CHALMERS
UNIVERSITY OF TECHNOLOGY

Biofilm Reduction Modelling in a Drip-flow Reactor

Developing a Method for Growing Biofilms in a Laboratory Setting, to Evaluate the Effects of an Anti-fouling Product Used in Paper Machines

Master's thesis in Biotechnology

EMIL FRITHIOFSON

Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2020

Master's thesis

Biofilm Reduction Modelling in a Drip-flow Reactor

Developing a Method for Growing Biofilms in a Laboratory Setting, to Evaluate the Effects of an Anti-fouling Product Used in Paper Machines

EMIL FRITHIOFSON

CHALMERS
UNIVERSITY OF TECHNOLOGY

Department of Biology and Biological Engineering
Chalmers University of Technology
Gothenburg, Sweden 2020

Biofilm Reduction Modelling in a Drip-flow Reactor
Developing a Method for Growing Biofilms in a Laboratory Setting, to Evaluate the Effects of an Anti-fouling Product Used in Paper Machines
EMIL FRITHIOFSON

© EMIL FRITHIOFSON, 2020.

Supervisor: Emma W. Janco, BIM Kemi Sweden AB
Examiner: Joakim Norbeck, Department of Biology and Biological Engineering

Master's Thesis
Department of Biology and Biological Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Cover: Sketch illustration of a paper machine.

Typeset in LaTeX
Gothenburg, Sweden 2020

Biofilm Reduction Modelling in a Drip-flow Reactor
Developing a Method for Growing Biofilms in a Laboratory Setting, to Evaluate the Effects of an Anti-fouling Product Used in Paper Machines
EMIL FRITHIOFSON
Department of Biology and Biological Engineering
Chalmers University of Technology

Abstract

Bacterial biofilms naturally form in paper machines, causing problems in production. Reducing the amount of biofilm formed is therefore of interest to the paper industry. To model biofilm reduction, a method was developed for growing biofilms in a laboratory setting at BIM Kemi Sweden AB. The goal was for the method to be used to evaluate effect of the anti-fouling product Bimogard produced by the company, a non-biocidal agent that reduces formation of process disrupting biofilms in paper machines. Initial experiments were carried out using petri dishes for cultivation, but the main part of the work was carried out using a drip-flow biofilm reactor. The biofilms were quantified by staining with safranin and measuring absorbance. Different process parameters for running the reactor were examined and improved, including what medium concentration to use and whether to inoculate with a mono-culutre of *Pseudomonas fluorescens* or a co-culture where *Bacillus subtilis* was also added. The final method, using the mono-culture and 7.5% of standard medium concentration, was used to evaluate the effects of adding Bimogard to the nutrient medium. The addition of Bimogard significantly reduced the amount of biofilm formed, but only at low concentrations.

Keywords: biofilm, drip-flow reactor, staining, quantification, Bimogard, anti-fouling, Pseudomonas fluorescens, Bacillus subtilis

Acknowledgements

Thanks to Emma W. Janco and everyone at BIM Kemi Sweden AB for the opportunity and assistance during the project. Thank you for making sure that I could continue my work despite the ongoing Covid-19 pandemic. Thanks also to Joakim Norbeck, for volunteering to be my examiner for the project. Thanks to my friends for listening to me venting about uncooperative bacteria. Lastly, thanks to my family for supporting me and showing interest in my work.

Emil Frithiofson, Gothenburg, June 2020

Contents

List of Figures xi

1 Introduction 1

2 Theory 3
- 2.1 Model Bacteria . . . 3
- 2.2 Formation of Biofilms . . . 3
- 2.3 Factors Affecting Biofilm Formation . . . 4
- 2.4 Biofilm Reduction . . . 5
- 2.5 Growing Biofilms in Petri Dishes . . . 6
- 2.6 Drip-flow Reactor . . . 6
- 2.7 Quantification of Biofilms . . . 7

3 Methods 9
- 3.1 Standard Medium . . . 9
- 3.2 Bacterial Strains and Culture Conditions . . . 9
- 3.3 Washing Protocol . . . 9
- 3.4 Overnight Cultures . . . 10
- 3.5 Biofilms in Petri Dishes . . . 10
- 3.6 Biofilm Biomass Quantification . . . 10
- 3.7 Drip-flow Reactor Operation . . . 10
- 3.8 Statistical analysis . . . 12

4 Results and Discussion 13
- 4.1 Biofilms Grown in Petri Dishes . . . 13
- 4.2 Initial Drip-flow Reactor Experiment . . . 14
- 4.3 Mono- vs. Co-culture Experiment . . . 15
- 4.4 Medium Concentration Experiment . . . 15
- 4.5 Anti-fouling Product Experiments . . . 16
- 4.6 Improvements and Future Studies . . . 18

5 Conclusion 19

Bibliography 21

List of Figures

2.1 Stages of biofilm formation. Bacteria attach to a surface, form micro-colonies and eventually EPS-encased structures (in this case pillars). . 4
2.2 Drip-flow reactor. A) Influent tubing. B) Influent port with rubber septum. C) Channel holding stainless steel plate. D) Air vent with filter. E) Effluent tubing. 7

3.1 Reactor setup. A) Effluent tubing. B) Drip-flow reactor. C) Influent tubing. D) Watson-Marlow 520S peristaltic pump. E) Marprene pump tubing. F) Medium containers with air filters. G) Glass flow breaks. 11

4.1 Mean biofilm formation on steel plates cultivated in petri dishes for 3 and 6 days as measured by absorbance at 530 nm, from three independent experiments (n=3). Biofilms consisted of either a monoculture of *P. fluorescens* or *B. subtilis*, or a co-culture of both. Controls were performed only once. The error bars represent ± one standard deviation. 13
4.2 Amount of biomass in *P. fluorescens* biofilms grown on steel plates in drip-flow reactor using 5% concentration medium, measured as the absorbance of safranin stain. Each bar represents the result from one channel in the same reactor run. 14
4.3 Mean amount of biomass in biofilms consisting of *P. fluorescens* or *P. fluorescens* and *B. subtilis* grown using 5% concentration medium, measured as the absorbance of safranin stain (n=2). The error bars represent ± one standard deviation. 15
4.4 Boxplot showing the biofilm formation when different concentrations of Bimogard was added to the nutrient medium; 0, 0.3, 3 and 30 ppm. Circles represents the median, crosses the mean and the edges of the boxes the highest and lowest values, for three independent experiments (n=3). *Significantly different from 0 ppm. 16

1 Introduction

In both natural and industrial settings, bacteria can cling together and attach to surfaces in what is known as biofilms [1]. Bacteria in a biofilm produce an extracellular matrix, which surrounds all the cells in the community [2]. The matrix consists of polysaccharides, proteins, nucleic acids and lipids, making the bacterial community into a slime that sticks to surfaces, and has many special properties including increased stress tolerance [3]. Typically, there are several types of bacteria in naturally formed biofilms [4].

