways, and fate conceptual diagram, by United States Geological Survey, 2023 (https://www.usgs.gov/media/images/microplastics-sources-pathways-and-fate-conceptual-diagram). In the public domain.*

Humans are exposed to microplastics largely by ingestion. There are several food items, where an abundance of microplastics have been found. Aquatic food products present the widest range of microplastic particles. However, the contaminated products include also various beverages such as soft drinks and milk. In addition, microplastics have been found in different fruits and vegetables. (Jin et al., 2021)

The density of the microplastic particles derives directly from the type of polymer it is made from but also the degree of ageing and weathering they have been exposed to in the environment (Koelmans et al., 2022). The density determines the particles' bioavailability in aquatic

environment. Lighter particles float and are prone to get carried away by wind or eaten by epipelagic fish. High density particles sink and while they are not spread by wind, this makes them available for consumption at all depths of the water column (S. L. Wright et al., 2013).

Plastics contain several additives such as stabilizers, fillers, plasticizers, colourants, flame retardants and curing agent, some of which are harmful for the environment (United Nations Environment Programme, 2021). Microplastics can distribute them in the environment, which can put the biota at risk. Therefore, microplastics do not only pose a risk as a physical particle, but they also have a chemical effect (Koelmans et al., 2022).

1.4. Biofilm and plastisphere

Bacteria can have two life phases: uni- or multicellular. Former includes planktonic bacteria, latter sessile bacteria forming biofilm (Berlanga & Guerrero, 2016). Biofilm is a complex structure of bacterial cells and extracellular polymeric substances (EPS) that keep the cells together. Bacteria in biofilms are more resistant to antibiotics and environment changes than single motile cells, therefore causing more harm in bacterial infections in humans and increasing the risk of contamination in hospitals (Vani et al., 2023). While bacterial biofilms are commonly described as surface attached colonies, not all biofilms need to be attached to a surface. They have also been observed to be non-surface attached aggregates. These biofilm forms are most seen in clinical and industrial settings but also in the environment (Sauer et al., 2022).

Plastisphere is a term describing the ecosystem that forms by bacterial colonization and biofilm formation on microplastics. The formation of biofilm can alter microplastic properties and in turn affect their behaviour in the environment. Furthermore, biofilm covered microplastics have a much higher sorption capacity for contaminants than clean microplastics (Liu et al., 2023). Contaminants can include antibiotics and heavy metals, which can change the microbial species distribution. The plastisphere creates a habitat that further promotes bacterial adhesion to the microplastics and therefore biofilm formation (Bartkova et al., 2021). The dense biofilm on the surface of microplastics also decreases the fragmentation and degradation rates of microplastic by protecting it from different chemical and physical processes like UV exposure and mechanical abrasion (Liu et al., 2023).

1.4.1. Biofilm and plastisphere formation

As microplastics adsorb contaminants, they can also adsorb nutrients and organic matter, which makes them ideal for bacterial colonisation and biofilm formation (Bartkova et al., 2021). Biofilm formation is a rapid but complex and controlled process employing specific genes, that regulate adhesion, chemotaxis, communication and matrix and fluid channels formation between the cells. (Dey et al., 2022)

Surface attached biofilm formation is often described in five general steps (**Figure 9**):

1) Reversible attachment: single planktonic bacterial cells make contact with a surface (Sauer et al., 2022).

2) Irreversible attachment: bacteria change their orientation to lay flat on the surface, reduce their flagella gene expression and start producing EPS (Rather et al., 2021; Sauer et al., 2022).
3) Biofilm maturation I: several cells thick clusters appear in the biofilm matrix made up of EPS (Sauer et al., 2022).
4) Biofilm maturation II: fully matured microcolonies appear; biofilm can acquire a shape structure where bacteria are arranged based on their metabolic needs and motility (Rather et al., 2021; Sauer et al., 2022).
5) Dispersion: matrix components degrade, releasing dispersed motile cells. (Sauer et al., 2022)

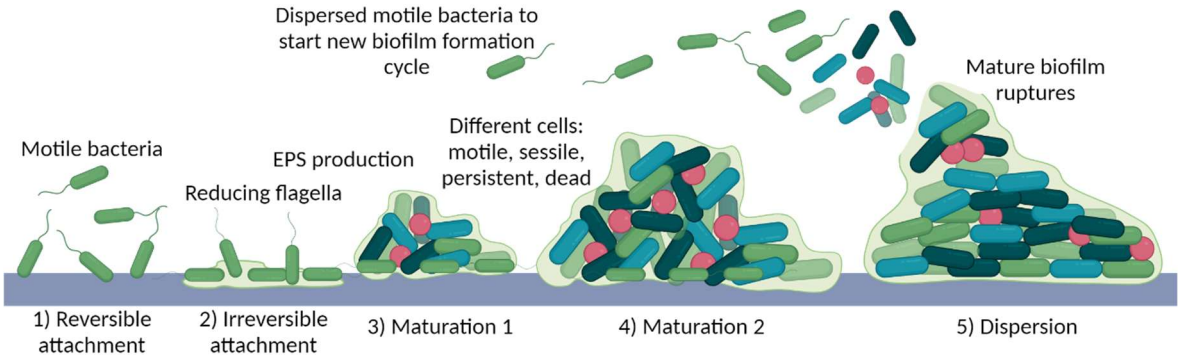

Figure 9 *Biofilm formation. For biofilm to be able to form, first (1) motile cells need to make contact with a surface, it is called reversible attachment. (2) When the attached bacteria start reducing their flagella and producing EPS, attachment becomes irreversible and (3) biofilm formation and maturation starts. (4) Biofilm can harbour different cells which can be motile, sessile, persistent, or dead. (5) When mature biofilm ruptures motile bacteria get dispersed and can start new biofilm. Created with BioRender.com*

This model, however, does not account for non-surface attached aggregates. Several different mechanisms can describe non-surface attached biofilm formation (**Figure 10**). For example, pieces of attached biofilm could get detached and take the form of non-surface attached aggregates – this process is referred to as sloughing. Another way takes place during cell division, where instead of dispersion of daughter cells, they stay with the mother cells by either adhesion molecules or EPS production. Also, adhesins could also be the reason planktonic cells stick together, which leads to formation of aggregates. In addition, host polymers can mediate aggregate formation. (Sauer et al., 2022).

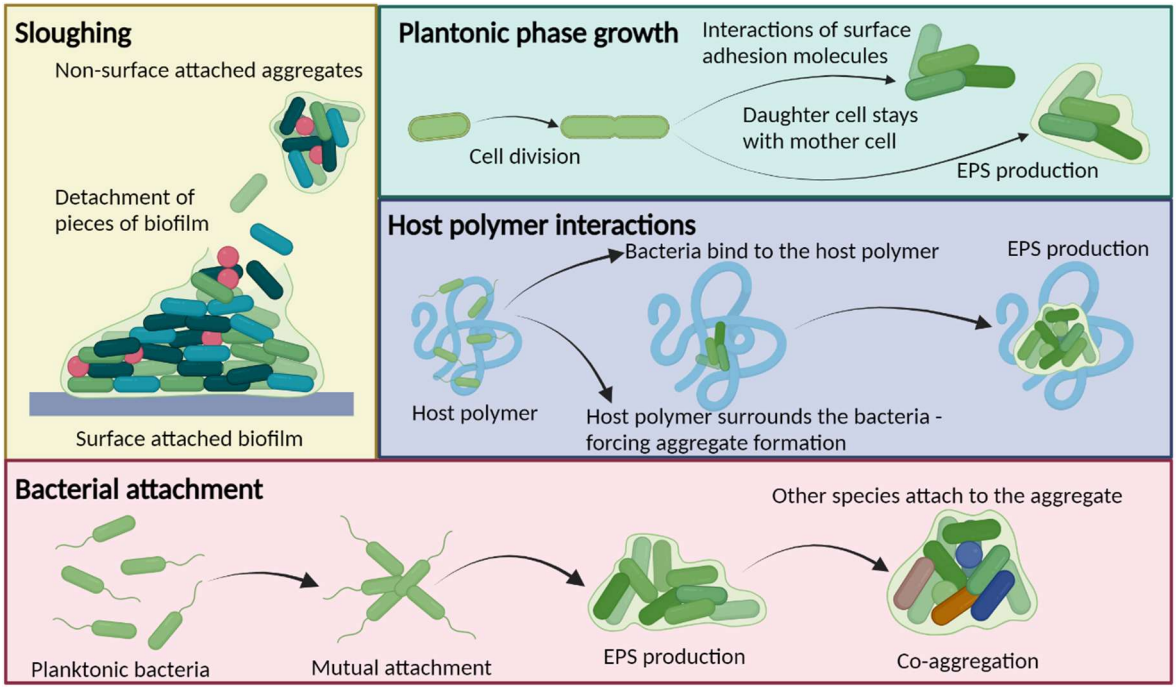

Figure 10 *Non-surface attached aggregates formation. Non-surface attached biofilm can form through sloughing, where pieces of mature surface attached biofilm become detached. Aggregates can form when daughter cells stay with mother cell after cell division; the attachment can be mediated through surface adhesion molecules or EPS production. Bacteria can interact with host polymers and create aggregates by either the host polymer surrounding them and forcing aggregate formation or by binding to the polymer. Motile bacteria can also form aggregates by mutual attachment. When aggregates are formed by more than one species of bacteria it is called co-aggregation. Created with BioRender.com*

1.4.2. Biofilm and plastisphere associated antibiotic resistance

It is important to understand, that biofilm itself does not give bacteria prolonged antibiotic resistance. When dispersed from resistant biofilm, bacteria restore their sensitivity to antibiotics, suggesting this is an adaptive resistance. However, biofilms and plastispheres are a good environment for spontaneous mutations and antibiotic resistance genes transfers. (Shree et al., 2023)

The antibiotic resistance of biofilm is related to the structure of the biofilm (Rather et al., 2021). EPS surrounding the bacteria can slow the antibiotic down from completely penetrating the bacterial cell. Also, outer zone of slow or non-growing bacteria can protect the inner live cells from antibiotic effects (Shree et al., 2023). The bacteria in biofilm can express different adaptive stress responses which includes differentiating into persister cells, which neither grow normally nor die when exposed to antibiotics (Rather et al., 2021; Shree et al., 2023). In addition to antibiotic resistance, biofilm is more resistant to disinfectants (Rather et al., 2021).

