Investigating effect of microplastic on antimicrobial resistance via droplet-based platform

Master's thesis

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Mikroplasti mõju uurimine antimikroobsele resistentsusele tilkpõhise platvormi kaudu

Magistritöö

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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 been previously presented for grading.

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The paper conforms to requirements in force.

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Chairman of the Defence Committee:

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Table of Contents

INTRODUCTION

Water-in-oil droplets refer to a dispersion system where water droplets are dispersed within an oil phase. In this system, water acts as the dispersed phase, while oil forms the continuous phase. There are two ways to make water-in-oil droplets: mixing and microfluidics. Droplet-based systems offer precise control and high-throughput capabilities for various research applications, including chemical and biological assays, drug discovery, single-cell analysis, and emulsion-based reactions.

Antibiotics are antimicrobial substances that are used to treat bacterial infections. Microbes develop mechanisms that protect them against the effects of antibiotics. There are several ways for bacteria to develop resistance towards antibiotics – one of them being biofilm formation. It has been suggested that microplastics in the environment may contribute to the development of antibiotic resistance in bacteria. Microplastics can act as a platform for bacteria to attach and form biofilm, thereby providing a protective environment for bacteria to resist antibiotics. The interactions between microplastics and biofilm formation are still an area of ongoing research. The exact mechanisms and consequences may vary depending on the type of microplastic, bacterial species, environmental conditions, and other factors.

The aim of this thesis is to investigate effect of microplastic on antimicrobial resistance via droplet-based platform. The methodology includes generating droplets, imaging them via microscope and analyzing them via free and user-friendly software.

The theoretical part of this thesis provides an overview of water-in-oil droplets and their application in different research fields with special focus on their use in microbiology. There is also a comparison between polydisperse droplets and monodisperse droplets. For the last part, a general description of microscopy and imaging analysis software is provided.

The second chapter describes the experimental part of the study. It consists of testing different microplastic, generation of droplets and bacterial incubation procedure. The experiments in this MSc thesis are part of the longer ongoing research carried out in the Microfluidics lab to investigate different environmental drivers of antimicrobial resistance.

Results from the three experimental components for investigating an effect of microplastic on antimicrobial resistance, are presented in the last chapter along with a discussion and conclusion.

1. LITERATURE OVERVIEW

1.1 Droplets in biotechnology

A droplet provides a small environment for conducting biological or chemical experiments. Droplets are formed by mixing two immiscible liquid phases – oil (continuous phase) and water (dispersed phase). Mostly, droplets are generated as a water-based confinement with oil as continous phase [1]. Water-in-oil droplets contain two immiscible liquid phases – oil (continous) and water (dispersed). Oil contains phospholipids which creates a lipid monolayer surrounding the water droplets. This way the two phases do not mix and the water droplets remain stable over time in the oil phase (**Figure 1**) [2].

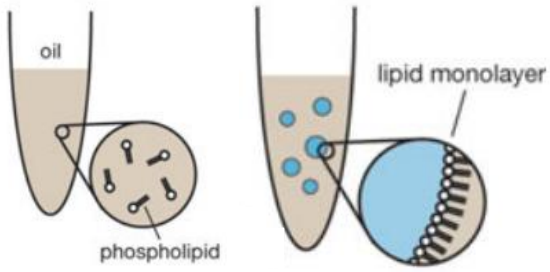

Figure 1. Schematic illustration of the formation of water-in-oil droplets. Water is added into the lipid-oil mixture. Once the droplet is formed, a lipid monolayer spontaneously forms on the surface of the water-oil interface [2].

1.1.1 Polydisperse droplets

Polydisperse droplets vary in size. To generate polydisperse droplets, two immiscible fluids, such as oil and water, need to be mixed together. There are several methods to generate polydisperse droplets, for an example inversion and vortex methods (**Figure 2**) [3]. These methods do not require special equipment and can be used in almost any laboratory. With the inversion method, droplets are generated by inverting the conventional test-tube and no additional equipment is required. Vortex method requires only a standard vortex mixer [4].

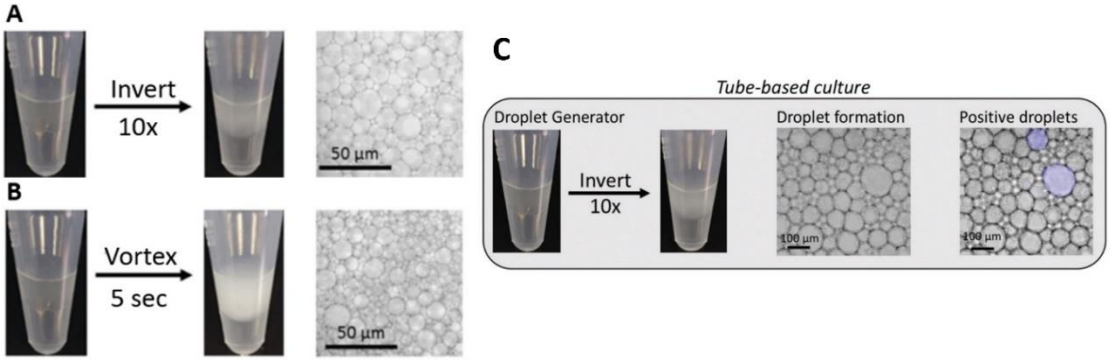

Figure 2. Generation of polydisperse droplets. Polydisperse droplets can be formed in two ways – (A) inversion method - test-tube is inverted 10x, (B) vortex method - test-tube is vortexed at least 5 seconds. Figure is based on modified image from Byrnes *et al.* (2018). (C) Polydisperse droplets can be used for cell culture and quantification [4].

The polymerase chain reaction (PCR) is a highly sensitive and specific method of detecting and quantifying diseases, which promotes more accurate diagnosis and treatment. Polydisperse droplet systems allow for accurate quantification of droplet digital PCR (ddPCR), which can reduce overall assay time while providing quantitive results. It has been shown that polydisperse droplet system with a statistical correction is comparable with microfluidic ddPCR. This method requires less equipment and time[5].

1.1.2 Monodisperse droplets

Monodisperse droplet emulsions are mixtures of two immiscible fluids – water and oil. Monodisperse droplets are uniform in size (**Figure 3**) [6]. Droplets are generated through microchannels in microfluidic chips using in two alternative options: passive and active. In passive generation, syringes and/or pressure-driven pumps are used, and droplets randomly enter the droplet formation region. Active generation uses acoustic, electrical, optical, or magnetic forces to guide the droplets into the droplet generation region and actively generate droplets. (dielectrophoresis, electrically controlled wetting) [1], [7]. As a result of this method, highly monodisperse droplets with diameters ranging from nanometers to micrometers are produced. In contrast to continuous-flow microfluidics, droplet-based microfluidics offers independent control over each droplet, creating microreactors that can be transported, mixed, and analyzed separately [8].

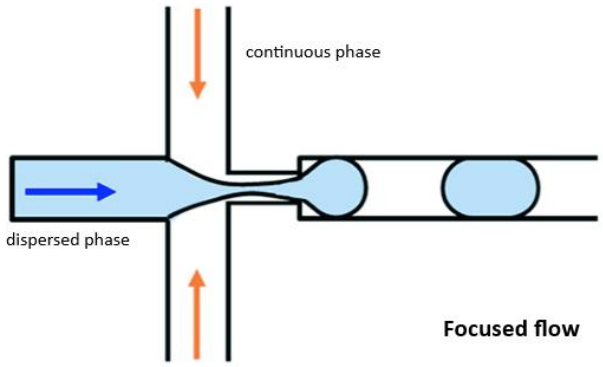

Figure 3. Generation of monodisperse droplets by pressure-driven flows in a flow-focusing microchannel. Monodisperse droplets microfluidic chips are used to generate monodisperse droplets. The continuous phase (oil) and the dispersed phase (water) are introduced into their respective channels of the microfluidic chip using syringe pumps or pressure-driven systems. At the junction, the fluids come into contact and form an interface. The geometry of the junction is designed to control the breakup of the dispersed phase into droplets [8], [9].

Droplet microfluidics has played as one of the most prominent roles in the development of biochemistry and molecular biology over the past three decades. In the last decade, droplet microfluidics have focused on finding high throughput results while reducing the amount of sample and reagents required, as well as increasing the assay's sensitivity and precision. This reduction makes the flow in the channels become laminar, represented by a low Reynolds number, which gives different properties, like immiscibility of the different phases of the fluid. Droplet-based

microfluidics provides a method to precisely manipulate analyze single biological molecules within femto-to-nanoliter droplets in high-throughput manner [10].

Several biological components can be analyzed using monodisperse droplet-based microfluidics (**Figure 4**) [10]:

1) **Field applications**: drug screening and diagnosis;
2) **Cellomics**: single-cell analysis;
3) **Genomics**: DNA sequencing, quantification of DNA etc.;
4) **Proteomics**: protein engineering, quantification of protein;
5) **Metabolites**: glucose and cancer metabolism.

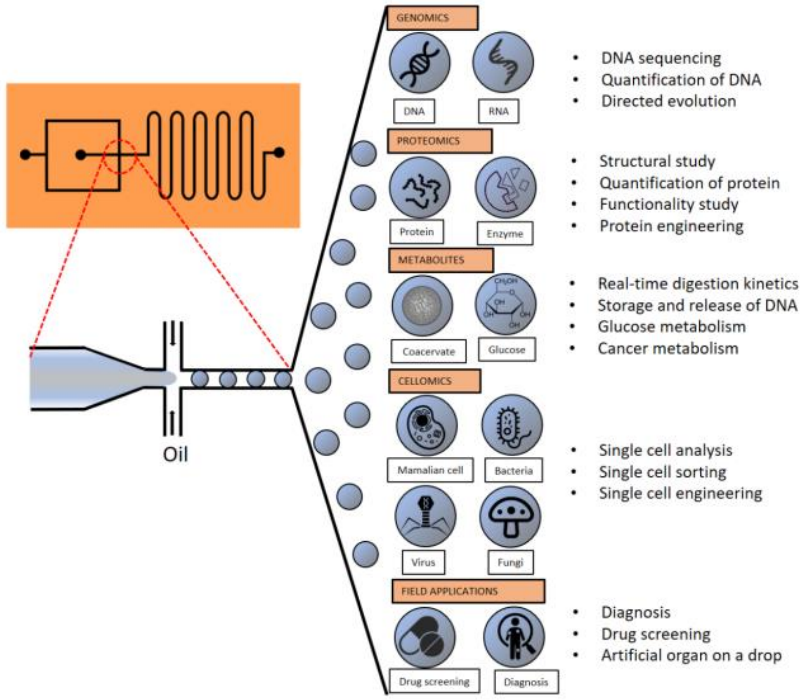

Figure 4. Diagram of biological components that can be analyzed and engineered using droplet-based microfluidics. Monodisperse droplets can be used in different research fields – genomics, proteomics, metabolites, cellomics, drug screening and diagnosis [10].