Biofilms can cause problems when they grow in places where they should not be, since they are difficult to remove [5] and have a high resistance to antibiotics [6]. For example, we use the mechanical action of a toothbrush to remove the biofilm that is plaque from our teeth, as it is not enough to rinse with water. Another place where biofilms can cause trouble is during the making of paper. The process conditions of paper machines make them favorable for growth of bacteria and formation of biofilms [7]. The biofilms have a negative effect on the paper production, with potential effects being reduced machine runability and end product quality [8]. For example, parts of biofilms growing in the paper machine can detach and end up in the final paper product, causing holes and spots. Therefore, there is an interest in finding solutions or products that remove or reduce biofilms that form in paper machines.

BIM Kemi Sweden AB, located in Stenkullen, Sweden, specializes in chemicals for pulp and paper industry. One product area of the company is anti-fouling agents, designed to reduce the formation of biofilms on surfaces in paper machines. To efficiently work with current products and develop new ones, there is a need for a laboratory model where the anti-fouling agents can be examined. This thesis work aims to provide a method where such products can be tested at the company, by providing a quantifiable laboratory model for biofilms. The model bacteria and growing conditions of the method need to be determined, as well as the method of quantification. The method should then be used to evaluate the effects of the anti-fouling product Bimogard.

2
Theory

This chapter explains some of the underlying theory and earlier results that the experimental work is based upon.

2.1 Model Bacteria

Pseudomonas fluorescens and *Bacillus subtilis* were selected as the biofilm forming model bacteria for this study. Both *B. subtilis* [8] and *P. fluorescens* [9] are common bacteria in industrial biofilms, and are safe to work with. Additionally, *P. fluorescens* is in the same genus as *Pseudomonas aeruginosa*, which has previously been used for drip-flow reactor method development [10]. However, *P. aeruginosa* is a pathogen [11], requiring safety precautions beyond what is available at BIM Kemi Sweden AB.

P. fluorescens will form biofilms on its own [9], whereas *B. subtilis* will not form biofilms in a mono-culture [8]. Prior testing at BIM Kemi Sweden AB indicated that a co-culture with both species may give thicker biofilms [12]. A mono-culture of *P. fluorescens* was therefore tested alongside a co-culture with both species. One culture was selected to be used for the model.

2.2 Formation of Biofilms

Outside of laboratory settings, bacteria exist mostly in biofilms [13]. The biofilms are formed when the bacteria start producing an extracellular matrix known as Extracellular Polymeric Substances (EPS), consisting of nucleic acids, lipids, polysaccharides and proteins [3]. The EPS forms a highly structured fibrillar network, which lends the bacterial community properties that the planktonic cells do not have [3]. This also gives the bacteria a better defense against the environment [14].

Formation of biofilms is controlled in three major steps. Step one is the initial attachment of bacteria to the surface, and step two is the formation of micro-colonies from the attached bacteria on the surface [15]. Step three is maturation of the biofilm. This includes control of the biofilm depth, and EPS-encased structures that form in the biofilm [15]. The stages are illustrated in figure 2.1.

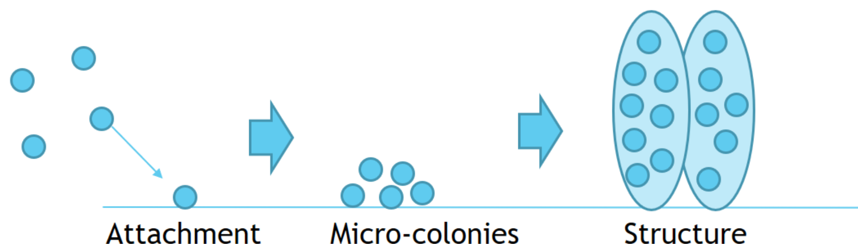

Figure 2.1: Stages of biofilm formation. Bacteria attach to a surface, form micro-colonies and eventually EPS-encased structures (in this case pillars).

Biofilm structure can be homogeneous or shaped into pillars with water channel for nutrient transport [15]. Eventually, the biofilm also releases cells to find new habitats [16], which can end up in the product when biofilms grow in paper machines.

Biofilm growth is associated with a change in gene expression, for example up-regulation of EPS producing genes [17]. The genetic pathways regulating biofilm formation differ between organisms, and one organism can have multiple pathways that control biofilm formation [18]. There are many different genes that have been identified as being essential for biofilm formation in different organisms, and many genes where an expression change is associated with biofilm formation [14].

2.3 Factors Affecting Biofilm Formation

An environmental factor which possibly contributes to biofilm initiation is nutrient availability in the environment [19]. Another factor which may contribute to the formation of biofilms is the stress the bacteria are subjected to, as many stress response regulators are also involved in biofilm formation [14].

The roughness and free energy of the surface can also have a significant effect on the formation of biofilms [20]. For this study, stainless steel metal plates were used as surfaces for developing the biofilms. Specially made polished steel plates were provided when purchasing the reactor, but additional plates were manufactured on-site. To ensure that the surface was as clean as possible, a cleaning protocol using sodium dodecyl sulfate, distilled water and sonication was employed every time the plates were used.

Another factor affecting the formation is shear stress, where the structure can be be affected [15]. High shear stress results in a patchy biofilm and low shear stress gives a more even biofilm [21]. High shear also makes the biofilms harder to reduce [22]. The drip-flow reactor used in this study provides a low shear environment [10], but different types of reactors exist for studying biofilms that form under different shear influence. A CDC reactor can be used to form biofilms under high shear, which has been suggested to be a better model for industrial settings specifically because the

biofilms are harder to reduce [23].