Biofilm is a reservoir of different antibiotic resistance genes and microplastics are capable of adsorbing these (Rather et al., 2021; Yu et al., 2022). Microplastics get contaminated when resistant bacteria carrying antibiotic resistance genes attach to the microplastic's surface. As the different bacterial cells accumulate on the microplastic, antibiotic resistance genes get transported by plasmids, prophages, or transposons to susceptible cells during cell-to-cell contact or DNA transfer

(Yu et al., 2022). Studies have shown an increased horizontal gene transfer via conjugation in the plastisphere. In fact, bacteria in plastisphere are more efficient in horizontal gene transfer comparison to single planktonic bacteria. (Marathe & Bank, 2022)

The contamination of microplastics by antibiotics and heavy metals lead to active selection of antibiotic resistance. Exposure to low levels of antibiotics do not only select for antibiotic resistance but can also induce horizontal gene transfer of antibiotic resistant genes. (Marathe & Bank, 2022)

2. Aims of the study

The main aim of this thesis was to investigate the influence of microplastic on bacterial antimicrobial susceptibility and autoaggregation in monodisperse droplets.

The specific objectives of this thesis were:

1) to find out if microplastic influences antimicrobial susceptibility in monodisperse droplets.
2) to see if the viability experiments are reproducible.
3) to determine if microplastic influences bacterial autoaggregation in monodisperse droplets.
4) to investigate the connection between bacterial susceptibility and autoaggregation.

3. Materials and methods

Current thesis is a part of a bigger experimental ecosystem in the Microfluidics group. The pipeline, used for analysing the experiments carried out for this thesis, has been developed by Merili Saar and Karoline Lindpere, who are master's and bachelor's students (respectively) in the group. They used images from two previous projects to develop and test their pipelines. The connection between theses is shown in **Figure 11**.

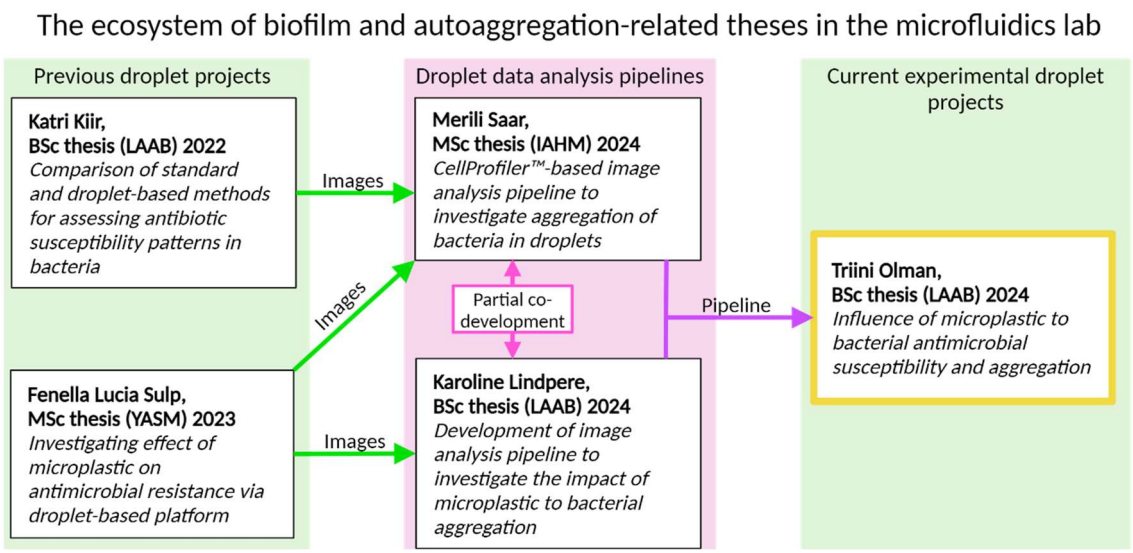

Figure 11 *The ecosystem of biofilm and autoaggregation related theses in the microfluidics lab. Current thesis and its position is marked with a yellow border. Created with BioRender.com*

The experiments for this thesis consisted of two parts (**Figure 12**). Experimental part that included (1) sample preparation, (2) droplet generation, (3) overnight incubation and (4) imaging with confocal microscope. Followed by analysis, first with (5) Ilastik to detect microplastic in droplets and then with (6) CellProfiler™ to identify droplets and measure texture and intensity. Results were combined in comma-separated values (csv) file and analysed in (7) Microsoft Excel.

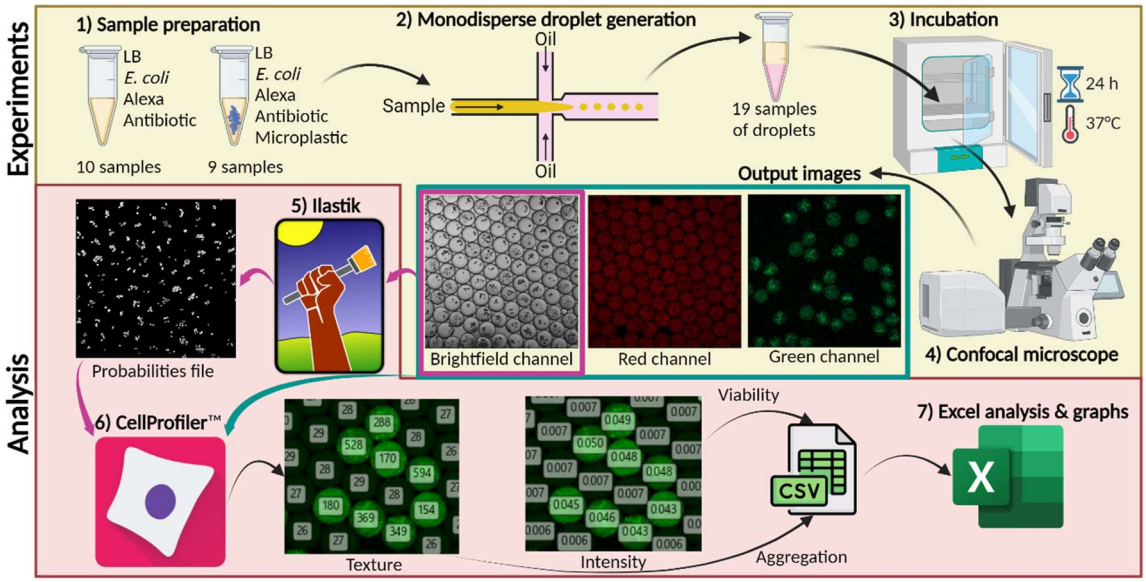

Figure 12 *Experimental pipeline of the thesis. Experiment part consisted of preparing droplet mixtures, generating droplets, incubating them, and imaging using confocal microscope. Analysis*

started with Ilastik program for microbeads detection from brightfield images. A specialised CellProfiler™ pipeline was used to measure bacterial texture and green fluorescent protein (GFP) intensity. The results were combined into a csv file and analysed as aggregation and viability, respectively. Created with BioRender.com

3.1. Monodisperse droplets generation

3.1.1. Bulk mixture preparation

Materials and equipment used to prepare bulk mixture:

1) Tube, 50 ml, pp, 30/115 mm, conical bottom, Cellstar®, blue screw cap, natural, graduated, writing area, sterile (Greiner Bio-One, Austria)
2) Microcentrifuge tube PP, 5ml, attached cap, transparent, grad., writ. area, np pcr ready, (Nerbe plus, Germany)
3) Inoculation loop, 1 µl, PS, white, sterile (Sarstedt, Germany)
4) Luria-Bertani (LB) broth (Biomaxima, Poland)
5) *Escherichia coli (E. coli)* JEK 1036 with chromosome-incorporated gene encoding the green fluorescence protein (GFP)
6) Dextran, Alexa Fluor™ 647, 10,000 MW, anionic, fixable (Invitrogen, Life Technologies Corporation), stock concentration 1mg/ml, diluted to 100 µg/ml
7) Microplastic beads: Invitrogen™ Polystyrene CML Latex Beads (Microspheres), 4% w/v, 10 µm (Thermo Fisher Scientific, USA)
8) Milli-Q (MQ) water
9) BioSpec-Mini Spectrophotometer (Shimadzu, Japan)
10) Test-tube shakers Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
11) Orbital shaker incubator ES-20 (Biosan, Latvia)
12) 20 – 200 µl ErgoOne® Single-Channel Pipette (Starlab, Germany)
13) 100 – 1000 µl ErgoOne® Single-Channel Pipette (Starlab, Germany)
14) 2 - 20µl ErgoOne® Single-Channel Pipette (Standard Cone) (Starlab, Germany)

For bulk mixture preparation *E. coli* JEK 1036 was inoculated in 50 ml Falcon tubes in 5 ml LB broth overnight in the orbital shaker incubator with the speed of 70 rpm at 37˚C. The bacterial growth was measured in optical density at 600 nm (OD_{600}) with the spectrophotometer and converted into colony forming units (CFU) using the conversion: $OD_{600}\ of\ 1.0 = 8 \times 10^8\ CFU$. The growth was then diluted to 3.5×10^7CFU.

Two different bulk mixtures were prepared – one with microplastic beads and one without. Both mixtures were prepared in 5 ml microcentrifuge tubes and contained LB broth, Alexa dye (final concentration in mixture 2 µg/ml) and *E. coli* JEK 1036 (final concentration in mixture 3.5×10^5CFU). For microplastic bulk mixture 4% microplastic beads (final concentration in mixture 0.4%) were added. For the other mixture equal volume of MQ water was added instead. Final volume of each mixture was 2 ml. All components were thoroughly vortexed and then pipetted together with appropriate automatic pipettes.