Despite their ability to quantify nucleic acids directly, digital nucleic acid amplification tests require specialized instruments to generate uniform compartments and read their fluorescent signals, which are costly, limiting the applications of these tests [11]. To overcome these limitations, polydisperse droplets have been used to detect nucleic acid, proteins etc. [3]. One of them being deep-learning-enabled polydisperse emulsion-based digital loop-mediated isothermal amplification (deep-dLAMP). This method is label-free and is low-cost. Using a deep learning algorithm, Deep-dLAMP performs LAMP reaction in polydisperse emulsions and determines the occupancy status of each emulsion in images by analyzing precipitated byproducts (**Figure 5**) [11].

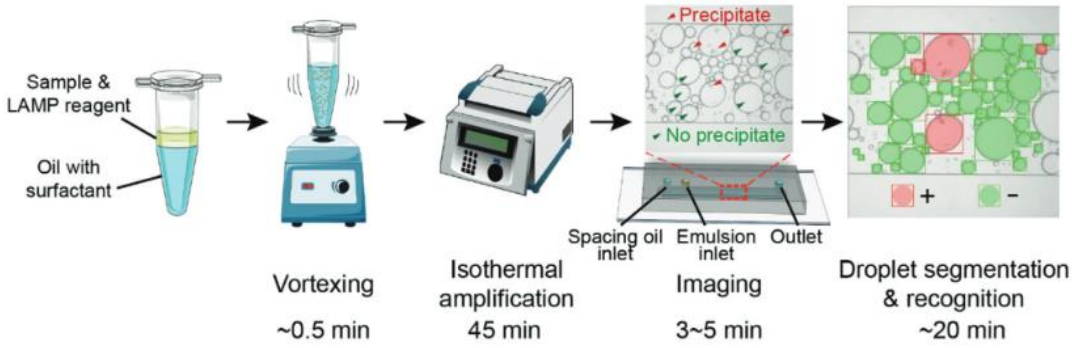

Figure 5. Digital loop-mediated isothermal amplification using polydisperse emulsions supported by deep learning (deep-dLAMP). The sample-reagent mix and oil with surfactant is vortexed to generate polydisperse droplets. Then droplets go through isothermal incubation to amplify nucleic acids of interest. In order to segment droplets based on precipitate presence, deep learning image analysis is utilized to analyze the images and determine the occupancy of the droplets. In order to calculate the nucleic acid concentrations, volumes and occupancy statuses are used [11].

1.2 Antibiotics and antimicrobial resistance

Antibiotics are antibacterial substances used to treat infections caused by bacteria and other microorganisms [12]. There are several antibiotics with different inhibitory mechanism, because each class works against specific bacteria. The inhibitory mechanism classes can be divided into three main classes (**Figure 6**)[13]:

1) **Cell wall synthesis inhibitors:** include antibiotics of class β-lactams and glycopeptides. Cell wall synthesis inhibitors are most used antibiotics for treating Gram-negative and also Gram-positive bacteria [14]. Inhibition of cell wall synthesis consists of inhibiting cell membrane – increases membrane permeability, which leads to protein mistranslation. β-lactams inhibit cell wall biosynthesis and blocks penicillin-binding proteins [15];
2) **Nucleic acid synthesis inhibitors:** work against folate synthesis, which blocks dihydrofolate reductase and prevents synthesis of the DNA; RNA polymerase, which blocks RNA synthesis; DNA gyrase, which blocks the DNA from supercoiling and replicating [15];
3) **Protein synthesis inhibitors:** are inhibiting 50S subunit, which inhibits peptidyl transferase; 30S subunit, which blocks translation and binding of tRNA [15].

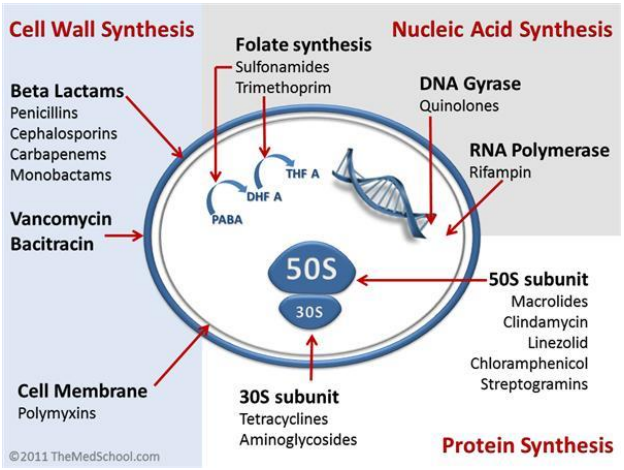

Figure 6. Antibiotic classification and mechanism. Inhibitory mechanisms can be divided into three groups – cell wall synthesis, nucleic acid synthesis and protein synthesis [13].

1.2.1 Cefotaxime

Cefotaxime (**Figure 7**) is a broad-spectrum beta-lactam antibiotic belonging to the third-generation cephalosporin group. Beta-lactam antibiotics are one of the most commonly prescribed drug classes [16]. Cefotaxime is used to treat a wide range of bacterial infections, including pneumonia, meningitis, urinary tract infections, skin infections, and infections of the blood, bones, and joints. It was first synthesized in 1976 and it is used to treat gram-positive, gram-negative and anaerobic bacteria [17].

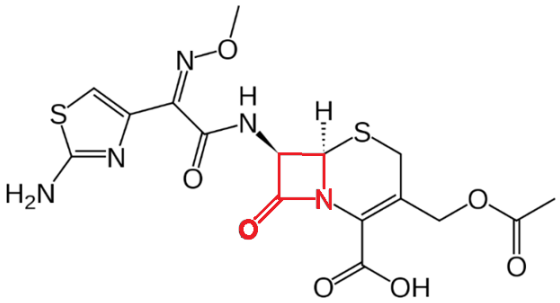

Figure 7. Cefotaxime structure. Beta-lactam ring is marked as red [18].

Cefotaxime works by binding penicillin-binding proteins (PBPs) via beta-lactam (**Figure 7**) rings and this inhibits the final transpeptidation step of peptidoglycan synthesis in cell wall [17]. Because of ongoing activity of cell wall autolytic enzymes in the absence of cell wall assembly, bacteria eventually lyse [17]. Due to its structural configuration of the molecule, cefotaxime provides an advantage of resistance to beta-lactamase degradation. The stability against β-lactamases is conferred by the syn-configuration of the methoxyimino moiety (**Figure 7**) [17], [19]. Cefotaxime and is usually administered intravenously or intramuscularly. The dosage and duration of treatment depend on the type and severity of the infection, as well as the patient's age, weight, and medical history [17]. Like all antibiotics, cefotaxime and can cause side effects, although not everyone experiences them. Common side effects include diarrhea, nausea, vomiting, and rash. More serious side effects, such as allergic reactions, liver and kidney problems, and blood disorders, are rare [17].

1.2.2 Minimal inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration (in µg/mL) of antibiotic that prevents visible bacterial growth. Effective antibiotics have low MIC values and as bacteria become less sensitive to antibiotics, MIC values increase [20]. The MIC curve is a graphical illustration of the relationship between the concentration of the antimicrobial agent and the growth response of the microorganism. On the x-axis is the logarithm of the drug concentration and on the y-axis is the growth response (such as optical density or turbidity). The curve shows at what concentration the antimicrobial agent inhibits the growth of the microorganism[21], [22]. With the increasing resistance of bacteria to antibiotics and the increasing frequency of unsuccessful treatment of infections, there is a need to identify the underlying causes of this problem. In addition, methods are being explored to reduce infection and improve the efficacy of

infection [21]. MIC helps to determine which class of antibiotic is most effective to treat the infection and to help in the fight to slow antibiotic resistance (**Figure 8**) [20], [22].

The MIC determination methods are [21]:

1) **Dilution methods**: in agar on a liquid medium;
2) **Gradient methods**: strips impregnated with a pre-defined concentration gradient of antibiotics.

All quantitation methods use Mueller-Hinton medium in agar or broth form to determine MIC values. In some cases, depending on the type of bacteria and antibiotics, additional supplementation may be required, for example 5% lysed horse blood or other compounds. Determination of MIC by dilution method requires the presence of antibiotics in the material. Antibiotics should first be dissolved to obtain a stock solution and then diluted to obtain the proper starting concentration. Water is both a solvent and a diluent for most antibiotics [21].

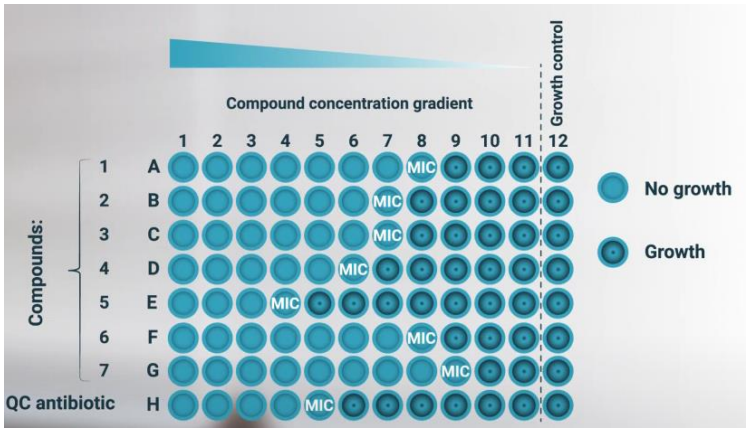

Figure 8. Schematic overview of MIC test in 96-well plate. Different antibiotic dilutions are made in order to determine the MIC value. The concentrations on the graph go from highest to lowest. QC antibiotic uses a reference strains with defined susceptibility to the antibiotic. Growth control is a bacteria without antibiotic [20].

1.2.3 Antibiotic resistance mechanisms in bacteria

Antimicrobial resistance (AMR) is the ability of microorganisms (such as bacteria, viruses, fungi, and parasites) to resist the effects of antimicrobial drugs that were previously effective in treating infections [23]. This occurs when microbes evolve mechanisms to defend themselves against antimicrobial agents, rendering the drugs less effective or completely ineffective [24]. AMR is a global public health threat that can lead to increased illness, disability, and death. It can make it harder to treat infectious diseases, such as pneumonia, tuberculosis, and bloodstream infections, and can also make routine medical procedures, such as surgery and chemotherapy, more dangerous due to the increased risk of infections that cannot be easily treated [24], [25].