The CDC reactor, however, provides less contact with the air than the drip-flow reactor, since the surfaces the biofilms form on are suspended vertically in liquid. This can also have an effect on the biofilm formation. Unpublished data from earlier testing at BIM Kemi Sweden AB, using stainless steel plates suspended vertically in liquid similar to a CDC reactor, showed that the bacteria used in this study will only form biofilms at the air-liquid interface [12].

2.4 Biofilm Reduction

To clean out bacteria from a paper machine, production has to be stopped, which is costly. It is more favourable if build-up of biofilms can be hindered while the machine is still in use. Biofilms are typically combatted in this way by adding biocides to kill the bacteria in the biofilms [9]. However, this can actually be counter productive. It can leave biomass behind, which can instead promote growth afterwards [9]. Additionally, biofilms have an inherent resistance to biocides compared to bacteria in suspension, through multiple mechanisms such as increased barriers for diffusion and the slower growth of the biofilms [24]. This means that high concentrations of biocides have to be used, which may also be harmful for the environment and personnel handling the chemicals. A sub-lethal dose of biocides may also contribute to the expressional change associated with biofilm formation, and give more biocide resistant bacteria [25]. A more recent approach to reducing biofilms in paper machines is to interrupt the interaction between the surface and the bacteria, which is a more environmentally friendly approach [26].

BIM Kemi Sweden AB produce an anti-fouling product under the name Bimogard, whose purpose is to reduce biofilm formation when added to paper machines. Bimogard is intended to be a non-biocidal solution that does not kill the bacteria. The main component of the product is lignin derivatives, a bi-product from wood processing, together with other surface active agents. The proposed mechanism for the product is that it hinders the initial attachment of bacteria to the surfaces in the paper machine, and makes the attachment weaker. The result of adding Bimogard to the paper machine is an overall decreased amount of biofilm.

In industrial applications, the normal concentration of added Bimogard is approximately 3 ppm. However, the pulp in paper machines is a mix of many fibers and particles. It is possible that the effective concentration of the product is lower than 3 ppm, since it could adsorb to other components in the pulp. This would leave less available surface active agents that can adsorb to the intended surfaces of the paper machine or act on the bacteria. It is worth to keep this in mind, since in laboratory experiments, the product will be added to a medium that does not contain pulp.

2.5 Growing Biofilms in Petri Dishes

Initial biofilms cultivation experiments were performed using petri dishes. To achieve the air contact that was needed to form biofilms, the petri dishes were placed in an incubator with shaking, and the bottom of the dishes just barely covered with liquid. This creates a "wave" continually going around the petri dish, providing the surface with both liquid and air contact. This method was evaluated to see if it could provide preliminary results as base for the later drip-flow reactor experiments. It was discovered early on that the biofilm could not attach to the smooth plastic surface of the petri dishes. Therefore, stainless steel plates were added to the petri dishes for the bacteria to grow on. Steel plates would also be used later on in the drip-flow reactor.

2.6 Drip-flow Reactor

Using a drip-flow bioreactor provides a way of growing biofilms under low shear at the air-liquid interface [10]. The drip-flow reactor consists of four channels that each fit one 75x25 mm stainless steel plate. The entire reactor sits in a rack at a 10° angle, so that the nutrient medium drips onto the plate and runs down along it. This results in a plug flow [10], meaning that the nutrient concentration is lower for the bacteria at the bottom of the plate. Medium is provided through an influent port with a rubber septum that is penetrated by a needle. The reactor also has a air vent with a filter. The reactor is pictured in figure 2.2.

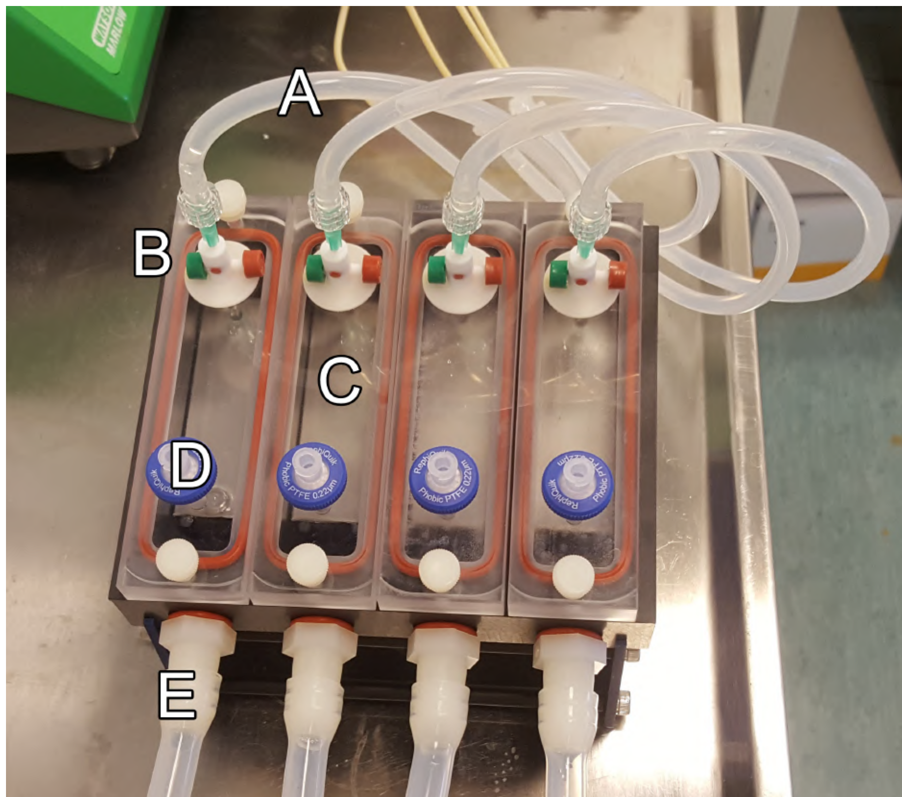

Figure 2.2: Drip-flow reactor. A) Influent tubing. B) Influent port with rubber septum. C) Channel holding stainless steel plate. D) Air vent with filter. E) Effluent tubing.