3.1.2. Antibiotic aliquots preparation

Materials and equipment used for antibiotic aliquots preparation:

1) ClearLine PP microtube, 1.5 ml, conical bottom, snap cap, non-sterile (Biosigma, Italy)
2) MQ water
3) Kanamycin Sulfate (White Powder), Fisher BioReagents, stock concentration 5 mg/ml (Thermo Fisher Scientific, USA)
4) Cefotaxime Sodium Salt, stock concentration 10 mg/mL (Carbosynth Limited)
5) Test-tube shakers Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
6) 20 – 200 µl ErgoOne® Single-Channel Pipette (Starlab, Germany)
7) 100 – 1000 µl ErgoOne® Single-Channel Pipette (Starlab, Germany)
8) 2 - 20µl ErgoOne® Single-Channel Pipette (Standard Cone) (Starlab, Germany)

Both antibiotics were diluted with MQ water to get nine different concentrations. For cefotaxime, the concentrations were 0.08; 0.1; 0.13; 0.18; 0.24; 0.32; 0.42; 0.56 and 0.75 µg/ml. For kanamycin, the concentrations were 0.5; 0.84; 1.4; 2.33; 3.88; 6.48; 10.8; 18.0 and 30.0 µg/ml. These concentrations were chosen based on previous experiments using 96-well plates and droplets in the Microfluidics lab.

The exact volumes of the antibiotic and MQ water were taken with appropriate automatic pipettes. The aliquots were preserved in 1.5 ml tubes in a freezer at -20˚C in between the experiments. Before the experiment, the aliquots were taken out to room temperature.

3.1.3. Droplet generation

Materials and equipment used for monodisperse droplet generation in addition to bulk mixtures and antibiotic aliquots:

1) Polytetrafluoroethylene (PTFE) tubing, inner diameter 0.5 mm, S1810-08 (Bola, Germany)
2) PTFE tubing, inner diameter 0.8 mm, S1815-04 (Bola, Germany)
3) ClearLine PP microtube, 1.5 ml, conical bottom, snap cap, non-sterile (Biosigma, Italy)
4) Microcentrifuge tube PP, 5ml, attached cap, transparent, grad., writ. area, np pcr ready, (Nerbe plus, Germany)
5) PDMS (poly-[dimethylsiloxane]) microfluidic chip (preparation is described in Appendix 1: PDMS chip preparation)
6) Novec HFE 7500 fluorocarbon oil with 2% concentration of perfluoropolyether (PFPE)−poly(ethylene glycol) (PEG)−PFPE triblock surfactant, synthesized according to protocol Holtze, et al., 2008, (obtained as a gift from Prof. Garstecki, ICHF PAN, Poland)
7) Novec HFE 7500 fluorocarbon oil (3M, USA)
8) Base 120 base module with two Nemesys S syringe pumps controlled by CETONI Elements software (CETONI, Germany)
9) 2.5 mL Gastight Syringe Model 1002 RN, Large Removable Needle, 22 gauge, 2 in, point style 2 (Hamliton, USA)
10) Olympus SZ61 stereo microscope (Evident, Japan)
11) INCU-Line® IL 23 digital incubator (VWR, UK)

Samples were prepared by pipetting together 180 μl of bulk mixture and 20 μl of antibiotic aliquot in 1.5 ml Eppendorf tube. This produced 19 samples – nine concentrations with microplastic beads, nine concentrations without microplastic beads and one control sample without microplastic beads or antibiotic (antibiotic was replaced by same volume of MQ water). The final antibiotic concentrations in droplets were 10x smaller than the aliquots.

Appropriate sizes of PTFE tubing were cut and connected with the microfluidic chip and syringes as shown in **Figure 13**. The pump system was controlled with corresponding software. The flow rate for just HFE 7500 oil was 120 μl /min and for HFE 7500 oil and 2% surfactant mixture was 170 μl/min. The generation of droplets in the microfluidic chip was monitored under the microscope. Droplets were collected into 5 ml microcentrifuge tubes and put into the incubator overnight at 37˚C.

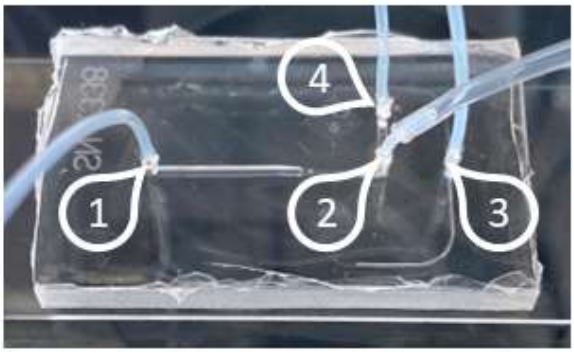

Figure 13 *PDMS microfluidic chip with tubes. 1 – sample inlet, connected to the oil syringe; 2 – droplet outlet, collecting droplets into a 5 ml tube; 3 – oil and surfactant mixture inlet, connected to the surfactant syringe; 4 – syringe for flushing the chip between samples and for stabilising pressure inside the chip, connected to an insulin syringe filled with HFE 7500 oil.*

3.2. Droplet imaging and image analysis

3.2.1. Droplet imaging

Materials and equipment used for droplet imaging:

1) Countess™ cell counting chamber slide (Invitrogen, Thermo Fisher Scientific)
2) LSM 900 confocal laser scanning microscope (Zeiss, Germany)
3) Zen 3.3 software (blue edition) (Zeiss, Germany)
9) 2 - 20μl ErgoOne® Single-Channel Pipette (Standard Cone) (Starlab, Germany)

The confocal microscope was used with objective Plan-Apochromat 10x (NA 0.45). Settings were as following:

1) LED light source Colibri 7
2) Diode lasers 488 nm and 640 nm
3) Green channel: excitation 395 nm, emission 502 nm
4) Red channel: excitation 653 nm, emission 668 nm
5) Bright-field channel: differential interference contrast (DIC)

18 μl of droplets and oil was pipetted into the cell counting chamber slide and placed under the confocal microscope. At least 40 images were scanned from each slide and exported as Tagged

Image Format (TIF). Exported images included individual channel images from all three channels as shown in **Figure 14**.

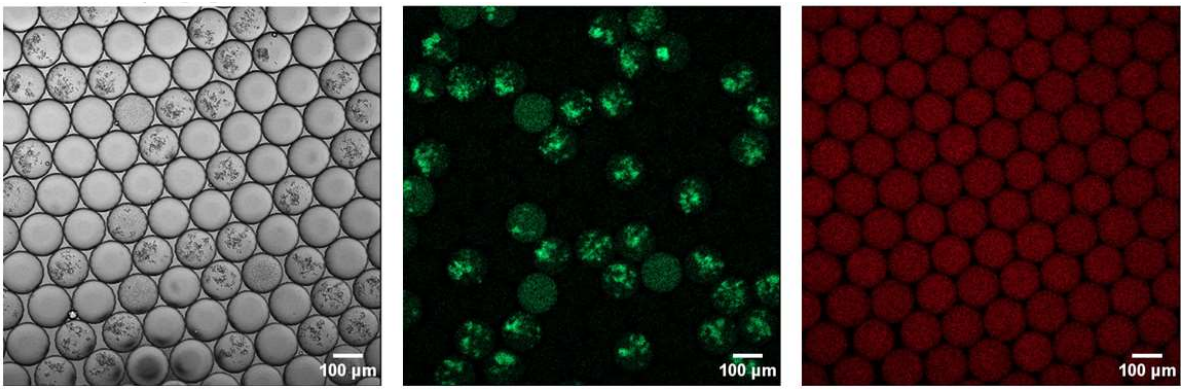

Figure 14 *Individual images exported from the confocal microscope. On the left is DIC channel image, in the middle is the green channel (bacterial GFP), on the right is the red channel (droplets dyed with Alexa).*

3.2.2. Ilastik software

Ilastik is a user-friendly image analysis, classification, and segmentation software. For this thesis it was used to identify microplastic beads in droplets. For that Pixel Classification + Object Classification workflow was selected. This workflow consisted of 8 modules (Berg, et al., 2019):

1) Input data: exported individual brightfield channel TIF images were uploaded here for algorithm training. All together six images were used: three from cefotaxime experiment and three from kanamycin experiment.
2) Feature selection: Ilastik provided three features which it uses to differentiate between different classes on pixels: colour/intensity, edge, and texture.
3) Training: one label is used to identify microplastic beads while the other is used to annotate everything else that is not a microplastic bead as shown in **Figure 15**.
4) Thresholding: thresholding values were selected for microplastic bead detection. A "simple" thresholding method was selected, and the value was set at 0.70 with a size filter from 150 to 10 000. The image is shown in **Figure 15**.
5) Object feature selection: standard object feature was selected.
6) Object classification: Ilastik shows identified object in white, where user can mark any objects wrongly identified, should Ilastik make any errors.
7) Object information export: desired export source is selected, and image settings are changed according to the source and final image format.
8) Batch processing: after training is complete, all brightfield images are uploaded and analysed.

Ilastik produces a probabilities output file for each label. In this case there are two labels – yellow (microplastic) and blue (everything besides microplastic), thus two output probabilities are produced per image input. Ilastik assigns each pixel a probability based on the prior training process in modules Training, Thresholding, and Object feature selection. In the probabilities file for yellow label (microplastic), every pixel is assigned a probability for being microplastic (seen as white), versus the probability of being background (seen as black). For further analysis of microplastic in

CellProfiler™ the probabilities file for microplastic is used as input (**Figure 15**), while the probabilities file for blue label (everything besides microplastic) is discarded.