There are several factors, that are accelerating the rate of AMR [25]:

1) **Misuse and overuse of antibiotics:** several surveys show that antibiotics will help with viral diseases, like flu or common cold. Also, in developing countries, where medicine and diagnostic is not fully developed, antibiotics are easily prescribed. Administering antibiotics, when they

are not needed, is a common misuse. Therefore, a lot of cases in developing countries with poor antibiotics quality are found [25];

2) **Agricultural use of antibiotics:** around 75% of the world's antibiotics are used on farmed animals [26]. Antibiotics are not only used to treat sick animals but are also widely added to healthy animal feed and drinking water as a preventative measure [25]. On the 28th of January 2022, the European Union's new laws come into force, banning farmed animals from being routinely fed a diet of antibiotics. As a result of the new regulations, only sick animals may be administered antibiotics [26];
3) **Increase in income levels**: between 2000 to 2015, antibiotic use arose 65%. This is due to the overuse of antibiotics in developing countries and which is the direct result of rising incomes. Also, this has led to an increase in animal protein consumption, meaning even more antibiotics are added to the animal food [25];
4) **Easy travel routes**: the modern and easy traveling routes for people, animals, and goods have also contributed substantially to the spread of antimicrobial resistance. It is highly likely that human travelers will return to their country colonized and infected as a result of exposure to resistant pathogens [25];
5) **Biological factors:** antimicrobial resistance can happen through mutation and bacterial evolution. Plasmids can contain a variety of resistance genes through transposons and insertion sequences. As a result, these plasmids are capable of being transferred to bacteria from other species and spreading antibacterial resistance throughout the bacterial population [25].

According to the WHO (the World Health Organization), in 2021, there were only 27 new antibiotics in clinical trial against priority pathogens (*Acinetobacter*, *Pseudomonas* and various *Enterobacteriaceae*) [27]. In 2017 the number of new antibiotics was increased up to 31 (thirty one). Only 6 out of 27 antibiotics fulfil at least one of the WHO's criteria for innovation. Despite the limited number of new antibiotics that reach the market, the lack of innovation undermines their effectiveness rapidly. On average resistance is reported to most new agents 2–3 years post market entry, but it takes approximately 10-15 years to get from the preclinical to the clinical stage. In order to accelerate and expand the pipeline for antibiotics, governments and the private sector must make urgent and concerted investments in research and development [28]. According to the WHO, antimicrobial resistance is among the top 10 health threats facing the world today. Approximately 1.27 million deaths were directly attributed to drug-resistant infections in 2019, according to recent estimates. Approximately 10 million deaths are expected to occur each year by 2050 (**Figure 9**) [23].

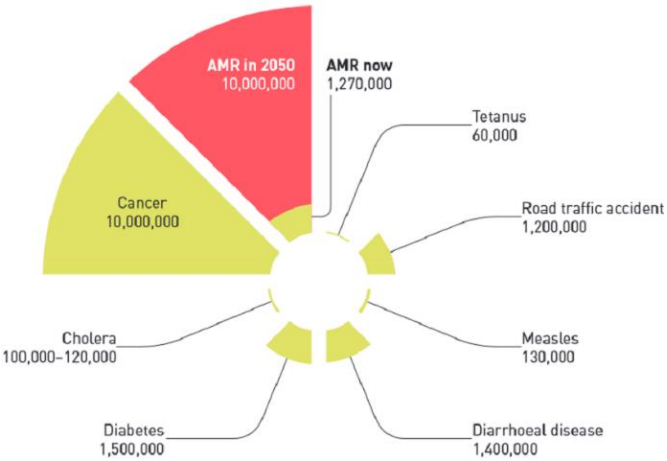

Figure 9. Predicted mortality from antimicrobial resistance compared to common causes of death today [23], [29].

Bacteria can develop resistance to antibiotics through various mechanisms. Some of the common mechanisms of bacterial resistance to antibiotics are (**Figure 10**) [30]:

1) **Mutation**: Bacteria can undergo genetic mutations that result in changes to their DNA, which can lead to resistance to certain antibiotics[31]. This can happen spontaneously or due to exposure to antibiotics [30], [31].
2) **Enzymatic degradation**: Some bacteria can produce enzymes that break down antibiotics, rendering them ineffective [31]. For example, beta-lactamase is an enzyme produced by some bacteria that can break down beta-lactam antibiotics, such as penicillin and cephalosporins [30], [31].
3) **Efflux pumps**: Some bacteria can pump antibiotics out of their cells before the drugs can reach their target site [31]. This is accomplished through specialized proteins called efflux pumps, which actively transport antibiotics out of the bacterial cell [30], [31].
4) **Target site modification**: Some bacteria can modify the target site of antibiotics, preventing the drugs from binding to their intended target. For example, some bacteria can modify their ribosomes, which are the targets of many antibiotics that inhibit protein synthesis [30], [31].
5) **Impermeable cell membrane**: Some bacteria can modify their cell membrane to prevent antibiotics from entering their cells. This can happen through a variety of mechanisms, such as modifying the structure of the cell membrane or reducing the number of channels through which antibiotics can enter [30], [31].
6) **Biofilm formation**: Some bacteria can form biofilms, which are communities of bacteria that are highly resistant to antibiotics. Biofilms can provide a physical barrier that prevents antibiotics from reaching the bacteria within, as well as a protective environment that can shield bacteria from the effects of antibiotics [30], [31].

These mechanisms can act alone or in combination to confer resistance to antibiotics. The development and spread of antibiotic resistance are major public health concerns, as they can lead to the emergence of bacterial infections that are difficult or impossible to treat with existing antibiotics [30].

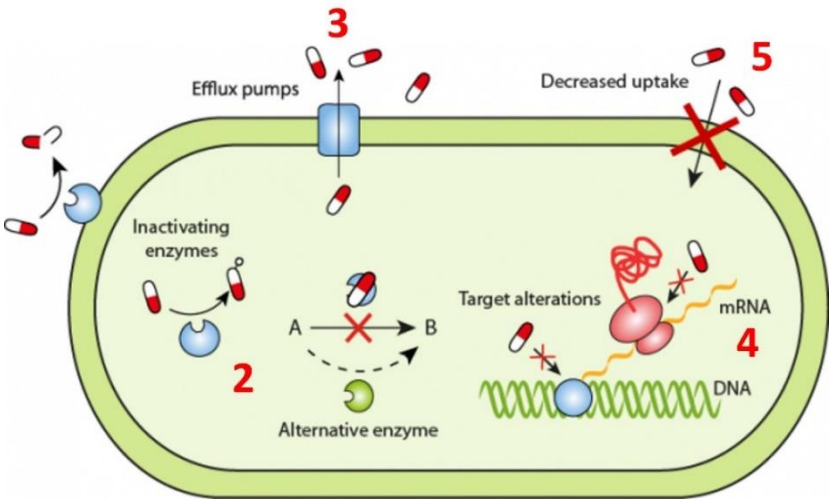

Figure 10. Different resistance mechanisms in bacteria. (2) Bacteria can produce or inactivate enzymes that break down antibiotics. (3) Bacteria can pump out antibiotics through efflux pumps. (4) Bacteria can modify the target site of antibiotics. (5) Some bacteria can modify their cell membrane to prevent antibiotics from entering their cell [31].

1.3 Micro- and nanoplastic

Microplastics are small (are larger than 1 µm and smaller than 5 mm in size) plastic particles that are present in the environment, including in oceans, rivers, soils, and air [32]. Most commonly found micro- and nanoplastic types are polypropylene, low-density polyethylene, polyethylene terephthalate, polystyrene, polycarbonate[33], [34]. Micro- and nanoplastic can be found in oceans, rivers, lakes, soil etc [35]. There is also growing recognition that micro- and nanoplastics (MNPs) present emerging health risks, particularly in food. Plastics passing through the gastrointestinal tract have been associated with disruptions of the gut microbiome [36].

Nanoplastics, on the other hand, are defined as plastic particles that are smaller than 1 um in size. Because of their smaller size, nanoplastics can potentially have greater impacts on the environment and human health than microplastics [37], [38]. For example, nanoplastics may be more easily taken up by organisms and transported throughout the body, potentially leading to toxicity and other health effects [36]. However, because nanoplastics are relatively new and their environmental impacts are not well understood, more research is needed to fully understand their potential risks and to develop strategies to ease their effects[36], [39], [40].

1.3.1 Microplastic in nature

Microplastics in the environment can have a range of impacts on ecosystems and wildlife. They can be ingested by a variety of organisms, including plankton, fish, and seabirds, which can lead to physical damage to their digestive systems and reduced feeding rates [34]. Microplastics can also act as a vector for the transport of pollutants and harmful chemicals, which can accumulate in the tissues of organisms and move up the food chain [41].

In addition, microplastics can have indirect impacts on ecosystems by altering physical and chemical properties of soils and water bodies [32], [33]. For example, microplastics in soil can reduce water infiltration rates and increase soil erosion, while microplastics in water can affect light penetration and nutrient cycling [33].

The presence of microplastics in the environment is a growing concern due to their potential impacts on ecosystems and human health [34], [36]. Efforts to reduce the release of microplastics into the environment include the development of biodegradable plastics, the reduction of plastic waste, and the implementation of policies and regulations to limit the use of single-use plastics [38], [41].

Pollution with micro- and nanoplastic is thought to cause risks to aquatic ecosystems [32], [37]. Microplastics (MPs), have increasingly been detected in various aquatic and marine environments, which has raised scientific interests and public health concerns [32], [42]. Microplastics may derive from manufactured raw plastics, including microbeads in facial cleanser, scrubbers and virgin plastic pellets [43], [44].

Microplastics may undergo complex transformations (e.g., aging, fragmentation, and aggregation) and interactions with environmental matrix or species such as bacteria and pollutants. The physicochemical properties of microplastics (e.g., shape, size, concentrations, surface charge, and hydrophobicity) affect the transformation, interaction, fate and bioavailability to aquatic organisms [44].

1.3.2 Microplastic related to antibiotic resistance

Research has suggested that microplastics in the environment could contribute to the development of antibiotic resistance in bacteria [45]. One study found that exposure to microplastics increased the ability of bacteria to survive exposure to antibiotics [46]. The researchers suggested that this could be caused by the surface of microplastics is suitable for bacterial growth. For instance, the surface can be an initial attachment substrate for the bacteria to make biofilms. Furthermore, the biofilms can provide a protective environment that allows bacteria to resist the effects of antibiotics [45], [47].