When running an experiment, the reactor is inoculated and then incubated without tilt in batch mode for a shorter duration to allow initial attachment of the bacteria to the surface. After this, it is run in continuous mode with a continuous flow. All the bacteria come from the initial inoculation, the medium itself should be sterile.

2.7 Quantification of Biofilms

In this study, the method of quantification was staining with safranin and measuring absorbance. Staining is a method that has been used to quantify biofilm biomass before, using for example crystal violet [27]. Crystal violet staining has been a common method, but safranin has emerged as a non-toxic alternative with high reproducibility [28]. These dyes both target the total amount of biomass, including the extracellular matrix, [29], but there are other approaches to quantification of biofilms. Staining with acridine orange allows for determining the number of attached cells by light microscopy [4] or fluorescence microscopy [30]. Thicker biofilms can also be quantified using confocal microscopy and image analysis [1]. However, the biomass staining absorbance measurement method is quite easy and suitable for work at BIM Kemi Sweden AB. Another simple method is detaching the biofilm from the metal plates and doing agar plate counts [31]. However, this is time-consuming and may not even accurately quantify all viable cells in the biofilm, as they may

not be culturable [32]. Additionally, even dead cells in a paper machine can cause trouble. Therefore, it should be more interesting to quantify the entire biomass.

The safranin is extracted from the biofilm using acetic acid, as this has been shown to be a very efficient way of releasing the stain [28]. The resulting solution can be analyzed with a spectrophotometer at 530 nm.

3
Methods

This chapter details the methodology used for the experimental work.

3.1 Standard Medium

Nutrient Broth No. 3 (Sigma-Aldrich, Sweden) was used as nutrient medium for all experiments. Medium was prepared and autoclaved with a final concentration of 13 g/l, according to supplier directions, for all purposes except for drip-flow reactor operation.

For drip-flow reactor medium, a 26 g/l stock solution was prepared. The stock solution was diluted directly into medium containers with distilled water to achieve desired concentration, since equipment limitations did not allow for sterilization of large amounts of medium.

For agar plates, 15 g/l of agar powder (Acros Organics, Belgium) was added to the medium before autoclaving. Warm agar solution was poured into triple vented petri dishes and allowed to set.

3.2 Bacterial Strains and Culture Conditions

Frozen strains of *B. subtilis* and *P. fluorescens* were sourced from the Culture Collection University of Gothenburg. Bacteria were revived in Nutrient Broth No. 3 medium and streaked on agar plates. Bacteria were kept on agar plates during the entire experiment and re-streaked onto new plates twice a week. *B. subtilis* plates were kept at room temperature, and *P. fluorescens* plates were kept at 30 °C. This provided similar growth, so that they could be re-streaked on the same days.

3.3 Washing Protocol

316L polished stainless steel plates (BioSurface Technologies Corporation, United States of America), dimensions 75x52x1 mm, were cleaned by sonicating twice in 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, Sweden) solution and twice in distilled water for a total of four washes. Plates were rinsed with distilled water between washes to remove all SDS. The steel plates were kept in 95% ethanol solution

until use.

After use, the plates were cleaned using regular dish detergent and paper, avoiding scratches on the surface. The plates were reused for multiple experiments.

3.4 Overnight Cultures

100 ml growth medium in an Erlenmeyer flask was inoculated with a colony picked from agar plates and placed in incubator overnight, 22±2 hours, at 30 °C and 75 rpm shaking. The overnight culture typically had an optical density (OD) between 1-2 at 600 nm.

3.5 Biofilms in Petri Dishes

Stainless steel plates were washed according to the protocol. Before placing the steel plates in the petri dishes, the ethanol was burned off. After the steel plates were placed in petri dishes, 10 ml of 10 times diluted overnight culture was added, barely covering the steel plates. For the experiments using a co-culture of bacteria, 5 ml of *B. subtilis* and 5 ml of *P. fluorescens* overnight culture was added, diluted approximately 10 times to reach the same OD. The petri dishes were placed in incubator at 30 °C and 75 rpm shaking. Cultivation time was 3 and 6 days. Plates were than stained according to biofilm biomass quantification protocol.

3.6 Biofilm Biomass Quantification

Steel plates were placed in centrifuge tubes and covered with 30 ml of a filtered 0.1% solution of Safranin O (Sigma-Aldrich, Sweden) in distilled water, and kept laying horizontally in the dark for 15 minutes. Plates were rinsed with distilled water, and the stain was dissolved in 20 ml of 30% acetic acid for 15 minutes. The absorbance of the solution was measured at 530 nm using a Thermo Scientific Spectronic 200 spectrophotometer.

3.7 Drip-flow Reactor Operation

Overnight cultures were prepared as described earlier. Stainless steel plates were cleaned according to the protocol described earlier, but were not placed in ethanol. The plates were inserted in the channels of the drip-flow reactor (BioSurface Technologies Corporation, United States of America), and the entire reactor was autoclaved with the steel plates inside. The effluent tubes were sealed using tubing clamps, and 15 ml of standard concentration nutrient medium was added to each channel of the sterilized reactor. Each channel was inoculated with 1 ml of the overnight culture. For the experiments using a co-culture of bacteria, 0.5 ml of *B. subtilis* and 0.5 ml of *P. fluorescens* overnight culture with similar OD was added.

The bacteria were incubated in batch mode for 6 hours in the reactor, without tilt. The reactor was then placed at a 10° incline and connected to the pump tubing. The reactor was allowed to run continuously for 48 h with the following setup.

Reactor setup consisted of four 10 l medium containers connected to the reactor via tubing with flow breaks inbetween, as described by Goeres et al. [10]. Medium containers were sterilized by filling partly with boiling distilled water and shaking. Container lids were outfitted with an air vent with a filter. Flow was provided by a Watson-Marlow 520S peristaltic pump set to 0.83 ml/min. For the incoming tubing, 3.1 mm inner diameter silicone tubing was used except for in the pump head, where 1.14 mm inner diameter marprene tubing was used. The effluent tubing was 7.9 mm inner diameter silicone tubing which was spliced down to 4.8 mm inner diameter silicone tubing at the end. After each experiment, the tubes were flushed with water and autoclaved. The biofilms were stained according to the biofilm biomass quantification protocol described earlier, and absorbance was measured. The entire reactor setup is pictured in figure 3.1.