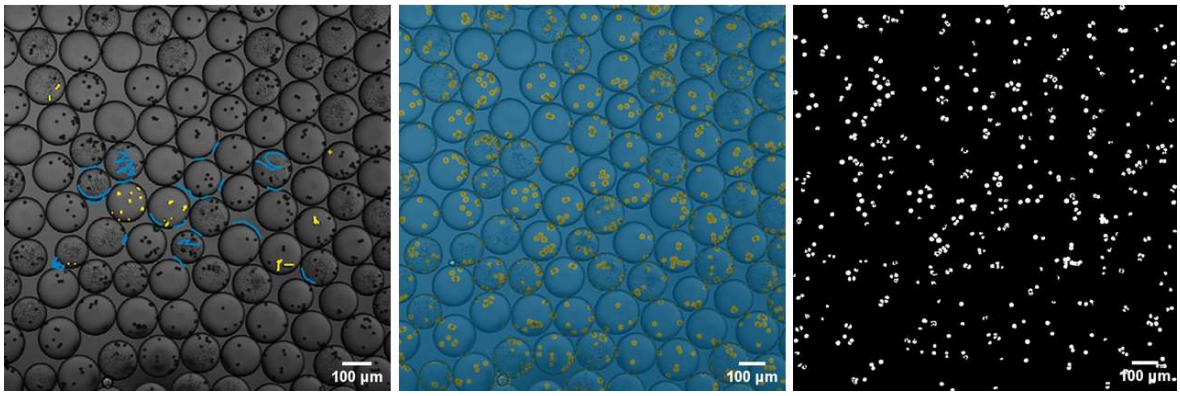

Figure 15 *Images from Ilastik training. On the left is an image from the training module: yellow and blue annotations were used to teach the program what are microbeads and what are not – yellow is for microbeads and blue is for everything else. In the middle is an image from thresholding module: program marks what it thinks are microplastics with yellow and everything else with blue. On the right is the Ilastik microplastic probabilities image used for CellProfiler™ analysis.*

3.2.3. CellProfiler™ software

CellProfiler™ is open-source modular high throughput image analysis software. "Pipeline" is a term used to describe the sequence of modules needed for an analysis (Carpenter et al., 2006). The pipeline was developed by Karoline Lindpere (bachelor's student LAAB) in partial co-development with Merili Saar (master's student IAHM). The CellProfiler™ pipeline used all individual channel images (brightfield, green, red) and Ilastik probabilities images.

First, a pipeline was used to set the threshold values for separating droplets that are empty/no-growth, have bacteria with homogeneous growth, and have bacteria with autoaggregation growth. In **Figure 16** is shown how CellProfiler™ set the texture values. The values were used to determine texture thresholds for the second pipeline, that classified the droplets based on the values into three groups: no growth, homogeneous growth and autoaggregation.

Modules used in both pipelines consisted of:

1) Images: all necessary TIF images were uploaded here.
2) NamesAndTypes: CellProfiler™ grouped all four images from the same tile together for analysing.
3) IdentifyPrimaryObjects: uses grayscale images (brightfield images) to identify droplets in the images.
4) MeasureObjectSizeShape: identified objects are measured.
5) FilterObjects: removes selected objects based on measurements, also removes objects on the edges of the images.
6) RelateObjects: associates objects with each other. Objects within a "parent" object become its "children".
7) MeasureTexture: measures the degree of texture from smooth to rough.
8) MeasureObjectIntensity: measures the intensity for identified objects.

9) ClassifyObjects: objects are classified into different classes according to the values user chooses.

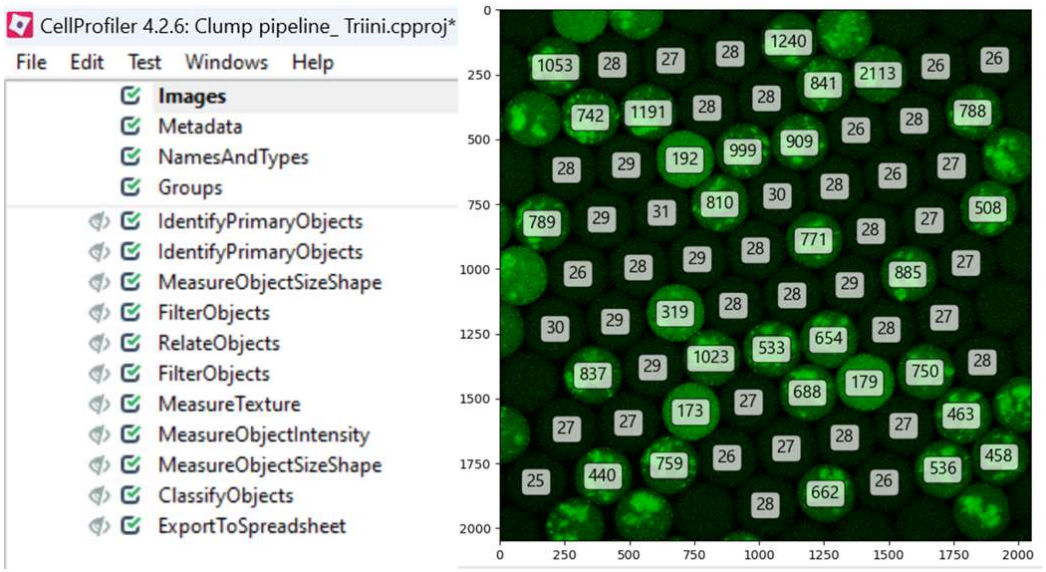

Figure 16 *Images from CellProfiler™. On the left is the pipeline used for the analysis after the threshold values were set. On the right is the workspace image from the pipeline used to set the thresholding values. The values show the degree of autoaggregation in droplets, the higher the number, the more autoaggregated bacteria in the droplets were.*

CellProfiler™ pipeline first divides the droplets into positive and negative, meaning droplets that contain bacteria and droplets that are empty. Then the positive droplets are further categorised by their texture into homogeneous and aggregated. Homogeneous growth means that the bacteria is evenly distributed in the droplet. Aggregated growth in droplets looks like the bacteria has clumped together, so there is no even green signal coming from those droplets. All the different droplets are shown in **Figure 17**

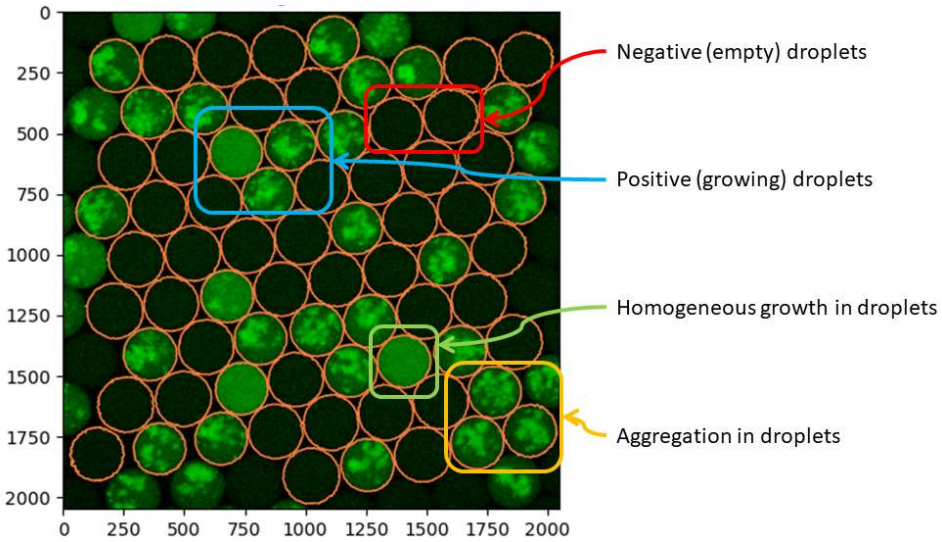

Figure 17 *Example of different droplets from same image as in* ***Figure 16****. Droplets identified by CellProfiler™ are circled with orange. Examples of negative (no bacterial growth) droplets are marked with red and positive (bacterial growth) droplets are marked with blue. Positive droplets are divided into two: homogeneously growing (marked with green) and aggregated (marked with yellow).*

4. Results and discussion

4.1. Microplastic reduced the viability of bacteria during treatment with cefotaxime, but not with kanamycin

First aim of this thesis was to investigate microplastic influence on bacterial antibiotic susceptibility. This was tested with two different antibiotics: cefotaxime and kanamycin. For both antibiotics nine concentrations were used. One experiment included two parallel experiments of every antibiotic concentration, one with microplastic and one without, and a control (without antibiotics and microplastics). Altogether, three experimental repeats were carried out for each antibiotic. Viability was determined by finding each sample's positive droplet fraction which was then normalized by dividing it with control's positive droplet faction.

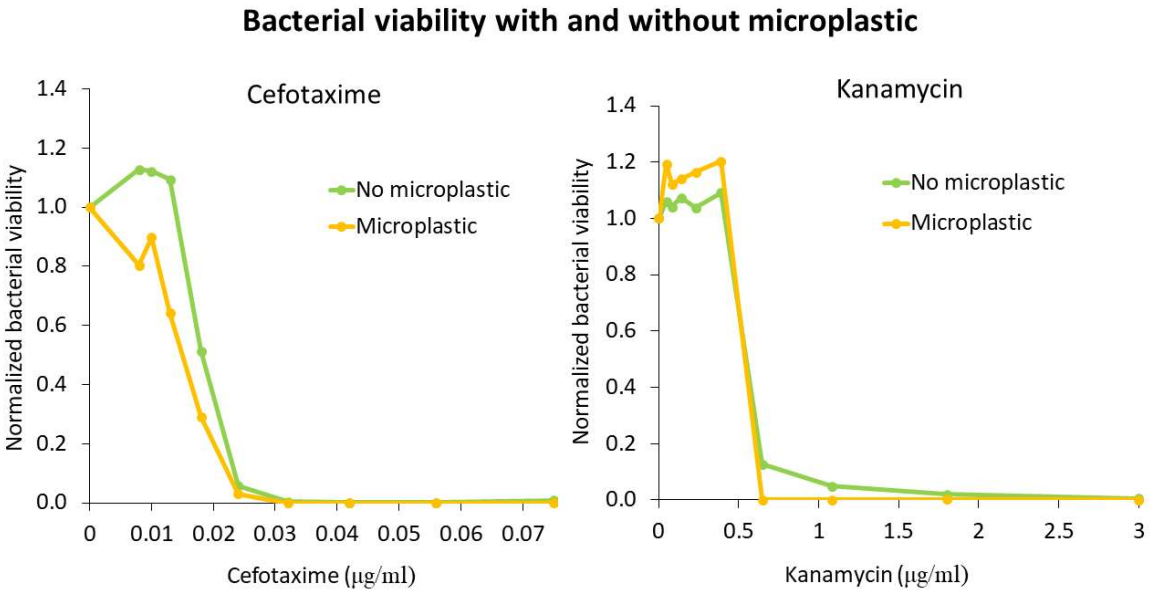

Figure 18 *Bacterial viability with and without microplastic. Viability of bacteria drops with increase of cefotaxime (left) and kanamycin (right) for samples with microplastic (orange) and without microplastic (green). On both graphs, the X-axis shows the antibiotic concentration in μg/ml and the Y-axis shows normalized bacterial viability. Microplastic made bacteria more susceptible to cefotaxime but had no effect with kanamycin.*

The terms susceptibility and viability are both used to describe bacterial response to antibiotics. Susceptibility describes the lack of resistance to antibiotics. Viability describes the ability to survive and grow successfully. Increase in susceptibility means decrease in viability – antibiotics can kill bacteria. Increase in viability means decrease in susceptibility – antibiotics are not able to kill bacteria.