In addition, microplastics in the environment can absorb and concentrate pollutants, including antibiotics and other chemicals. This can create an environment in which bacteria are exposed to low levels of antibiotics over long periods of time, which could promote the development of antibiotic resistance [45].

More research is needed to fully understand the relationship between microplastics and antibiotic resistance. However, the potential for microplastics to contribute to the development and spread of antibiotic resistance highlights the need for strategies to reduce the release of microplastics into the environment and to improve the management of plastic waste [45].

Plastics have been accumulated offshore and in the deep oceans at an unprecedented scale. Plastic does not biodegrade in the environment, but fragments into smaller fragments as it interacts with

the environment. As a result of the presence of the plastisphere, microbes are able to attach and produce biofilms [47]. Microbial communities have colonized the plastisphere, which has become a reservoir for both antibiotic and metal resistance genes (ARGs and MRGs) [47]–[49]. This combination of being surrounded by pollutants while being protected by biofilm can lead to possible change in the microbial species distribution. Heavy metals are circulating in the environment as a result of industrial activities such as mining, fertilizer use, etc. Pollution with heavy metals drives the selection of MRGs and correlates with an increase in the occurrence and amount of ARGs (**Figure 11**) [47].

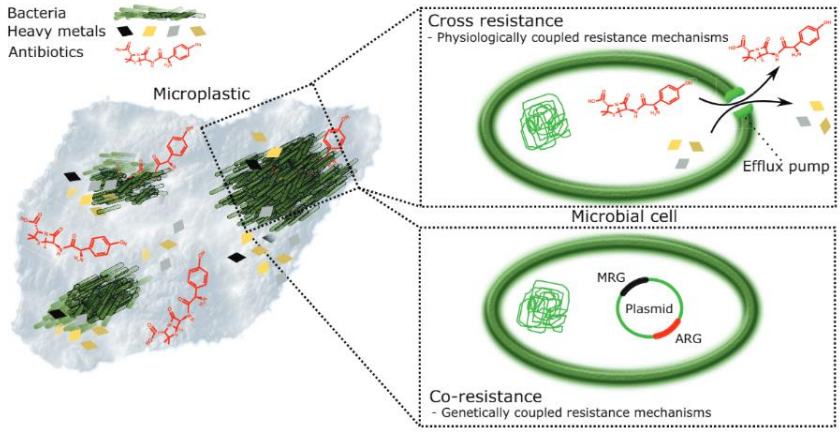

Figure 11. Microplastic can create an environment for heavy metals and antibiotics. These ecosystems can promote antimicrobial resistance through two pathways: i) **Cross-resistance**, where heavy metal and antibiotic resistance mechanisms are integrated physiologically (efflux pumps); ii) **Co-resistance**, in which resistance genes for both antibiotics and metals are present on the same genetic element and are genetically correlated[47].

2. AIMS OF THE STUDY

The main objective of the thesis was to investigate effect of microplastic on antimicrobial resistance via droplet-based platform.

The specific aims of the current study were:

1) to determine whether droplets remain stable with encapsulated microplastic;

2) to evaluate whether MIC curves are similar in mono- and polydisperse droplets;

3) to find out if microplastic has an effect on antimicrobial susceptibility in droplets.

3. MATERIALS AND METHODS

Overview of how each aim is addressed:

Aim 1 – 1 µm and 10 µm microplastic were encapsulated in droplets to determine droplet stability over time

Aim 2 – Standard MIC test is performed in droplets (both mono- and polydisperse) and 96-well plate to determine if MIC curves are similar within mono- and polydisperse droplets

Aim 3 – MIC test is performed in polydisperse droplets while encapsulated with 10 µm microplastic to determine the effect on antimicrobial susceptibility

3.1 Droplet generation and analysis (Aim 1)

3.1.1 Generation of polydisperse droplets

Materials and equipment used for generating polydisperse droplets:

1) 5 mL test-tube
2) Dextran, Alexa FluorTM 647, 10,000 MW, anionic, fixable (Invitrogen, Life Technologies Corporation), stock concentrations 100 µg/mL
3) Luria-Bertani (LB) Broth (Biomaxima, Poland)
4) Oil: Novec HFE 7500 fluorocarbon oil (3M, USA)
5) Surfactant: droplet microfluidics surfactant synthesized according to protocol [50] (Obtained as a gift from Prof. Garstecki, ICHF PAN, Poland)
6) Test-tube shakers Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
7) Polystyrene 1 um and 10 um microplastic spheres 10% solids (+- 0.5%) in milli-Q Ultrapure water (Obtained as a kind gift from Margit Heinlaan, National Institute of Chemical Physics and Biophysics, Tallinn)
8) Countess™ Cell Counting Chamber Slides (ThermoFisher Scientific)
9) Orbital shaker incubator ES-20 (Biosan, Latvia)

Polydisperse water-in-oil droplets were generated for testing droplet stability while polystyrene microplastic spheres were encapsulated inside. The droplets were generated by mixing together two immiscible liquids: water-based and oil-based. Water phase consisted of polystyrene, pure water and Alexa fluorescent dye at concentration of 2 µg/mL. The dye was added to simplify further droplet image analysis. Oil phase consisted of HFE 7500 hydrocarbon oil with 2% of surfactant. Surfactant is necessary to keep the droplets stable over time.

Then the tube was vortexed for 5 seconds to emulsify the solutions into polydisperse droplets. Droplets were incubated at 37^{o}C overnight.

3.1.2 Generation of monodisperse droplets

Materials and equipment used for generating monodisperse droplets:

1) 5 mL test-tube
2) Dextran, Alexa FluorTM 647, 10,000 MW, anionic, fixable (Invitrogen, Life Technologies Corporation), stock concentrations 100 µg/mL
3) Luria-Bertani (LB) Broth (Biomaxima, Poland)
4) Oil: Novec HFE 7500 fluorocarbon oil (3M, USA)
5) Surfactant: droplet microfluidics surfactant synthesized according to protocol [50] (Obtained as a gift from Prof. Garstecki, ICHF PAN, Poland)
6) Test-tube shakers Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
7) Syringe Pump system
8) Polystyrene 1 um and 10 um microplastic spheres 10% solids (+- 0.5%) in milli-Q Ultrapure water (Obtained as a kind gift from Margit Heinlaan, National Institute of Chemical Physics and Biophysics, Tallinn)
9) PDMS (poly-[dimethylsiloxane]) microfluidic chip
10) Orbital shaker incubator ES-20 (Biosan, Latvia)

Monodisperse droplets are generated using pump system, that consists of two syringe pumps – oil and surfactant. Pump system is controlled with computer. Monodisperse droplets are generated using microfluidic chip (**Figure 12**).

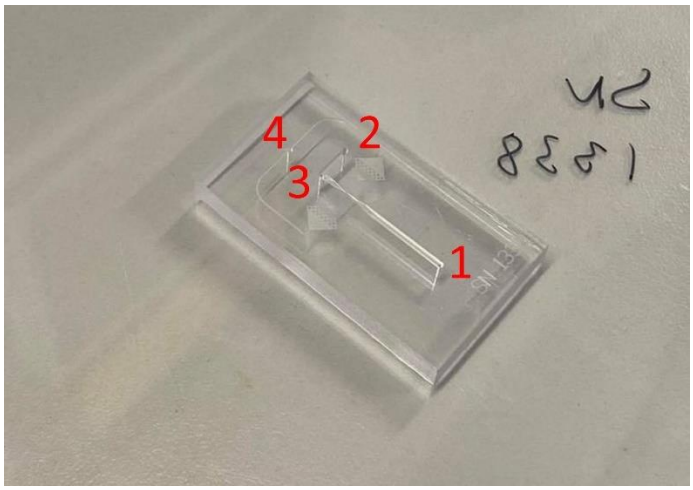

Figure 12. PDMS microfluidic chip for making monodisperse droplets. 1 – Samplet inlet, connected with oil pump; 2 – plastic syringe is connected for flushing out sample and stabilizing pressure; 3 – droplet outlet; 4 – surfactant inlet, connected to surfactant pump.

3.1.3 Analysis of droplets

Droplets were imaged with LSM 900 Laser Scanning Microscope (Zeiss, Germany) running on ZEN 3.3 (blue edition) software with the following settings:

1) Objective Plan-Apochromat 10x (NA 0.45)

2) LED light source Colibri 7
3) Diode lasers 488 nm and 640 nm
4) Green channel: excitation 395 and emission 502
5) Red channel: excitation 653 and emissioon 668
6) Bright-field channel: DIC

Before droplets were analyzed, 18 µL of droplets mix was pipetted into the slide chamber. Droplets were imaged by the confocal microscope. The scanned pictures from the microscope were taken for further analysis in CellProfiler™ (CP) software. With CP it is possible to detect and measure size, shape, intensity and texture of target objects in images. Program user must construct an image analysis pipeline, consisting of modules with different functions [51].

The general CP pipeline for the analysis of polydisperse droplet images was developed by the microfluidics group and is shown on **Figure 13** [51]:

1) **IdentifyPrimaryObjects** module identifies target objects. This module needs grayscale images containing bright objects on a dark background;
2) **MeasureObjectSizeShape** module calculates area and shape of identified objects;
3) **FilterObjects** module removes all objects that do not fulfill parameters from previous modules, which are then discarded. It may also remove objects touching the image border;
4) **MeasureObjectIntensity** module measures intensity features for each object.

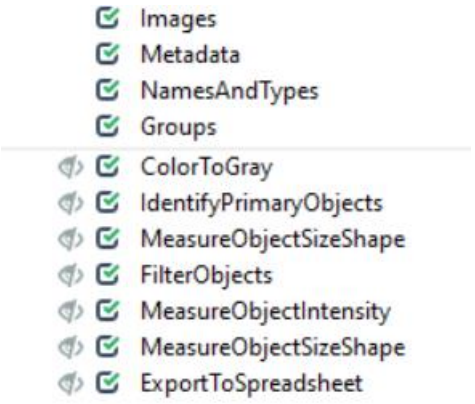

Figure 13. Pipeline for analyzing polydisperse droplets in CellProfiler.

EasyFlow Analytical Web App was used to analyze droplet data from CellProfiler. Easyflow requires only droplet label, size and signal data for a quick profile analysis, in this thesis antibiotic concentration (µg/mL), Volume (nL), Mean Intensity [52]. An Excel .csv file was uploaded for the calculations and visualization of the results (**Figure 14**). In EasyFlow bin numbers and threshold was adjusted to generate figures of the results.