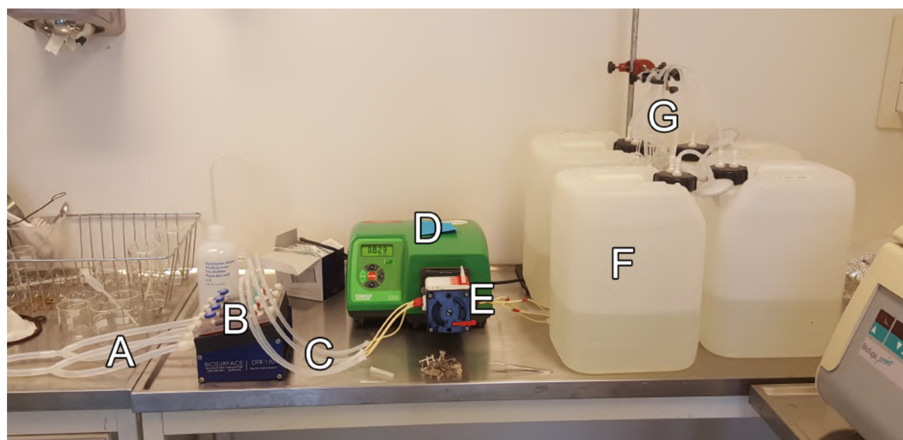

Figure 3.1: Reactor setup. A) Effluent tubing. B) Drip-flow reactor. C) Influent tubing. D) Watson-Marlow 520S peristaltic pump. E) Marprene pump tubing. F) Medium containers with air filters. G) Glass flow breaks.

Multiple experiments were performed in the reactor. Initially, the reactor was run without any variable to confirm that biofilms would form, where 100 ml concentrated medium was added to the medium to reach a concentration that was approximately 5% of the standard concentration. Then, experiments were performed in order to improve the method used. The effect of using a co-culture compared to the mono-culture was examined using the 5% medium. The effect of different medium concentrations was tested by adding 20, 50, 100, 150, 200 and 300 ml concentrated medium, corresponding to approximately 1, 2.5, 5, 7.5, 10 and 15% of standard concentration. Finally, the effect of adding 0, 0.3, 3 and 30 ppm of Bimogard was evaluated by doing three repeats, using the 7.5% medium. The parameters for the experiments were updated based on the results from previous experiment.

When doing repeats with Bimogard, the concentrations in the channels were switched around between experiments to minimize impact that the individual channels may have on the results. Four different concentrations were tested each time. This was done to account for the natural variability between repeats.

3.8 Statistical analysis

The results from the repeated experiments using Bimogard were analyzed using a two sided, paired ratio t-test with the null hypothesis that the mean ratio between the two populations is equal to 1. The alternative hypothesis is that the ratio is not equal to 1.

$$\begin{aligned} H_0 &: \mu_{ratio} = 1 \\ H_A &: \mu_{ratio} \neq 1 \end{aligned} \tag{3.1}$$

The sample size is 3 for every treatment, and each sample is paired with the reference that belongs to the same reactor run, i.e. the reference from the first run is paired to the 0.3 ppm treatment from the first run. Three tests were done, comparing the different treatments to the reference. The test is executed by log-transforming the data and performing a regular mean difference paired t-test. The difference will represent the ratio because of the log transformation. The test results were examined at $\alpha = 0.05$, where the result is considered significant if $P < \alpha$.

4

Results and Discussion

This chapter presents the results from the experimental work and discussion regarding the results.

4.1 Biofilms Grown in Petri Dishes

There was some formation of biomass on the steel plates that were cultivated in petri dishes. However, it was difficult to see before staining, and the biomass was very weakly attached to the plates. This made it difficult to quantify, since it would partly slide off during the staining procedure. The results of the experiment are shown in figure 4.1.

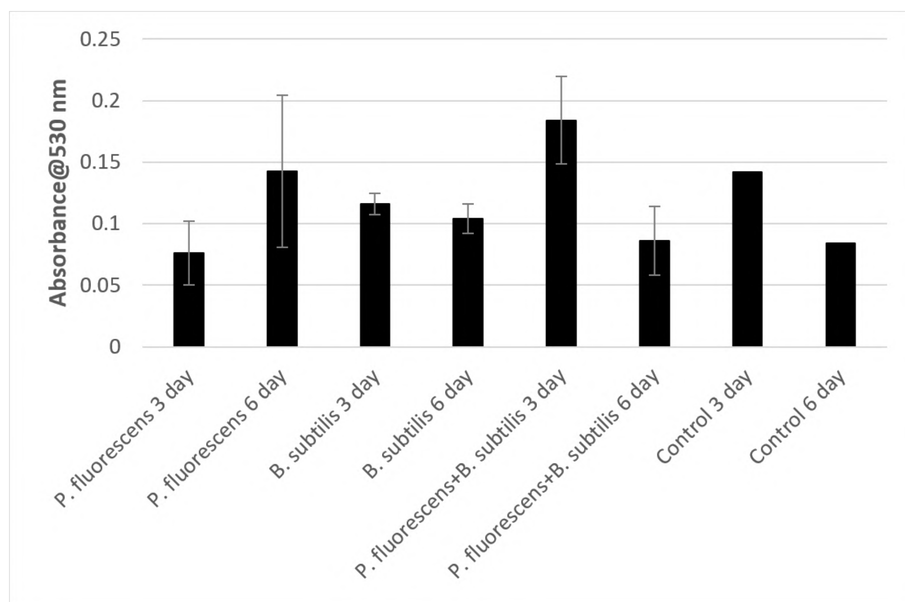

Figure 4.1: Mean biofilm formation on steel plates cultivated in petri dishes for 3 and 6 days as measured by absorbance at 530 nm, from three independent experiments (n=3). Biofilms consisted of either a monoculture of *P. fluorescens* or *B. subtilis*, or a co-culture of both. Controls were performed only once. The error bars represent ± one standard deviation.