Microplastic increased the bacterial susceptibility to cefotaxime when compared to parallel experiment without microplastic. The viability of bacteria with microplastic was lower than without microplastic from the first antibiotic concentration used (0.008 μg/ml). Bacterial growth was fully inhibited from 0.032 μg/ml for both parallels. This trend presented itself in all three repeats (Appendix 2: Bacterial viability for all experiments).

Microplastic had no effect on bacterial viability with kanamycin. The bacterial viability was stable for both parallel experiments up until antibiotic concentration of 0.388 μg/ml, after which a sudden drop of viability was observed. While it looks like microplastic had higher bacterial viability, the difference does not seem large enough to claim significance. After the drop at 0.388 μg/ml microplastic samples' bacterial growth was fully inhibited. The parallel experiments without microplastic had low positive droplet signals until the last concentration of 3 μg/ml. However, these results could be experimental artefacts due to a missing control with microplastic. The need for a second control was identified only during analysis, which is why it is missing from the original experiment design.

In conclusion, antibiotic effects on bacteria seem to change in the presence of microplastic. However, the change in susceptibility seemed significant only for cefotaxime, where microplastic parallel experiments were consistently more susceptible to the antibiotic. With kanamycin, there did not seem to be a difference in susceptibility between parallel experiments with and without microplastics. Further experiments need a proper statistical analysis to state if the differences observed are significant.

According to literature, microplastics provide a surface for bacteria to adhere to and form biofilm, which would make them less susceptible to antibiotics (Marathe & Bank, 2022). However, the exact effects depend on the specific plastic type and bacteria (Yi et al., 2021). A decrease in susceptibility was not observed in this experimental setup. Quite the opposite, the experiments with cefotaxime showed increase in susceptibility.

This might be, because the microplastics used in the experiments were primary – meaning, manufactured as that size with pristine and smooth round surface. In the environment, microplastics would have undergone fragmentation and change in shape and chemical structure during aging. There are studies that indicate that aged microplastics promote and accelerate biofilm formation in comparison to unaged microplastics (Bao et al., 2022; Shan et al., 2022). Other ways microplastics can promote resistance are either by being contaminated with low concentrations of antibiotic and selecting for adaptive resistance in bacteria or by being contaminated with antibiotic resistance genes, which induces horizontal gene transfer to susceptible bacteria (Marathe & Bank, 2022). The microplastics used for this thesis were pristine. Furthermore, only one species of bacteria without any resistance genes was used in the experiments, so there could not have been horizontal gene transfer of resistance genes.

However, it has been shown that polystyrene microplastics of smaller size (5 μm) inhibit the cell growth of *E. coli* (Yi et al., 2021). This size is similar to the microplastic bead size used in this thesis (10 μm). Furthermore, there has been research, that suggests that plastic particles attaching to bacterial cells can cause oxidative stress resulting in cell wall depression and potential cellular malfunction (Wang et al., 2023; Yi et al., 2021). Since cefotaxime targets cell wall synthesis, then microplastic induced cell wall stress would make it easier for the antibiotic to inhibit bacteria. This could explain why microplastic samples had less viability for cefotaxime but no change in viability for kanamycin, since it targets protein synthesis. However, microplastic inhibiting cell growth and functions are better described for microalgae (X. Zheng et al., 2021) and mammalian cells (Banerjee & Shelver, 2021).

The differing effects from microplastic influence on bacterial susceptibility to given antibiotic, could come from the specific antimicrobial mechanism the antibiotic has. Kanamycin and cefotaxime are from separate antibiotic classes and have a different mechanism of action against bacteria. To confirm this, more experiments with antibiotics from other structural or action mechanism classes should be done. In addition, the experiments should be repeated with secondary microplastics to see, if aged microplastics give different results for viability as well as with different types and sizes of microplastics and even nanoplastics.

4.2. Viability experiments were reproducible with kanamycin, but not with cefotaxime

Second aim of the thesis was to determine if the viability experiments were reproducible. Antibiotic aliquots were prepared similarly for both antibiotics: made at the beginning of the first experiment and frozen at -20˚C for other two repeats. This was done to lessen the possibility of pipetting errors when making the aliquots three separate times and to save time. However, cefotaxime experiments were carried out over a period of two months, while kanamycin experiments were done in less than a month. In both cases, the experiments are numbered chronologically.

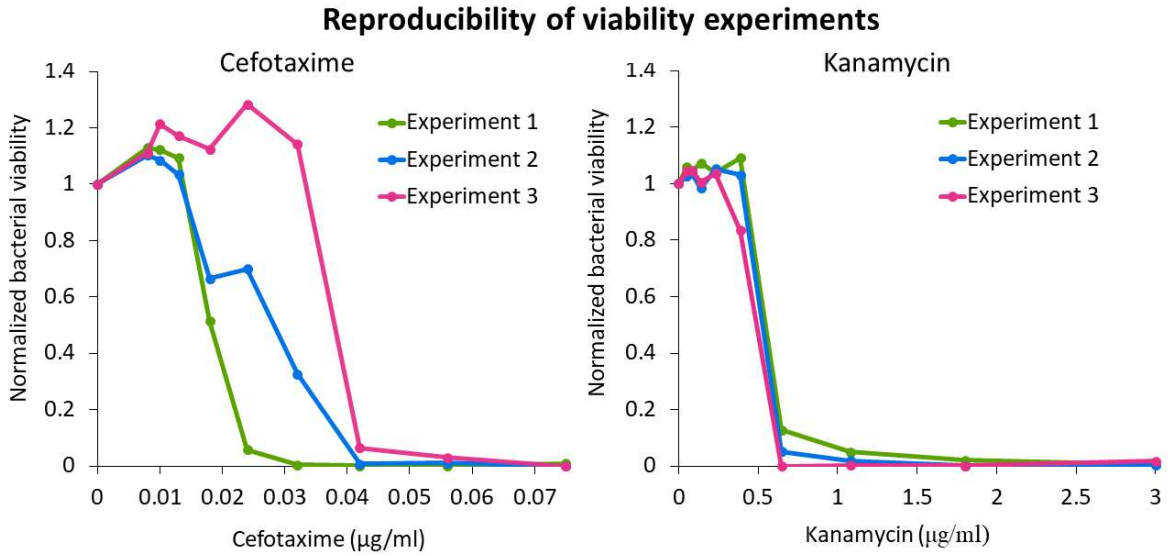

Figure 19 *Reproducibility of viability experiments for samples without microplastic. Change in susceptibility between experiments is showed for cefotaxime (left) and kanamycin (right). X-axis shows antibiotic concentration in µg/ml and Y-axis shows normalized bacterial viability. First, second and third experiment are marked with different colours (in order: green, blue, pink). Cefotaxime experiments had varying susceptibility trends in different experiments, while kanamycin remained stable throughout the repeats.*

In cefotaxime experiments, the susceptibility trend did not stay consistent throughout the repeats and shifted towards less susceptible with each repeat; this was not seen for kanamycin. In the first cefotaxime repeat, the drop in viability happened after concentration of 0.013 µg/ml. However, in the last repeat, the drop happened at 0.032 µg/ml. This trend for changes in susceptibility did not present itself in kanamycin experiments. No change of susceptibility was observed in repeats, all three followed a similar pattern. The viability of bacteria was stable in the first five concentrations (0.05 to 0.388 µg/ml) after which the viability dropped.

It is unlikely that the bacteria became resistant to cefotaxime since a fresh overnight culture was used each time. This suggests a change with the antibiotic itself. While kanamycin experiments were carried out in a shorter time span, this is not likely to be the main reason for the stability. The time between first and last kanamycin experiment was roughly the same as the time between first and second cefotaxime experiment. However, kanamycin showed no signs of change while cefotaxime already had a change in bacterial viability. These trends were consistent for both antibiotics in microplastic containing parallel experiments as well (Appendix 3: Reproducibility for microplastic samples).

Literature confirms that kanamycin can be stable for up to 12 months, while cephalosporins (β-lactam antibiotics subgroup including cefotaxime) lose a significant amount of activity after only two months frozen at -20˚C and are best kept at -70˚C instead (Berendsen et al., 2011; Okerman et al., 2007). Therefore, going forward all cefotaxime aliquots should be kept at -70˚C for long time storage, however, the exact timeframe needs to be determined. Alternatively, cefotaxime aliquots could be prepared fresh in the beginning of each experiment at the risk of pipetting inaccuracies or used within a shorter period. The literature suggests that cephalosporins have their activity frozen at -20˚C for a few weeks, but not longer than a month (Okerman et al., 2007), however, the exact time frame for the specific conditions in the Microfluidics lab needs be determined.

4.3. Microplastic reduced bacterial autoaggregation in droplets

Third aim of this thesis was to investigate the microplastic influence on the texture of the bacteria. The term texture is used to describe how the bacteria is growing inside the droplet – is it evenly distributed throughout the droplet (homogeneous) or forming “clumps” (aggregation). In these experiments only autoaggregation could occur, since there was only one strain of bacteria forming the aggregates.

For this analysis, only previously identified positive droplets were used and the growth inside the droplets was categorised as homogeneous or aggregated with a specialised CellProfiler™ pipeline. In this thesis the data is presented as the fraction of positive droplets that demonstrate bacterial autoaggregation. Fraction of droplets with autoaggregation + fraction of droplets with homogeneous growth = 1. For clarity, data is shown only for the datapoints that contained at least 20 positive droplets.