Antibiotic concentration	Volume (nL)	Mean Intensity
0.075	0.647167409	0.016689979
0.075	0.76473053	0.017841743
0.075	0.945381076	0.033800118
0.075	0.61355387	0.019019771
0.075	0.928868983	0.019838948
0.075	1.23648056	0.022286237
0.075	0.514907332	0.018544525
0.075	1.363007376	0.022821696

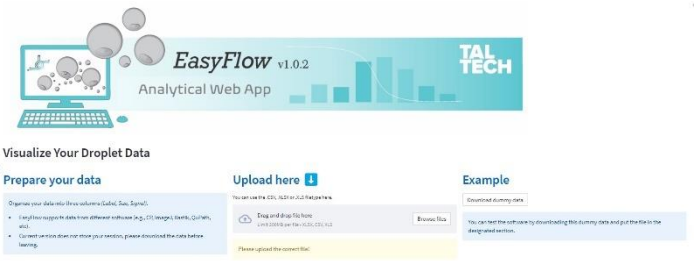

Figure 14. EasyFlow Analytical Web App. An Excel .csv, .xlsx or .xls file is uploaded to the EasyFlow Analytical Web App for data analysis [52].

3.2 Antibiotic MIC test

3.2.1 Antibiotic MIC test in polydisperse droplets (Aim 2)

Materials and equipment used for MIC test in polydisperse droplets were described in 3.1.1, in addition: Cefotaxime Sodium Salt (Carbosynth Limited), stock concentration 10 mg/mL.

Droplets were generated as described in 3.1.1. Nine different antibiotic concentrations were made for the experiment. Cefotaxime's 9 concentrations were – 0.075, 0.056, 0.042, 0.032, 0.024, 0.018, 0.013, 0.010, 0.008 µg/mL. For droplet generation, both water and oil phase was pipetted together into the same 5 mL test-tube:

1) 20 µL cefotaxime;

2) 200 µL oil and 2% surfactant;

3) 180 µL mix:

- 4410 µL of LB
- 90 µL of 100 µg/mL Alexa dye
- 46 µL *E.coli*

In total there were 10 tubes for each antibiotic – with 9 different antibiotic concentrations and 1 control (without antibiotic).

3.2.2 Antibiotic MIC test in monodisperse droplets (Aim 2)

Materials and equipment used for MIC test in monodisperse droplets were described in 3.1.2, in addition:

1) Cefotaxime Sodium Salt (Carbosynth Limited), stock concentration 10 mg/mL

Monodisperse droplets were generated the same way as in described in 3.1.2. The same cefotaxime concentrations were used in the experiment as described in 3.2.1.

3.2.3 Antibiotic MIC test in 96-well plates (Aim 2)

Materials and equipment used for MIC test in 96-well plates:

1) 5 mL test-tube

2) Dextran, Alexa FluorTM 647, 10,000 MW, anionic, fixable (Invitrogen, Life Technologies Corporation), stock concentrations 100 µg/mL
3) Cefotaxime Sodium Salt (Carbosynth Limited), stock concentration 10 mg/mL
4) Luria-Bertani (LB) Broth (Biomaxima, Poland)
5) Test-tube shakers Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
6) 96-well plates
7) Tecan plate reader
8) *Escherichia coli* JEK 1036 with chromosome-incorporated gene encoding the green fluorescence protein (GFP) [53]
9) Orbital shaker incubator ES-20 (Biosan, Latvia)

For the MIC test in 96-well plates the same 9 different cefotaxime concentrations were used. Rows 1-9 represent antibiotic concentrations from lowest to highest for both antibiotic (kanamycin and cefotaxime). In the 10 row was pure LB (negative control) and row 11 was *E. coli* (positive control) (**Figure 15**). Rows 1-9 consisted of 100 µl of bacteria and 100 µl of cefotaxime, 10 row 200 µl of LB and 11 row 100 µl LB and 100 µL bacteria. 96-well plates were incubated at 37°c overnight.

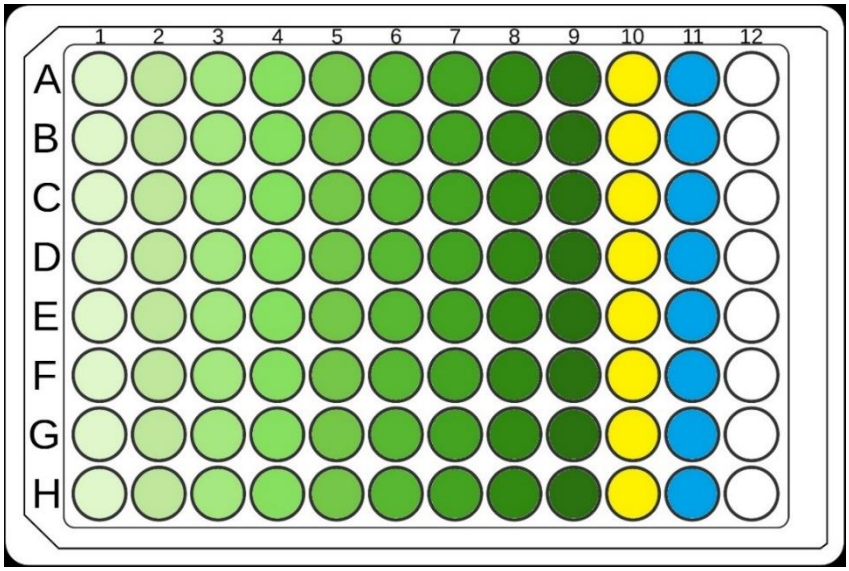

Figure 15. Scheme of the 96-well plate experiment [10]. Light green to dark green represents cefotaxime concentrations from lowest to highest. Yellow represents LB (negative control), blue bacteria (positive control).

3.2.4 Antibiotic MIC test with polystyrene microplastic spheres in polydisperse droplets (Aim 3)

Materials and equipment used for generating polydisperse droplets were described in 3.1.1, in addition:

1) Cefotaxime Sodium Salt (Carbosynth Limited), stock concentration 10 mg/mL
2) Polystyrene 10 um microplastic spheres 10% solids (+- 0.5%) in milli-Q Ultrapure water (Obtained as a kind gift from Margit Heinlaan, National Institute of Chemical Physics and Biophysics, Tallinn)

Droplets were generated as described in 3.1.1. Cefotaxime's 9 concentrations were used – 0.075, 0.056, 0.042, 0.032, 0.024, 0.018, 0.013, 0.010, 0.008 μg/mL. For droplet generation, both water and oil phase was pipetted together into the same 5 mL test-tube:

1) 20 μL antibiotic
2) 200 μL oil and 2% surfactant
3) 180 μL mix:
    - 2023 μL of LB
    - 45 μL of 100 μg/mL Alexa dye
    - *23* μL *E.coli*
    - 150 μL Polystyrene 10 um microplastic spheres in milli-Q Ultrapure water

In total there were 20 tubes – 9 different cefotaxime concentrations and 1 control (without antibiotic) with polystyrene 10 um microplastic spheres, other 9+1 tubes without polystyrene 10 um microplastic. Droplets were incubated at 37°C overnight.

Protocol for the analysis of polydisperse droplets in CellProfiler was described in 3.1.3.

4. RESULTS AND DISCUSSION

4.1 Mono- and polydisperse droplets remain stable with encapsulated microplastic

In this thesis the first aim was to test if droplets remain stable with encapsulated microplastic. For this two different kind of droplet types were used – monodisperse and polydisperse droplets. Stability of droplets was tested by comparing average droplet diameter after 24h incubation.

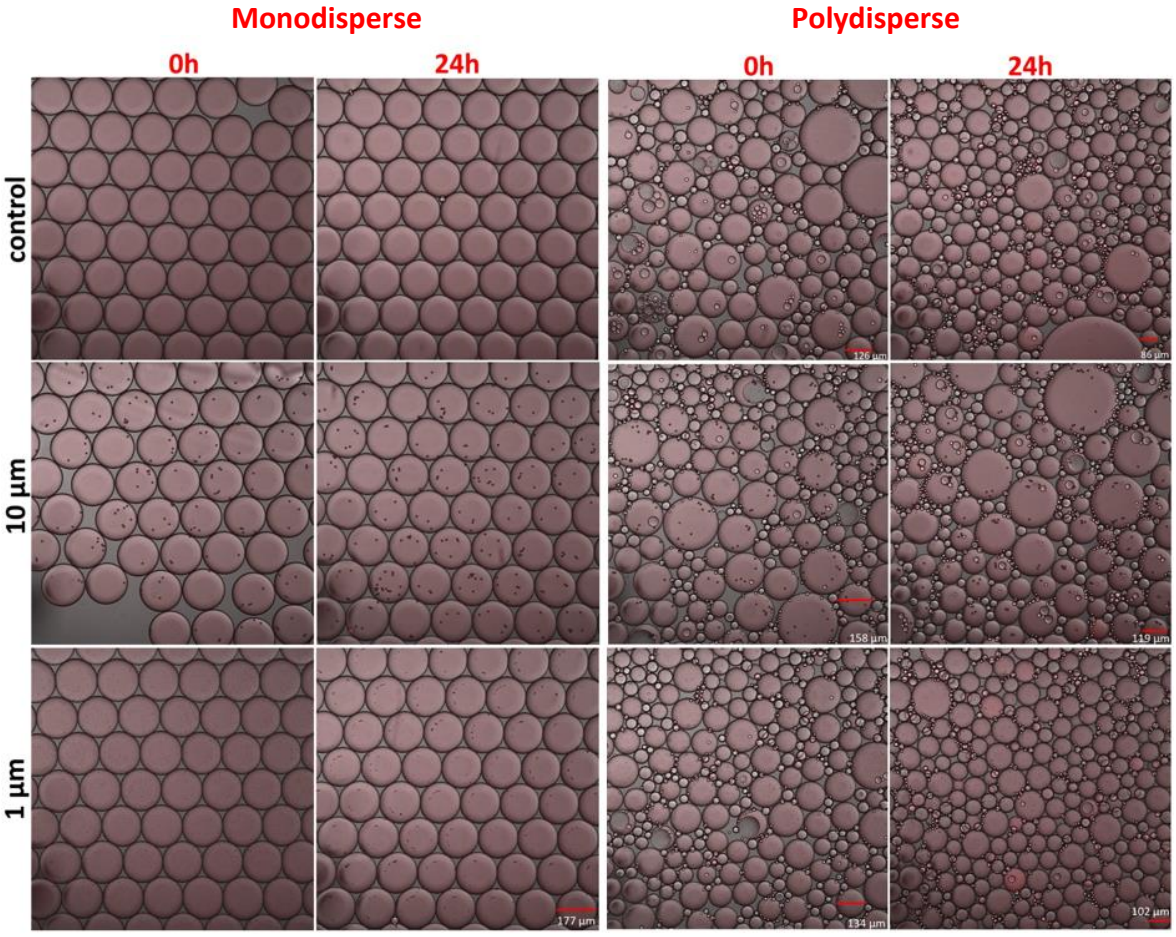

Figure 16. Mono- and polydisperse droplets control, 10 µm and 1 µm at 0h and 24h. Droplets were imaged after the droplet generation at 0h. After 24h incubation at 37°C droplets were again imaged using confocal microscope. Control had no microplastic encapsulated inside the droplets. 1 and 10 µm polystyrene microplastic spheres were used in this experiment. Scale bars indicate droplet diameter in µm.