Overall, the method using petri dishes was not very effective. The results were inconsistent, and there was a lot of variability. As mentioned, *B. subtilis* should not

be able to form biofilms by itself. The absorbance detected from the control plates is also in the same range as the others, when it should be lower since no biofilms should form. Possibly, there was no biofilm actually attached to the plate surfaces. What is then detected could be unattached biomass, or various biomolecules in the medium.

The inconsistent results from staining meant that the experiments could not be used to gather data for the drip-flow reactor experiments. This could have been useful, as the reactor only allows for four experiments at a time. For example, concentrations of added anti-fouling product could have been tested beforehand using this method.

4.2 Initial Drip-flow Reactor Experiment

Initial experiment showed that *P. fluorescens* mono-culture biofilms successfully formed in the reactor using 5% concentration medium. The biofilms were thick enough that they could be seen on the plates before staining, unlike in the petri dishes. This supports the theory that there was no biofilm actually attached to the surfaces of the plates in the petri dishes. The results of staining the biofilms from the drip-flow reactor are shown in figure 4.2.

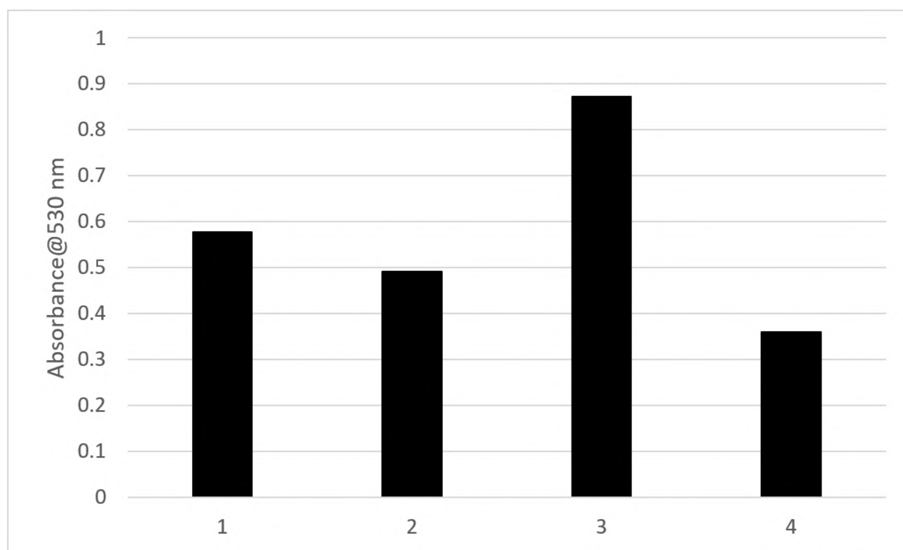

Figure 4.2: Amount of biomass in *P. fluorescens* biofilms grown on steel plates in drip-flow reactor using 5% concentration medium, measured as the absorbance of safranin stain. Each bar represents the result from one channel in the same reactor run.

The recorded absorbance values here are higher than the values from the petri dishes. There is quite a bit of variability between the different channels, but it shows that the method appears to be working.

4.3 Mono- vs. Co-culture Experiment

In the experiment comparing the mono-culture of *P. fluorescens* to the co-culture of *B. subtilis* and *P. fluorescens* using 5% concentration medium, the mono-culture gave a slightly higher mean amount of biomass. The results are illustrated in figure 4.3

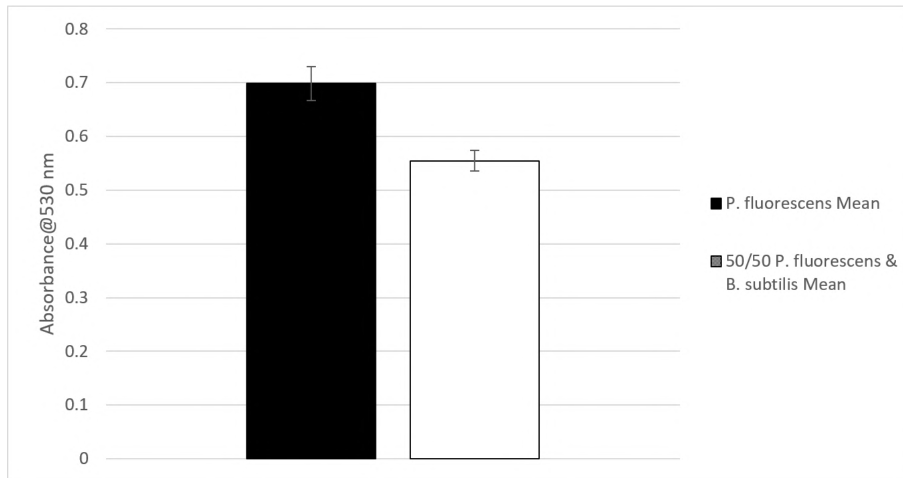

Figure 4.3: Mean amount of biomass in biofilms consisting of *P. fluorescens* or *P. fluorescens* and *B. subtilis* grown using 5% concentration medium, measured as the absorbance of safranin stain (n=2). The error bars represent ± one standard deviation.

The addition of *B. subtilis* to the bacterial culture did not seem to improve the formation of biofilms. Therefore, it was decided that the mono-culture of *P. fluorescens* would be used, since this simplifies the experimental procedure and saves time. It may still be interesting to examine the effect of anti-fouling products on biofilms with multiple species since that is how they normally form, though it was not in the scope for this study to examine both.

4.4 Medium Concentration Experiment

The most immediate results from running the reactor using different concentrations were the visual appearances of the biofilms. On the plates which were fed low concentrations of medium, the biofilms were very thin and almost hard to see. On the other hand, high concentrations seemingly gave high amounts of biomass, but the biofilms were more patchy and not as consistent in appearance.

Unfortunately, the amount of biomass varied quite a lot. It was not apparent what the difference was between the highest concentrations due to the varying recorded absorbance. This made it hard to draw conclusions from the experiment.