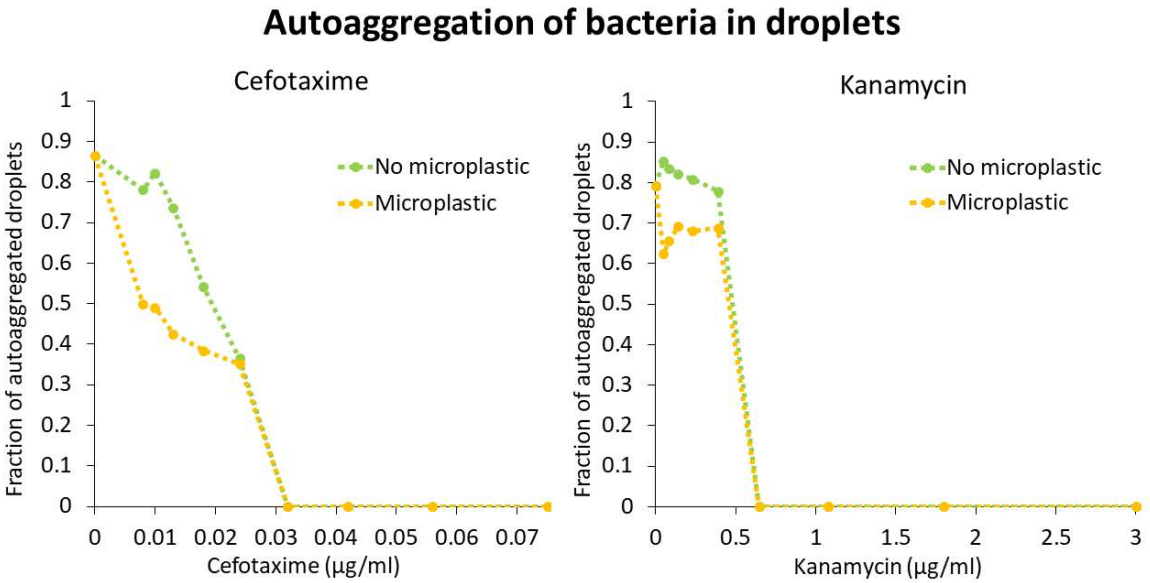

Figure 20 *Autoaggregation comparison between samples with microplastic (green) and without microplastic (orange). Changes in autoaggregation for cefotaxime (left) and kanamycin (right). On both graphs, the X-axis shows antibiotic concentration in µg/ml and on the Y-axis is shown the fraction of autoaggregated droplets. In both cases, samples without microplastic had more autoaggregation than microplastic containing samples.*

More autoaggregation was observed in parallel experiments that did not contain microplastic for both antibiotics. For cefotaxime's first parallel experiments (0.008 µg/ml) the fraction of autoaggregated bacteria containing droplets without microplastics was almost 0.8, while in the microplastic containing parallel experiment, the fraction was around 0.5. With kanamycin, a similar pattern of more autoaggregation without microplastic repeated itself. Without microplastics the fraction of autoaggregated bacteria was around 0.8 to 0.9, while in microplastic parallel experiments the fraction was around 0.6 to 0.7. The difference was not as big as in cefotaxime, but it was still observable. In conclusion, microplastic did not facilitate more autoaggregation with either antibiotic. This trend was also present in the repeats (Appendix 4: Autoaggregation of bacteria in droplets).

Microplastics have been seen as excellent adhesion substrates, however, this depends on the polymer type, surface properties and particle sizes. Some polymer types favour bacterial adhesion due to their hydrophobicity (Nath et al., 2023; Z. Zheng et al., 2023). *E. coli* has been shown to be able to bind to polystyrene by its flagella, making it a potential primary colonizer, however, this was seen for smaller microplastic sizes than used in this thesis (Nath et al., 2023). The possible reasons for less aggregation in microplastic samples are similar to the reasons discussed in viability experiments (Chapter 4.1) – the used microplastic was primary, but adhesion and biofilm formation occurs better on secondary and aged microplastics (Bao et al., 2022; Shan et al., 2022).

It is impossible to distinguish autoaggregation from biofilm formation with the currently used pipelines in the Microfluidics group. The current pipeline is only able to differentiate homogeneous growth and bacterial autoaggregates (clumps). For a pipeline to be able to give information about actual biofilm formation, it needs to connect the autoaggregates to microplastic beads. This pipeline is currently still in the development to be used in future experiments.

An oversight was made in the designing of the experiments because the texture difference observation was added later to the analysis. The texture comparison analysis would have benefited from a second control sample made with microplastic beads, to get the baseline change in texture with no antibiotic present. While this would not have changed the data gathered and presented, it would have given an insight to just microplastic influence to bacterial growth and aggregation.

4.4. Increase of antibiotic concentration reduces viability and autoaggregation of bacteria

The final aim of this thesis was to investigate the connection between bacterial susceptibility and autoaggregation. For the last part of this analysis, previously gathered results about bacterial viability and autoaggregation as a response to antibiotic concentration increase were combined.

While bacterial viability and autoaggregation are presented on the same graph and Y-axis, it is important to understand that they are two separate indicators. Bacterial viability shows normalized positive droplet fractions for samples. Autoaggregation shows the autoaggregation fraction of those positive droplets. This is why in **Figure 21** autoaggregation line can sometimes cross the viability line.

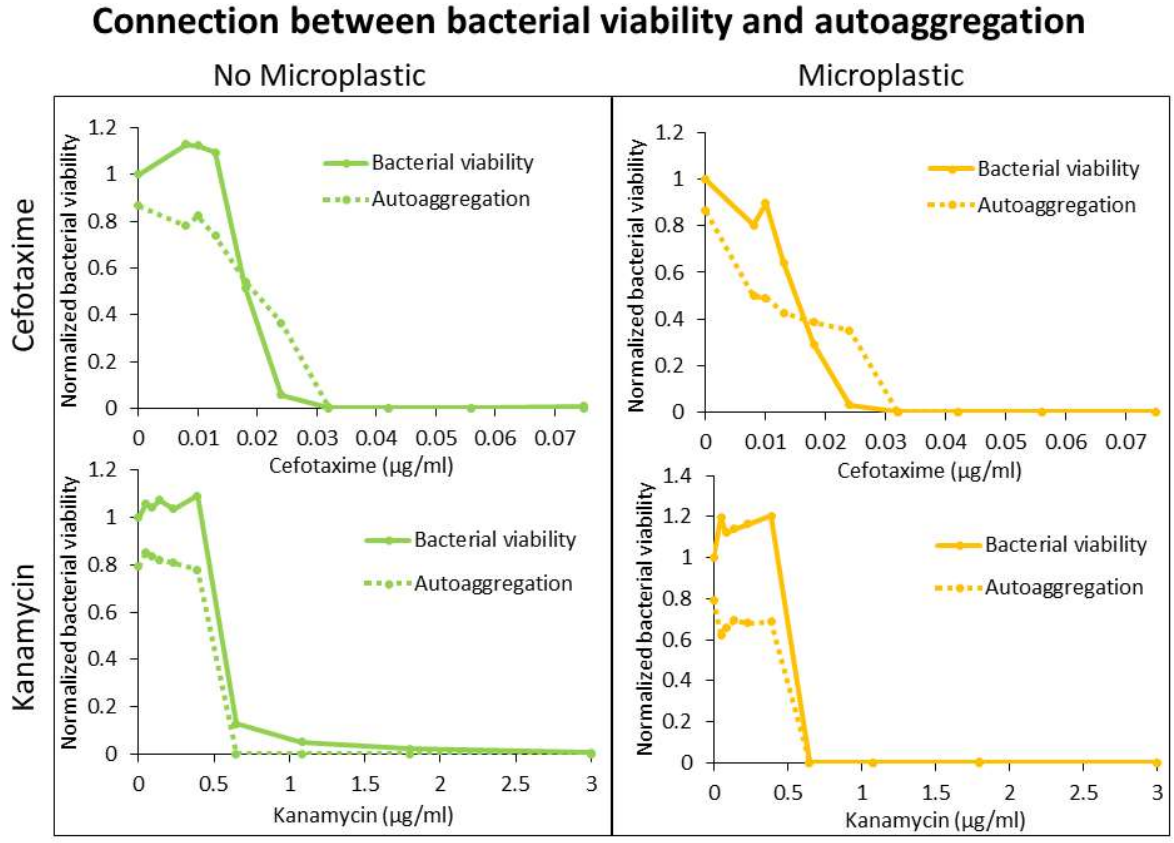

Figure 21 *Connection between bacterial viability and autoaggregation. Changes in bacterial viability and autoaggregation are shown for cefotaxime (upper row) and kanamycin (lower row), as well as for samples without microplastic (left column) and with microplastic (right column). X-axis shows antibiotic concentration in µg/ml. Y-axis shows fraction of droplets – for viability it means normalized positive droplets fraction, for autoaggregation it means autoaggregation fraction in positive droplets. No microplastic samples bacterial viability is shown with a solid green line and*

autoaggregation is shown with a dotted green line. Microplastic samples are shown similarly, but only in orange.

In case of cefotaxime, the autoaggregation rate followed the susceptibility trend. Both autoaggregation and viability decreased with the increase of antibiotic concentration. The viability trend was different for samples with and without microplastic (see also **Figure 18**), however, the autoaggregation followed the trend of viability in both cases. While the viability experiments in cefotaxime were not reproducible, the same trend for aggregation to follow the viability drop was still present in all repeats.

For kanamycin the autoaggregation rate remained stable for both parallel experiments with the increase of antibiotic up until the drop in viability. Autoaggregation did not seem to change with the increase in antibiotic. Comparing kanamycin experiments' viability and autoaggregation trend, it looks like the autoaggregation follows the viability trend. Up until the concentration of 0.388 μg/ml both viability and autoaggregation rates are stable. The same trend is repeated in microplastic containing samples.

In conclusion, increase of antibiotic concentration decreases the bacterial viability in all tested cases – both antibiotics, with and without microplastics. There was also a decrease in autoaggregation that seemed to follow the viability trend. Microplastic enhanced the antibiotics' effect in viability for cefotaxime and in aggregation for both antibiotics.