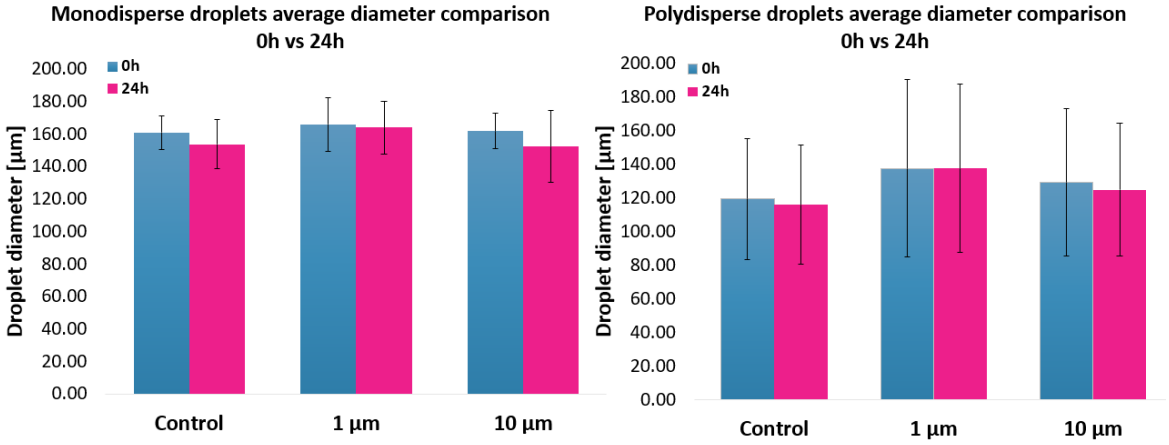

Figure 17. Mono- and polydisperse droplets average diameter comparison at 0h vs 24h. Graphs: Control, 1 µm and 10 µm (x-axis); Average droplet diameter in µm (y-axis) – shows average droplet diameeter in µm (mono- and polydisperse). Error bars indicate average diameter standard deviation. Blue marks 0h and pink after 24h incubation.

Monodisperse droplets encapsulated with 1 µm microplastic did not show any significant differences at 0h or 24h. At 0h the average diameter for monodisperse droplets with 1 µm microplastic was 165.88 µm with standard deviation of 16.35 and after 24h the diameter was 163.88 µm and standard deviation 16.13. With monodisperse droplets encapsulated with 10 µm microplastic the average diameter at 0h was 161.81 µm and standard deviation of 11.04. After 24h the diameter was 152.35 µm and standard deviation 21.89. Control monodisperse droplets had no microplastic encapsulated inside the droplets. The average diameter at 0h was 160.76 µm and standard deviation 10.32. After 24h the diameter was 153.73 µm and standard deviation 15.01. Every monodisperse droplet experiment had ~1600 droplets it total and exact numbers can be found in a **Table 1** in **Appendix 1**. The number of droplets depends how much sample and oil/surfactant is pipette into the slide and also how well CellProfiler's pipeline detects droplets.

In polydisperse droplets no significant differences were found between the control, 1 µm and 10 µm droplets. The most similar were polydisperse droplets encapsulated with 1 µm microplastic – at 0h the average droplet diameter was 137.33 µm with standard deviation of 52.73. After 24h the droplet average diameter was 137.52 µm with standard deviation of 50.22. Droplets encapsulated with 10 µm microplastic had an average diameter 128.96 µm at 0h with standard deviation of 43.75. After 24h the diameter was 124.76 µm and standard deviation 39.43. Control droplets had no microplastic inside. The average diameter at 0h was 119.28 µm with standard deviation of 36.08. After 24h the diameter was 115.92 µm and standard deviation 35.42. Every polydisperse droplet experiment had ~1600 droplets it total and exact numbers can be found in a **Table 1** in **Appendix 1**.

The results indicate that polystyrene microplastic spheres have no effect on droplet stability, Since the encapsulated microplastic did not have a noticeable effect on the droplet size distribution. For monodisperse droplets the average diameter was consistently around 165 µm with standard deviations of approximately 16 µm after 24h incubation at 37oC. For both mono- and polydisperse

droplets the standard deviations for 1 µm and 10 µm microplastic groups remained relatively consistent throughout the 24-hour period. Number of droplets was also similar after 24h, although this number is also highly dependant on how many droplets are scanned by the microscope and how many are detected via the software. In conclusion, these results confirm that microplastic can be used, without affecting the droplet stability, for further and more complicated experiments involving bacteria and antibiotics for aim three of this thesis.

4.2 MIC curves are similar in mono- and polydisperse droplets

The second aim of the thesis was to check if droplet generation method affects antibiotic MIC results. Monodisperse and polydisperse droplets were with different antibiotic concentrations and their MIC results were compared. Effect of droplet method was evaluated on similarity of the MIC curves. Additionally, MIC was also generated via 96-well plate in order to compare both droplet methods to the laboratory standard MIC bulk method.

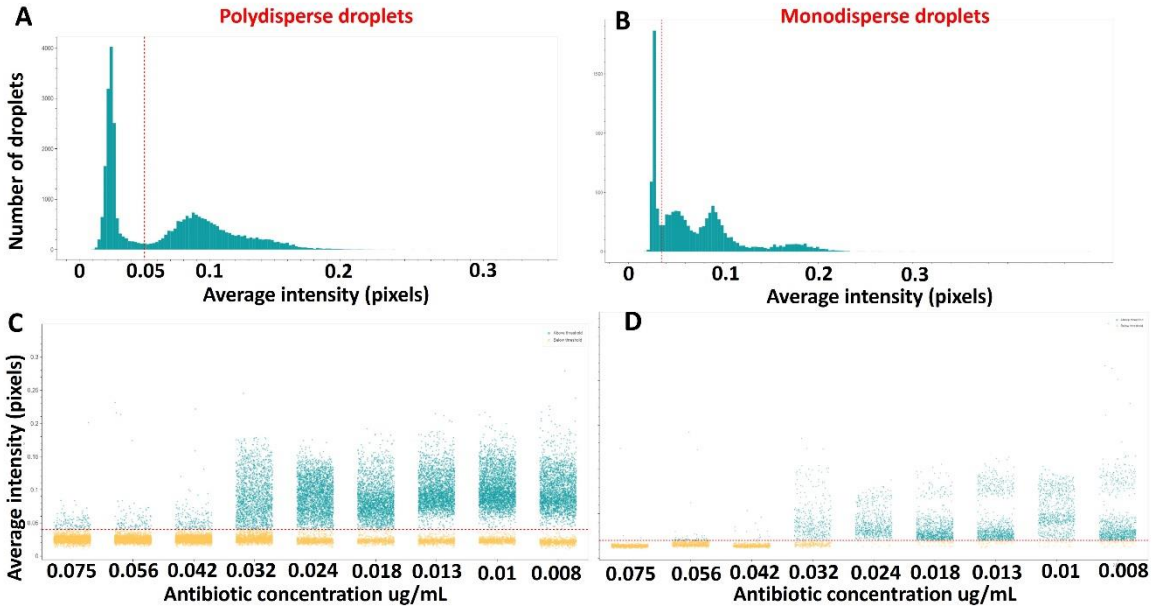

Figure 18. Mono- and polydisperse droplet distribution and comparison of experimental conditions. Graphs: A) Average Intensity (pixels) (x-axis) vs Number of droplets (y-axis) – shows polydisperse droplet signal distribution, threshold is 0.04; B) Average Intensity (pixels) (x-axis) vs Number of droplets (y-axis) – shows monodisperse droplet signal distribution, threshold is 0.04; C) Antibiotic concentration µg/mL (x-axis) vs Average intensity (pixels) (y-axis) – shows polydisperse droplet distribution at 9 different antibiotic concentrations (highest concentration is on the left); Antibiotic concentration µg/mL (x-axis) vs Average intensity (pixels) (y-axis) – shows monodisperse droplet distribution at 9 different cefotaxime concentrations.

Graph (**Figure 18**) threshold is shown as a red line on each figure (A-D). Negative and positive droplets are classified according to threshold. Analyzing the figures manually allows us to determine the threshold. Lowest bin between two peaks is determined as the threshold value. On figures C-D, yellow color indicates negative droplets and blue positive droplets, meaning bacteria grew inside the droplet. Exact number of droplets in total can be found in **Table 2** in **Appendix 2**.

When comparing mono- and polydisperse droplet results (**Graphs A-B**), positive and negative droplet results are similar. Poly- and monodisperse droplet distribution had a threshold of 0.04, meaning droplets below the threshold are empty droplets (without bacteria) and over the threshold are positive droplets. Both had two clearly distinguishable fluorescence peaks.

The same results are also confirmed in Graphs C-D. In poly- and monodisperse droplets the bacteria started to grow from the concentration 0.032 µg/mL. In three highest concentrations the growth was zero or minimal.