Another effect of the differing medium concentration could be noticed in the medium containers themselves. In the medium containers with the highest concentrations,

10 and 15%, there was a noticeable bacterial smell. With higher concentration of medium, there is increased opportunity for contaminating organisms to grow in the medium. This is especially important to consider in the method of this study, as the possibility for sterilization was limited. Therefore, the medium concentration should not be too high.

The 7.5% medium was selected as the concentration to be used for the final experiments, though it was not conclusive what the ideal concentration is. However, below 5% is probably not recommended as the growth using theses concentrations was low.

4.5 Anti-fouling Product Experiments

The results of measuring the amount of biofilm grown under the effect of Bimogard as the absorbance of safranin are illustrated as a box plot in figure 4.4.

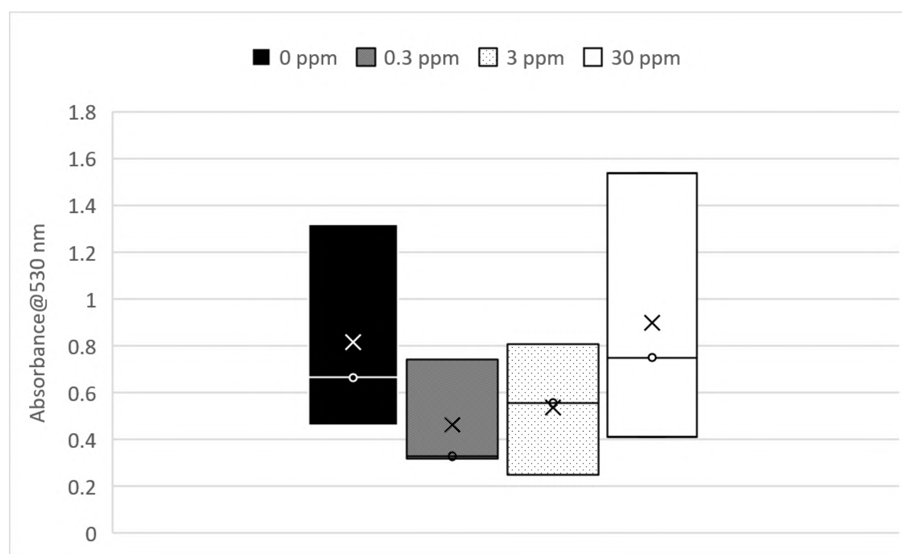

Figure 4.4: Boxplot showing the biofilm formation when different concentrations of Bimogard was added to the nutrient medium; 0, 0.3, 3 and 30 ppm. Circles represents the median, crosses the mean and the edges of the boxes the highest and lowest values, for three independent experiments (n=3). *Significantly different from 0 ppm.

The mean amount of biomass initially decreases with the addition of Bimogard, after which it starts increasing as the concentration of Bimogard rises. Large variations between individual repeats were observed, but the difference between each dosage level generally followed the same pattern in each reactor run: a low amount of biofilm for 0.3 ppm Bimogard, which then increased with increasing dosage to eventually reach similar amounts to the untreated biofilms. The reference mean, where no Bimogard was added, was 0.815. With 0.3 ppm, the mean was 0.463, which is 57% of the reference mean. This treatment also had a statistically significant effect (P=0.0294). With 3 ppm the mean was 0.537, 66% of the reference

mean. The effect of this treatment was not significant (P=0.334). With 30 ppm the mean was 0.900, 110% of the reference mean. The effect of this treatment was also not significant (P=0.868). However, it is important to note that the sample size is small (n=3), meaning that the statistical power is not strong.

At lower concentrations, the product appears to have its intended effect. The biofilm biomass is lower than the reference value. This reflects the results of adding Bimogard to paper machines in production, where the concentration is approximately 3 ppm.

The reason that the mean biomass amount eventually gets higher could be that the anti-fouling product puts the bacteria under some stress when the dosage is too high, which increases the incentive to form biofilms as a way to improve the defense against the stress. An alternative hypothesis is that Bimogard contains one or several compounds that are beneficial to the bacteria, enhancing their growth as the concentration increases.

Adding Bimogard to the medium also resulted in a change in the visual appearance of the biofilm, specifically for the biofilms grown in 30 ppm of the product. The steel plates with these biofilms had large chunks of biomass attached to the surface. It was instantly apparent which of the channels that had been subjected to 30 ppm of Bimogard each time.

The reason that the biofilms appear as chunks on the steel plates could be that the Bimogard makes it difficult for the bacteria to initially attach like they would normally do. The biofilm would then only be able to grow well in the few spots where bacteria manage to attach, which would be where the chunks appear.

The chunks were more loosely attached to the surface than the biofilms on the other plates. This could mean that they would be more prone to detaching and ending up in the product in a paper machine. However, it could also mean that they would never reach enough biomass to cause problems. The higher flow in a paper machine could cause the bacteria to detach before growing to a significant thickness.

0.3 ppm of Bimogard is the only tested concentration that significantly affects the amount of biofilm formed. This is interesting, because the concentration that is added to the paper machines in production is approximately 3 ppm, which did not have a significant effect here. The effective concentration in production may however, as mentioned, be lower. It is interesting that the product is effective at reducing the amount of biofilm formed at such low concentrations. This is of course an advantage for both the producer and the consumer since the volumes of product that need to be made and handled will be smaller.

4.6 Improvements and Future Studies

The experiments in this study were all made using the same reactor run-time, 48 hours. It is possible that altering the run-time could yield better results. This could also be tested in conjunction with varying the medium concentration. For example, a longer run-time could be used to give the biofilms more time to grow when using lower concentrations. The opposite could also be tested, where a higher medium concentration is used with a shorter run time. Improving run-time could also increase the throughput of the method. The working temperature could also be increased to match that of a paper mill, which could give different results.

A big issue with this study was the fact the the medium could not be sterilized. The water was distilled, but after this stored in a metal container. It is likely that the medium was contaminated from the start, and that the efforts to sterilize the medium container were fruitless. Many times during the experiments, some type of contaminating biomass could be seen in the influent tubes. This means that the biofilms can not be guaranteed to consist of purely *P. fluorescens*. Possibly, this is why the amount of biomass could vary so much between experiments. This was especially apparent in the experiments where Bimogard was evaluated. The approximate pattern was similar every time, but the magnitude of the stain absorbance varied.