Considering the sudden drop of viability in kanamycin samples, further experiments should include at least one concentration between 0.388 and 0.648 μg/ml. This would help to determine where exactly the viability changes and if the aggregation would follow similar gradual drop in viability in that region. Going forward, more experiments with different antibiotics should be done, since cefotaxime and kanamycin had different outcomes. This could help determine, why they have different results in both viability and aggregation rates.

4.5. Takeaways from experiments for the future

The experiments revealed that while microplastic has an influence on bacterial viability with only cefotaxime, it alters autoaggregation for both cefotaxime and kanamycin. These experiments were the first attempt to investigate antibiotic susceptibility, its connection to autoaggregation and microplastics in monodisperse droplets with multiple repeats in the Microfluidics group. Since there was no previous experience, the design of the experiments was not perfect in hindsight.

With current knowledge the future experimental protocol would include a second control with microplastics, to observe if microplastics by themselves have any influence on bacteria. There would also be one or two extra concentrations in kanamycin experiments between 0.388 and 0.648 μg/ml, to be able to see where exactly the loss of viability happens and what influence it has on autoaggregation. Cefotaxime storage would be moved to -70°C. There should be a separate study on cefotaxime stability in -20°C and -70°C over a longer period.

The droplet technology itself also has room for improvements. One experiment takes three consecutive days to execute. While this is due to microbiological reasons and cannot be changed (the bacteria need to be incubated twice overnight), there is room to improve other protocols to

simplify the work needed to be done during those days. Preparing one single repetition with 19 samples (control plus nine parallel antibiotic concentrations with and without microplastic) takes an experienced technician under perfect circumstances around three hours. Imaging with the confocal microscope takes 15 minutes per sample, so about five hours for all the samples. This is followed by data analysis. Due to the large number of images produced in the experiments, both Ilastik and CellProfiler™ analysis took several hours each.

Further automation of microfluidic technology can potentially reduce the time cost of the workflow. For example, to improve the droplet generation step, more advanced microfluidic setups could be designed, so that right antibiotic concentrations would get automatically incorporated into droplets, which would eliminate the need to prepare bulk mixtures. That would save time and limit possible mistakes and inaccuracies related to human error. To eliminate the pipetting of droplets for imaging purposes, special incubation chambers can be designed, that can be used for both incubation and imaging. This would minimize the risk of cross-contamination as well as droplets getting damaged.

The analysis process has many manual steps, with final data analysis and graph creation being fully manual in Microsoft Excel. However, the main limiting factor in analysis, are the software themselves. This cannot be fixed with a better pipeline, since Ilastik analysis relies on manual training of the programme (supervised machine learning). There is a need for more automated software. However, there has been development in automating data analysis and graph creation. A member of the Microfluidics group, Immanuel Sanka (PhD) created a custom-developed data visualization tool “EasyFlow”, which can visualize distributions of droplet size and signal as well as the relationship between them from a csv file (Sanka et al., 2023). This is a great tool and a big step towards simplifying data analysis and graph making, however, to be applicable to more advanced experiments, like in this thesis, it needs more development.

Abstract

Antibiotics are substances that have antimicrobial activity against bacteria. The structure of the antibiotic determines the specific mechanism. However, bacteria can have or acquire counter mechanisms to survive the effects of antibiotics. This is called antibiotic resistance and is a growing problem in healthcare. One such mechanisms is bacterial aggregation which leads to biofilm formation – bacteria aggregate together and develop adaptive resistance mechanisms. One driver of biofilm formation is microplastic since it offers a surface for the bacteria to adhere to. Microplastic has become ubiquitous to the environment and can be found everywhere. Microplastic colonized by bacteria is often referred to as plastisphere. It can harbour many bacterial species and it has a high activity for horizontal gene transfer, which spreads antimicrobial resistance to previously susceptible bacteria.

The main aim of this bachelor's thesis was to investigate the influence of microplastic on bacterial antimicrobial susceptibility and autoaggregation in monodisperse droplets. The specific objectives were (1) to find out if microplastic influences antimicrobial susceptibility in monodisperse droplets. This investigation revealed that microplastic enhances bacterial susceptibility to cefotaxime but has no effect with kanamycin. (2) To see if the viability experiments are reproducible. The results showed that cefotaxime storing conditions of aliquots need to be revised to generate reproducible experiments; this was not seen for kanamycin. (3) To determine if microplastic influences bacterial autoaggregation in monodisperse droplets. It was seen that microplastic decreased the autoaggregation rate for both tested antibiotics. (4) To investigate the connection between bacterial susceptibility and autoaggregation. Increase of antibiotic decreased bacterial viability as well as autoaggregation for both with and without microplastic parallel experiments.

In conclusion, the results of this thesis show that microplastic influences both bacterial viability and autoaggregation. While the change in autoaggregation rate was similar for both tested antibiotics, viability trend was only affected in the case of cefotaxime. These results help to understand the influence and risks of plastispheres in our environment.

Kokkuvõte

Vesi-õlis tilkasid valmistatakse vee faasi dispersioonil õli faasi. Need tilgad on nagu väikesed katseklaasid, võimaldades üksikuid mikroobirakke eraldi mikrokeskkondadesse eraldada. Vesi-õlis tilku saab valmistada käsitsi või spetsiaalset aparatuuri kasutades. Käsitsi valmistatud tilgad on polüdispersed ehk erinevate suurustega. Spetsiaalset aparatuuri kasutades (mikrofluidika pumbad ja kiibid) saab toota ühesuuruseid ehk monodispersed tilkasid. Nende eeliseks on lihtsam analüüsiprotseduur, kuid miinuseks ajakulukam tilkade valmistamine.

Antibiootikumid on ained, mis omavad antibakteriaalset aktiivsust. Antibiootikumi struktuur määrab ära selle spetsiifilise toimemehhanismi. Bakteritel omakorda võib olla või nad võivad omandada mehhanisme, et antibiootikumide mõjule vastu pidada ehk omandada antibiootikumiresistentsus. See on kasvav probleem tervishoiusüsteemis, kuna resistentsete bakterite põhjustatud infektsioonid alluvad halvemini ravile ning põhjustavad tihtipeale pikaajalist haiglas viibimist.

Mikroplastid on plastiku fragmendid, mis on väiksemad kui 5 mm. Mikroplast on looduses üldlevinud ja neid võib leida kõikjalt keskkonnast. Need võivad looduses olla kas primaarsetena või sekundaarsetena. Primaarsed on toodetud mikroskoopilise suurusena, kuid sekundaarsed fragmenteeruvad suurematest plastiku tükkidest. Mikroplastik pakub bakteritele soodsat kohta, kuhu kinnituda. See põhjustab bakterite omavahelist agregatsiooni ja viib biofilmi tekkeni. See põhjustab bakterites adaptiivset resistentsust, mistõttu on biofilmist keerulisem lahti saada. Mikroplastikut, mis on koloniseeritud bakterite või teiste mikroorganismide poolt kutsutakse ka plastisfääriks. Seal võib olla mitmeid erinevaid mikroorganismide liike. Vaid mõnel neist võib olla resistentsusgeen, kuid plastisfääris on kõrgendatud horisonaalse geenivahetuse aktiivsus, seega levivad antibiootikumi resistentsusgeenid kiiresti muidu tundlikele bakteritele.

Selle bakalaureuse töö peamiseks eesmärgiks oli uurida mikroplasti mõju bakterite elulisusele ja autoagregatsioonile monodispersetes tilkades. Konkreetsed ülesanded olid (1) vaadelda mikroplasti mõju bakterite antibiootikumitundlikkusele monodispersetes tilkades. Tulemused näitasid, et mikroplast võimendab bakterite tundlikust tsefotaksiimile, kuid mitte kanamütsiinile. (2) Näha, kas elulisuse eksperimendid on reprodutseeritavad. Tsefotaksiimi lahjenduste hoiustamise tingimused tuleb üle vaadata ja neid muuta, et saavutada reprodutseeritavus; sama probleemi ei esinenud kanamütsiini puhul. (3) Välja selgitada, kas mikroplast mõjutab bakterite autoagregatisooni monodispersetes tilkades. Tulemused näitasid, et mikroplast vähendab autoagregatsiooni hulka mõlema kasutatud antibiootikumi puhul. (4) Uurida seost bakterite elulisuse ja autoagregatsiooni vahel. Antibiootikumi kontsentratsiooni tõus langetas nii bakterite elulisust kui ka autoagregatsiooni nii mikroplastiga kui ka mikroplastita paralleelsetes eksperimentides

Kokkuvõttes, selle bakalaureusetöö tulemused viitavad, et mikroplastil on mõju nii bakterite elulisusele kui ka autoagregatsioonile. Kuigi mikroplasti mõju autoagregatsioonile oli antibiootikumide vahel sarnane, siis elulisust mõjutas mikroplast ainult tsefotaksiimi puhul. Saadud tulemused aitavad paremini aru saada plastisfääri mõjust ja riskidest keskkonnale.

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Appendices

Appendix 1: PDMS chip preparation

Materials and equipment used for PDMS chip preparation:

1) SYLGARD™ 184 Silicone Elastomer Kit (DOW, USA)
2) Perfluorooctyltrichlorosilane, 97% (Thermo Fisher Scientific, USA)
3) Novec™ 1720, electronic grade coating (3M, USA)
4) DRY-Line® Prime, drying oven with natural convection (VWR, UK)
5) Disposable biopsy punch with plunger, 1 mm (KAI Medical, Japan)
6) BD-20 high frequency generator (Electro-Technic Products, USA)

First, a mould of a glass chip was made. For this, the base and the curing agent of the silicone kit were combined with a ratio of 10:1 – the same mixture was later used to make the actual chip. Mixture was placed in a vacuum dome to get rid of bubbles for 30 minutes. The glass chip was encased in the mixture and heated at 120˚C for 1 hour. After the mixture solidified, the glass chip was removed, and the mould got hydrophobic treatment with silane in a vacuum dome for 3 hours.

The mould was used to create the chips used in the experiments. The silicone mixture was poured into the mould and heated at 120˚C for 1 hour. Then the chip was carefully removed from the mould and inlet/outlet channels were punched through with a 1 mm biopsy needle. A high frequency generator was used to fuse the chip onto a microscopy slide. Chip was treated with Novec™ 1720 to make it hydrophobic. After letting the chip dry at least an hour, it was ready to be used.