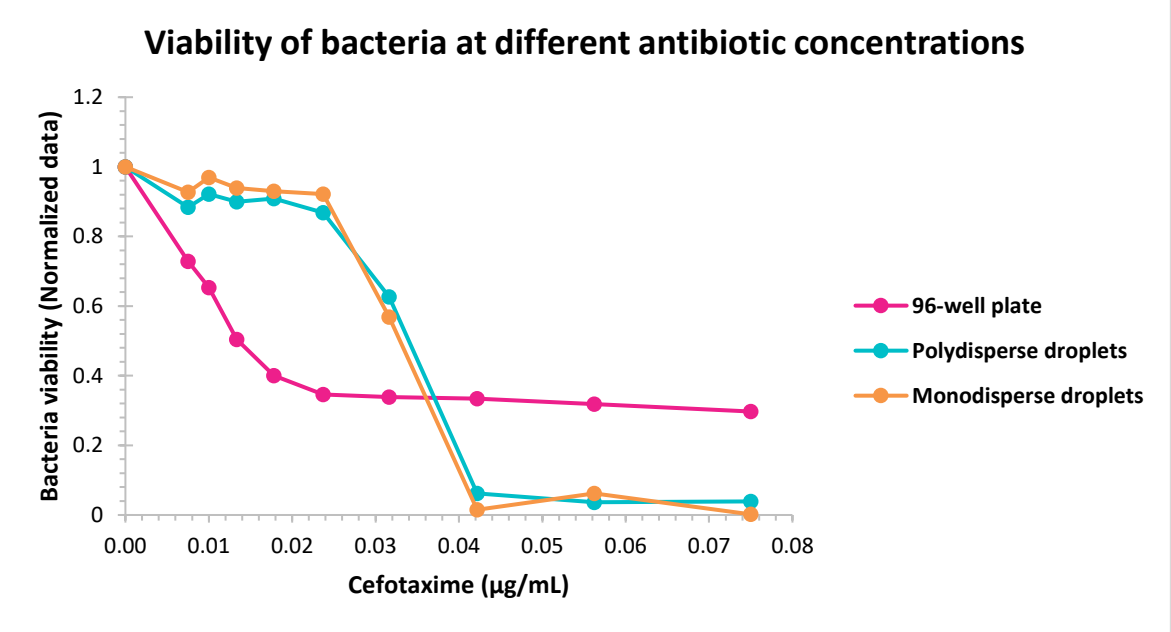

Figure 19. Cefotaxime MIC curves in 96-well plate, mono- and polydisperse droplets. On x-axis is cefotaxime concentrations µg/mL. On y-axis is bacteria viability – control without cefotaxime is 1 meaning bacteria is 100% viable. Other data points are obtained from 1/ (each cefotaxime concentration positive droplet fraction).

Poly- and monodisperse droplet MIC curves had no significant difference (**Figure 19**). In both the bacteria's viability was low in three highest cefotaxime concentrations (0.075, 0.056, 0.042 µg/mL). Below 0.042 µg/mL the viability started to rise until it reached almost to the same level as control (no antibiotic). 96-well plate had a higher MIC due to bacteria being more resistant as a population/group than as individuals (i.e. single cell inside droplets). In the future more repeats with higher antibiotic concentrations included of the 96-well plate is needed.

In conclusion, mono- and polydisperse MIC results are similar, so in further experiments polydisperse method is used, because droplet generation is easy and quick and no need of special equipment to make droplets. The experiment also highlights that single-cell research is important in studying population heterogeneity and discovering unique characteristics of individual cells. In droplets a single bacterium gets encapsulated inside the droplet, which enables seeing the antibiotic effect on individual bacterium. In 96-well plates a single well consists of a whole bacteria population, meaning it does not provide direct observation of individual bacterium. In droplets

direct observation and analysis of individual bacterial cells, provides more detailed information about their phenotypic behavior and responses to specific enivornmental stimuli (e.g. antibiotic).

4.3 Microplastic increase the antimicrobial resistance

In this study the aim was to see if microplastic affects antibiotic resistance in bacteria. The 10 µm microplastic was chosen for this experiment due to having more surface area for bacteria to form the biofilm, to see the microplastic in the droplets and thus the possible biofilm formation clumping around the microplastic. Polydisperse droplets were generated with different antibiotic concentrations and their MIC results were compared. Half of the tubes (10 tubes in total) had 10 µm microplastic inside the polydisperse droplets. Effect of the 10 µm microplastic was evaluated on differences of the MIC curves.

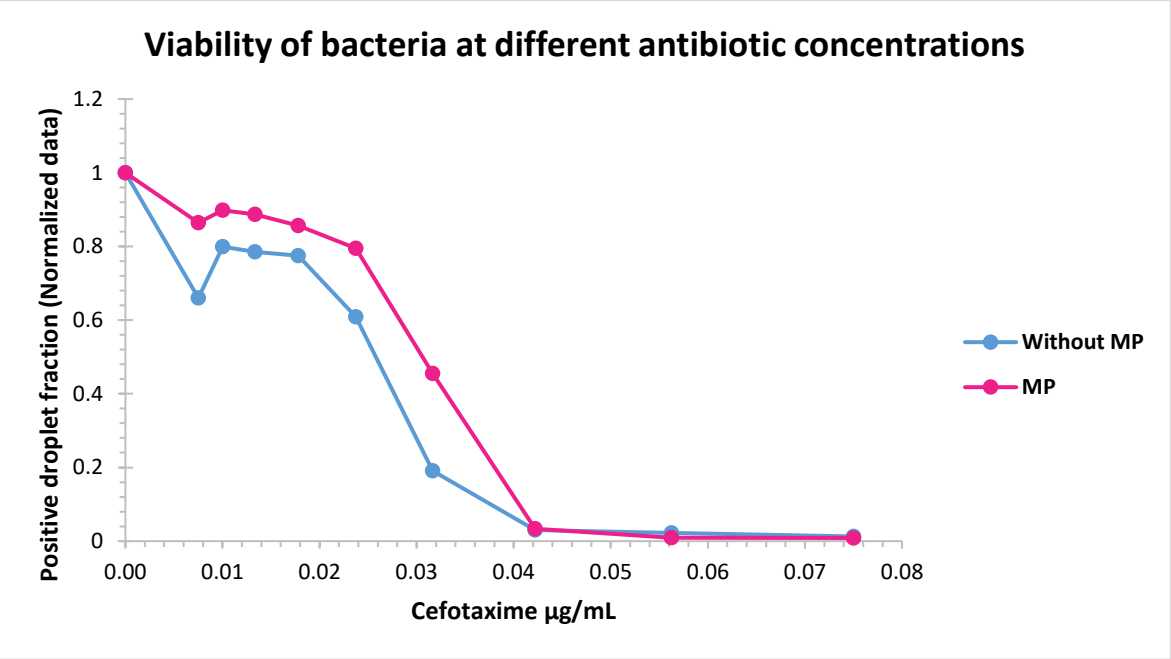

Figure 20. Viability of bacteria at different antibiotic concentrations. Droplets encapsulated with microplastic had more positive droplets compared to droplets without microplastic at different antibiotic concentrations. X-axis shows the cefotaxime concentration (µg/mL) and y-axis presents positive droplet fraction.

Microplastic seems to increase resistance to cefotaxime (**Figure 20**). From the concentration 0.04 ug/mL the positive droplet fraction is higher than droplets without microplastic. Two highest concentrations (0.075 and 0.056 µg/mL) had the lowest positive droplets for both cases. Overall the polydisperse droplets encapsulated with 10 µm microplastic had a higher number of positive droplets.

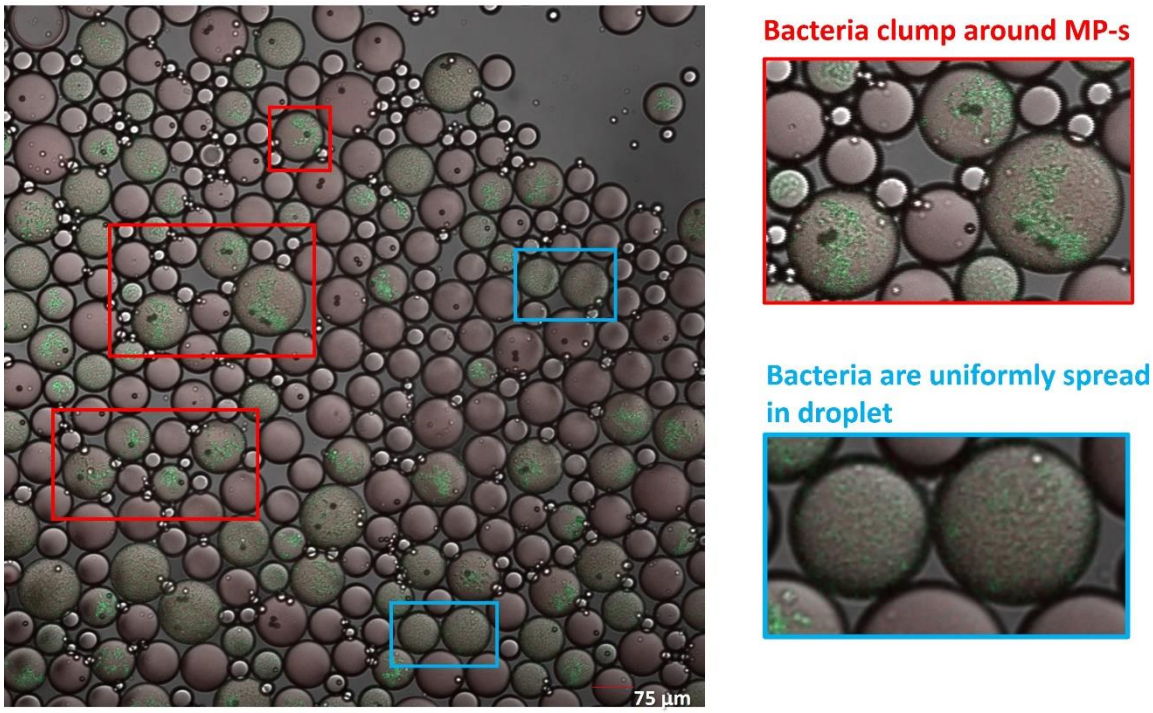

Figure 21. Bacteria clump around microplastic. Image shows polydisperse droplets encapsulated with 10 µm polystyrene microplastic spheres (Cefotaxime concentration 0.008 µg/mL). **Red** box shows an example how bacteria tend to clump around microplastic, **blue** shows that droplets without microplastic the growth of bacteria is more uniformly spread.

Bacteria clump around polystyrene microplastic spheres compared to droplets without microplastic (**Figure 21**). It indicates that the bacteria are becoming less responsive or resistant to the inhibitory effect of cefotaxime. The change in MIC response may be explained by possible biofilm formation. Biofilms are structured communities of bacteria that are encased within a protective matrix. Microplastics in water can form a suitable base for biofilm formation due to their large and rough specific surface area. Microplastic can become colonized within 24h depending on a variety of factors [40], [45]. Due to formation of biofilms, the growth environment for bacteria could be modified, potentially affecting the MIC values and the resulting higher MIC curves. Microplastic can also potentially adsorb and accumulate antimicrobial compounds in the environment (droplets). When bacteria come into contact with microplastics carrying adsorbed antimicrobials, it may influence the local concentration and availability of the antimicrobial agents. This could alter exposure to antimicrobials and thus impact the MIC value and MIC curve (**Figure 20**).