Appendix 2: Bacterial viability for all experiments

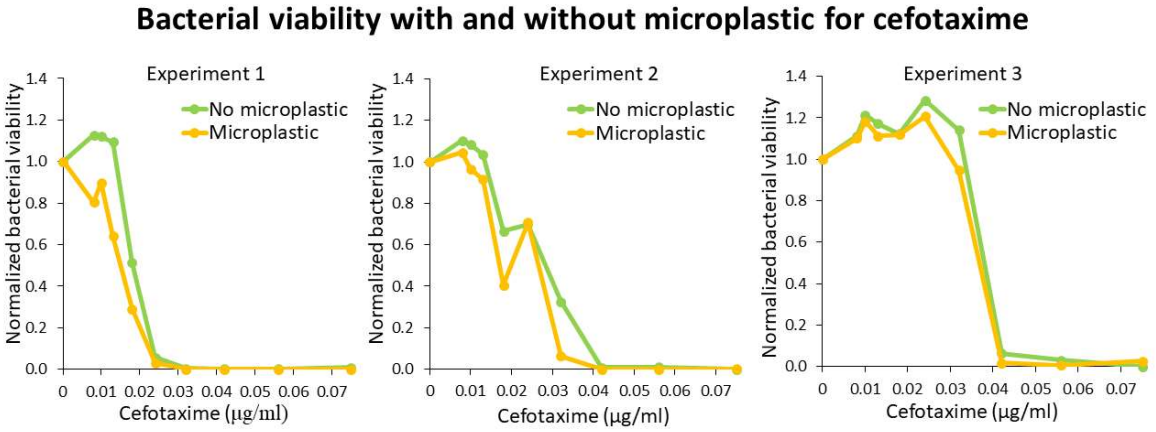

Figure 22 *Bacterial viability comparison between samples with microplastic (green) and without microplastic (orange) for cefotaxime. X-axis shows antibiotic concentration in µg/ml and Y-axis shows normalized bacterial viability.*

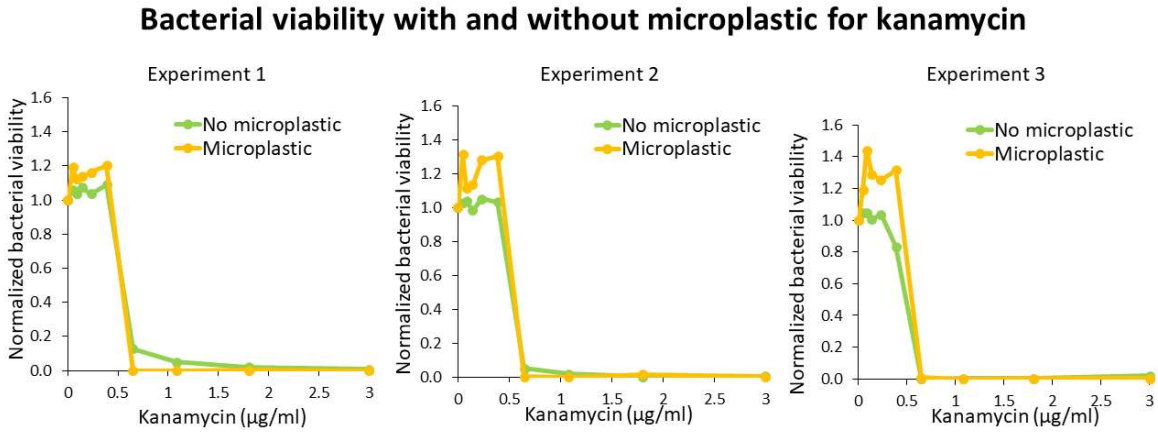

Figure 23 *Bacterial viability comparison between samples with microplastic (green) and without microplastic (orange) for kanamycin. X-axis shows antibiotic concentration in µg/ml and Y-axis shows normalized bacterial viability.*

Appendix 3: Reproducibility for microplastic samples

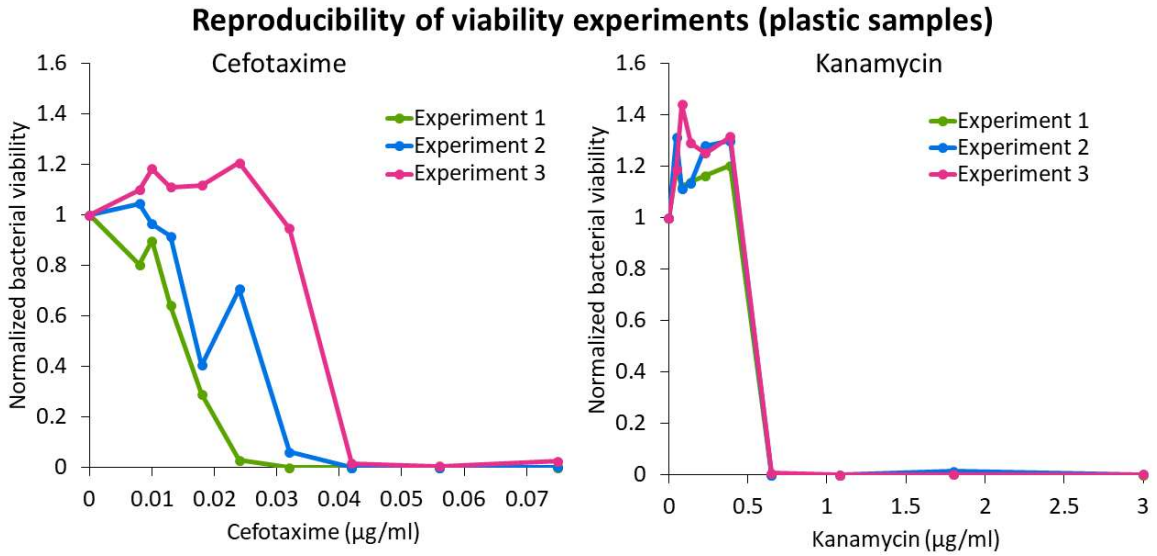

Figure 24 *Reproducibility of viability experiments. Susceptibility changes in all three repeats (samples with microplastic). Change in susceptibility between experiments is showed for cefotaxime (left) and kanamycin (right). X-axis shows antibiotic concentration in µg/ml and Y-axis shows normalized bacterial viability. First, second and third experiment are marked with different colours (in order: green, blue, pink).*

Appendix 4: Autoaggregation of bacteria in droplets

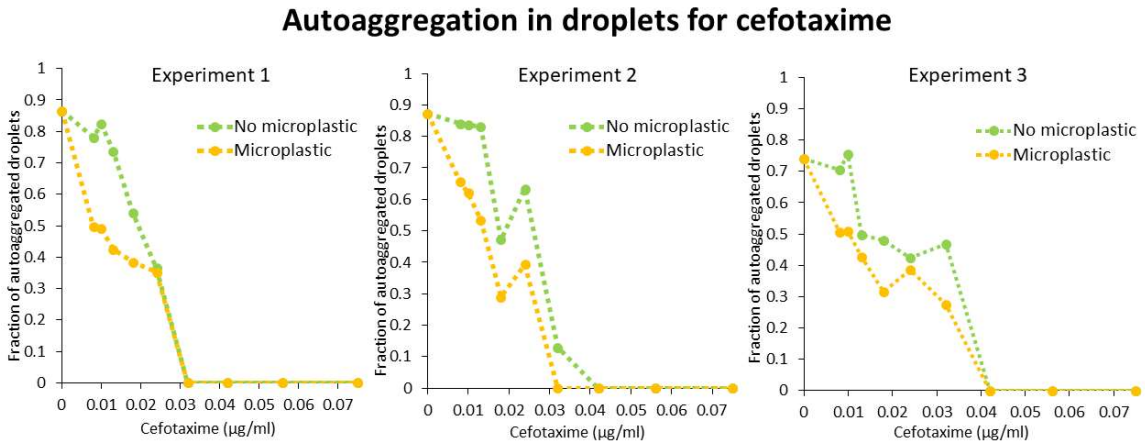

Figure 25 *Autoaggregation comparison between samples with microplastic (green) and without microplastic (orange) for cefotaxime. X-axis shows antibiotic concentration in µg/ml and Y-axis shows the fraction of autoaggregated droplets.*

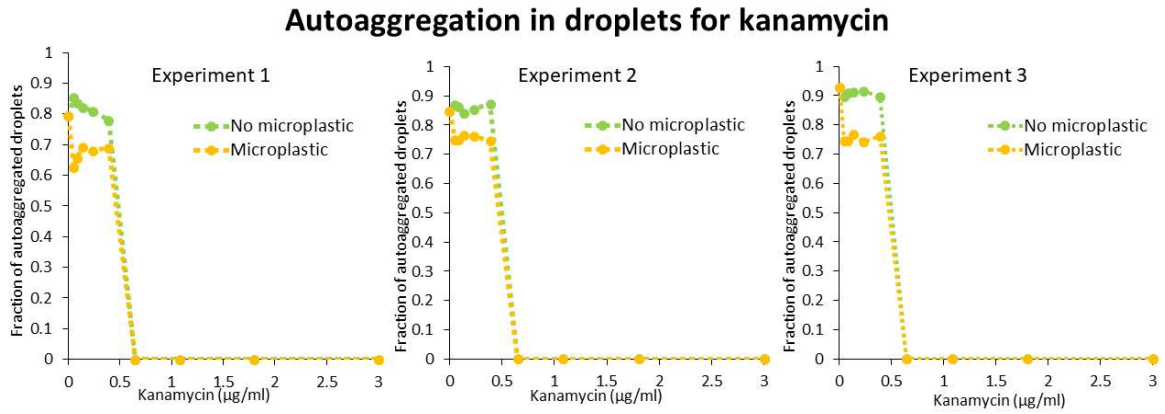

Figure 26 *Autoaggregation comparison between samples with microplastic (green) and without microplastic (orange) for kanamycin. X-axis shows antibiotic concentration in µg/ml and Y-axis shows the fraction of autoaggregated droplets.*

Lisa
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