It's important to note that the specific effects of microplastics on MIC curves may vary depending on the type of microplastic, the bacterial species involved, the antimicrobial agent being tested, and the environmental conditions. Further research is needed to better understand the potential interactions between microplastics and microbial susceptibility to antimicrobial agents.

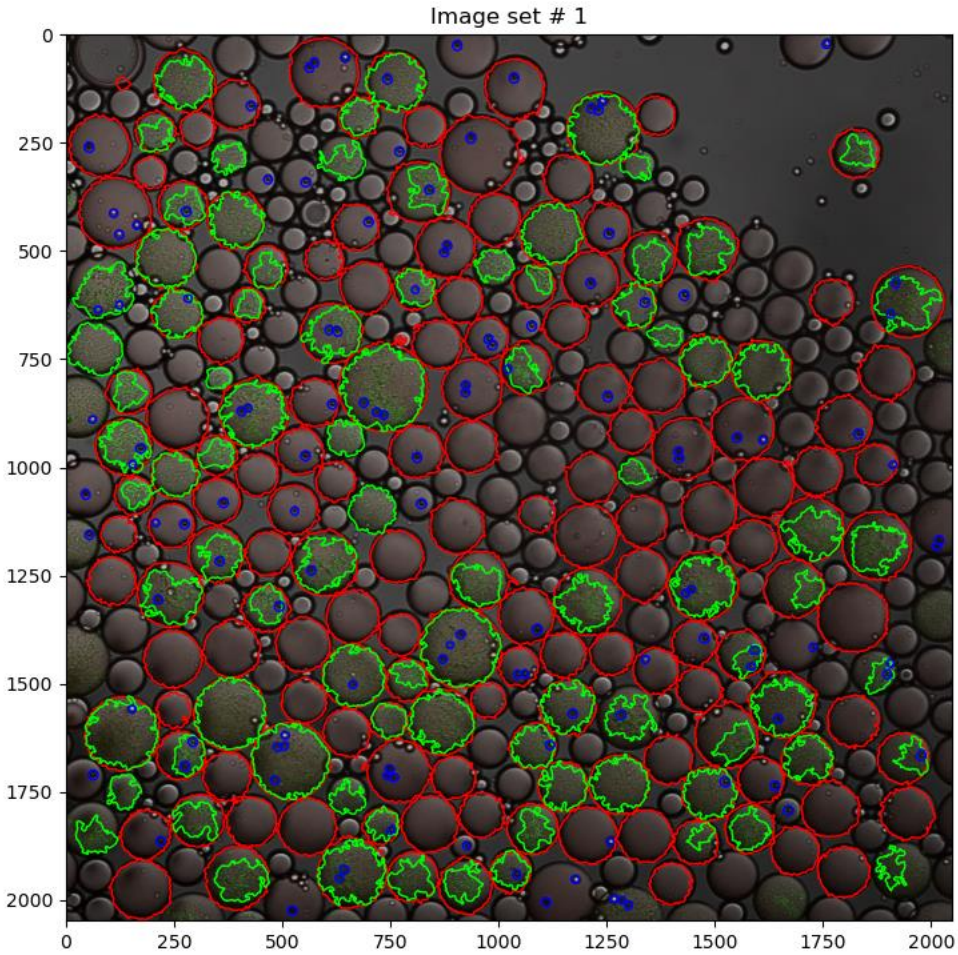

Figure 22. Example of potential future analysis of images gathered during this MSc thesis (e.g. CellProfiler based pipelines currently developed in the microfluidics group) can be used to detect clumped bacteria around microplastic. Blue - microplastic, green - bacteria, red – droplets without bacteria.

It was out of scope for this MSc thesis to look deeper into the relationship between microplastics and, their size and number in droplets. In the future CellProfiler in combination with an additional software called Illastik, could be used to detect clumping of bacteria around microplastic. However, in the Microfluidics group this pipeline for detection is still in the development, with the intention of it being used in future experiments.

ABSTRACT

Antibiotics are a type of antimicrobial substances designed to target bacterial infections. The antibiotic inhibitory mechanisms can be divided into three classes – cell wall, nucleic acid and protein synthesis inhibitors. Antimicrobial resistance is the ability of bacteria to resist antimicrobial treatments. There are several mechanisms how bacteria can develop resistance to antibiotics – mutation, enzymatic degradation, efflux pumps, target site modification, impermeable cell membrane and biofilm formation. Some bacteria can form biofilms, which are highly resistant to antibiotics. Bacteria can attach to the surface of microplastics and form biofilms. Microplastic can be found in oceans, rivers, lakes, soil, meaning bacteria is easily exposed to it.

The main purpose of this Master's thesis was to investigate effect of microplastic on antimicrobial resistance via droplet-based platform. The specific aims were (i) to determine whether droplets remain stable with encapsulated microplastic; (ii) to evaluate whether droplet generation method affects antibiotic MIC results; (iii) to find out if microplastic has an effect on antimicrobial resistance droplet-based platform.

Result 1: Mono- and polydisperse droplets were encapsulated with 1 um and 10 um polystyrene microplastic spheres in order to test stability. After 24h of incubation at 37˚C, mono- and polydisperse droplets remain stable.

Result 2: Next mono- and polydisperse droplets were compared for cefotaxime's MIC results. Mono- and polydisperse droplets resulted in similar MIC results and for further experiment polydisperse droplets were used as they are faster to generate.

Result 3: Polystyrene microplastic spheres in size of 10 µm were used to test if they have an effect on antimicrobial resistance. Droplets with encapsulated 10 µm microplastic had a higher MIC curve, meaning microplastic affected antibiotic susceptibility. It indicates that the bacteria are less responsive to the antibiotic. As a result, the antibiotic becomes less effective.

In conclusion, experiments carried out during this MSc thesis hint that microplastic has an effect on antimicrobial resistance. Bacteria started to clump around microplastic and there were more positive droplets with increase of antibiotic concentration in comparison to control experiment without the microplastic. It could be that microplastic in droplets enhanced the clumping of bacteria, which is the early stage of biofilm, and this in turn led to increased resistance to antibiotic.

KOKKUVÕTE

Antibiootikumid on antimikroobsed ained, mis on loodud batkeriaalsete infektsioonide vastu võitlemiseks. Antibiootikumide toimemehhanismid saab jaotuda kolme klassi – rakuseina, nukleiinhapete ja valgu sünteesi inhibeerimine. Antimikroobne resistentsus on bakterite võime muutuda vastupanuvõimeliseks antimikroobsetele ravimeetoditele. Antibiootikumi resistentsus võib tekkida mitmel viisil – mutatsioonide tekkimine, ensümaatiline lagundamine, pumpade kaudu antibiootikumi rakust eemaldamine, antibiootikumi toime sihtkoha muundamine ja biofilmi teke. Biofilm on mikroorganismide kooslus. Üheks võimalikuks biofilmi moodustamise kohaks looduses on mikroplastiku pind. Looduses esineb väga palju mikroplastiku reostust. Mikroplastikut võib leida põhimõtteliselt igalt poolt: ookeanidest, jõgedest, järvedest, pinnasest ning ka organismidest. See tähendab, et bakterid puutuvad kergesti kokku mikroplastikuga ning saavad sellel moodustada biofilme.

Magistritöö peamine eesmärk oli uurida mikroplasti mõju antimikroobsele resistentsusele tilgapõhise platvormi abil. Konkreetsed eesmärgid olid: (i) kindlaks teha, kas tilgad jäävad stabiilseks koos kapseldatud mikroplastikuga; (ii) hinnata, kas mono- ja polüdispersetes tilkades on antibiootikumitundlikkuse tulemused sarnased; (iii) selgitada välja, kas mikroplastikul on mõju antibiootikumitundlikkusele.

Tulemus 1: Tilkade stabiilsuse testimiseks sisaldasid mono- ja polüdispersed tilgad 1 µm ja 10 µm mikroplastikut. Pärast 24-tunnist inkubeerimist 37°C juures jäid nii mono- kui ka polüdispersed tilgad stabiilseks.

Tulemus 2: Järgmisena kasutati mono- ja polüdisperseid tilkasid, et kindlaks teha, kas need mõjutavad tsefotaksiimi MIC-tulemusi. Mono- ja polüdispersed tilgad andsid sarnaseid tulemusi, edasiste katsete jaoks kasutati polüdisperseid tilkasid.

Tulemus 3: Järgmisena uuriti 10 µm mikroplastiku mõju antimikroobsele tundlikkusele. Tilgad, milles oli kapseldatud 10 µm mikroplastik, andsid tulemuseks kõrgema MIC-kõvera, mis tähendab, et mikroplastik mõjutas antibiootikumide tundlikkust. See näitab, et bakterid on vähem tundlikud antibiootikumi suhtes. Selle tulemusena antibiootikumi tõhusus väheneb.

Kokkuvõtvalt mõjutab mikroplastik antimikroobset resistentsust. Tilkades tehtud katsed näitasid, et bakterid hakkasid mikroplastiku ümber kogunema ja olid siis vastupidavamad antibiootikumide toimele. Tilkades sisalduv mikroplast võis suurendada bakterite kokkukleepumist, mis on biofilmi varajane staadium ning see omakorda suurendas resistentsust antibiootikumide suhtes.

Acknowledgements

I would like to express my sincere gratitude to my supervisors Simona Bartkova and Ott Scheler for their support and knowledge of my study. Their guidance and motivation helped me in all the time of research and writing of the thesis.

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Appendix 1

	Monodisperse droplets			Polydisperse droplets		
	Control	**1 µm**	**10 µm**	**Control**	**1 µm**	**10 µm**
0h	1975	1679	1847	2377	862	1339
24h	1538	1490	1421	2228	1166	1775

Table 1. Number of droplets in mono- and polydisperse droplets at 0h and 24h.

Appendix 2

	Polydisperse				Monodisperse			
Cefotaxime concentrations µg/mL	**Droplets in total**	**Negative droplets**	**Positive droplets**	**Positive %**	**Droplets in total**	**Negative droplets**	**Positive droplets**	**Positive %**
0.008	3371	793	2578	76	1073	86	987	92
0.01	4107	835	3272	80	868	33	835	96
0.013	3390	752	2638	78	1050	72	978	93
0.018	3135	671	2464	79	1100	85	1015	92
0.024	3628	905	2723	75	694	11	683	98
0.032	3256	1491	1765	54	732	319	413	56
0.042	3558	3367	191	5	931	917	14	2
0.056	4012	3885	127	3	1053	988	65	6
0.075	4292	4146	146	3	1632	1629	3	0

Table 2. Poly- and monodisperse droplets in total.

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