To ensure that the results that led to the final method in this study are correct and repeatable, they need to be verified by doing the experiments again. The experiments in this work were not repeated many times, due to the time restraints. To get a more reliable picture of the effects of Bimogard, more experiments also need to be done. More concentrations could be tested to get a continuous picture of the effects of concentrations that range from below 0.3 ppm and above 30 ppm. It would also be interesting to look into the variance of untreated biofilm biomass.

Doing experiments with the drip-flow reactor is time consuming, and throughput is quite low. An alternative to get a large sample size in a quicker way could be to grow biofilms in multi-well plates. The multi-well plates can be placed in a shaker to simultaneously grow many biofilms under low shear conditions [27]. In that case, other model bacteria could be selected that are capable of growing away from the air-liquid interface. It would also be interesting to examine the effects of Bimogard on biofilms formed under high shear instead of low shear, as this has been suggested to better model industrial settings [23]. A CDC biofilm reactor could be used to achieve this, but this reactor also gives low air contact compared to the drip-flow reactor.

5
Conclusion

The method that is detailed in this study successfully provides a way to grow and quantify the biomass of a *P. fluorescens* biofilm. The method does not require advanced tools beyond a spectrophotometer and a drip-flow reactor, and is relatively simple to execute. It is however fairly time-consuming, and the through-put is low. There is still room for improvements to be made, but the method should be able to be used in future development and testing of anti-fouling products at BIM Kemi Sweden AB.

The addition of Bimogard to the medium could significantly reduce the amount of biofilm formed in the drip-flow reactor. This shows that the product does indeed have an anti-fouling effect like it is intended to. The biofilm reducing effect was significant at a low concentration of 0.3 ppm. However, increasing the concentration of Bimogard gave a non-significant change, with increasing mean amount of biomass. With the highest examined concentration, 30 ppm, the mean biomass amount was comparable to the untreated biofilms. Hence, it is important to find the correct dosage level for industrial applications.

Bimogard is already in use as a solution for the problem with biofilms in paper industry. These results, and especially the method, allow for further development and improvement of Bimogard and similar products. The products can help the move away from harmful biocides and towards a sustainable approach, since they are created as a bi-product in wood processing.

Bibliography

[1] A. Heydorn, A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll, and S. Molin. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology*, 146(10):2395–2407, 2000.

[2] Hera Vlamakis, Yunrong Chai, Pascale Beauregard, Richard Losick, and Roberto Kolter. Sticking together: building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology*, 11(3):157–168, 2014.

[3] Hans-Curt Flemming. EPS—Then and Now. *Microorganisms*, 4(4):41, 2016.

[4] DE Nivens, RJ Palmer Jr, and DC White. Continuous nondestructive monitoring of microbial biofilms: a review of analytical techniques. *Journal of Industrial Microbiology*, 15:263–276, 1995.

[5] O M Väisänen, A Weber, A Bennasar, F A Rainey, H.-J Busse, and M S Salkinoja-Salonen. Microbial communities of printing paper machines. *Journal of Applied Microbiology*, 84:1069–1084, 1998.

[6] Zohra Khatoon, Christopher D. McTiernan, Erik J. Suuronen, Thien Fah Mah, and Emilio I. Alarcon. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon*, 4(12):e01067, 12 2018.

[7] Ann Cathrin Olofsson, Malte Hermansson, and Hans Elwing. N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. *Applied and Environmental Microbiology*, 69(8):4814–4822, 2003.

[8] M. Kolari, J. Nuutinen, and M. S. Salkinoja-Salonen. Mechanisms of biofilm formation in paper machine by Bacillus species: The role of Deinococcus geothermalis. *Journal of Industrial Microbiology and Biotechnology*, 27(6):343–351, 2001.

[9] M. Simões, M. O. Pereira, and M. J. Vieira. Monitoring the effects of biocide treatment of Pseudomonas fluorescens biofilms formed under different flow regimes. *Water Science and Technology*, 47(5):217–223, 2003.

[10] Darla Goeres, Linda R Loetterle, Diane Walker, Darla M Goeres, Martin A Hamilton, Nicholas A Beck, Kelli Buckingham-Meyer, Jackie D Hilyard, Lindsey A Lorenz, Diane K Walker, and Philip S Stewart. A method for growing a biofilm under low shear at the air–liquid interface using the drip flow biofilm reactor. *Nature Protocols*, 4(5):783–788, 2009.

[11] Nazanin Saeidi, Choon Kit Wong, Tat Ming Lo, Hung Xuan Nguyen, Hua Ling, Susanna Su Jan Leong, Chueh Loo Poh, and Matthew Wook Chang. Engineering microbes to sense and eradicate Pseudomonas aeruginosa, a human pathogen. *Molecular Systems Biology*, 7(1):521, 1 2011.

[12] Emma W. Janco. Biofilm Experiments (Unpublished Raw Data), 2019.

[13] Benjamin Mielich-Süss and Daniel Lopez. Molecular mechanisms involved in Bacillus subtilis biofilm formation. *Environmental Microbiology*, 17(3):555–565, 2015.

[14] Kimberly K Jefferson. What drives bacteria to produce a biofilm? *FEMS Microbiology Letters*, 236(2):163–173, 7 2004.

[15] Nicola R. Stanley and Beth A. Lazazzera. Environmental signals and regulatory pathways that influence biofilm formation. *Molecular Microbiology*, 52(4):917–924, 4 2004.

[16] J.B. Kaplan. Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. *Journal of Dental Research*, 89(3):205, 2010.

[17] Christophe Beloin and Jean Marc Ghigo. Finding gene-expression patterns in bacterial biofilms. *Trends in Microbiology*, 13(1):16–19, 1 2005.

[18] George O'Toole, Heidi B. Kaplan, and Roberto Kolter. Biofilm Formation as Microbial Development. *Annual Review of Microbiology*, 54(1):49–79, 10 2000.

[19] George A. O'Toole and Roberto Kolter. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology*, 28(3):449–461, 4 1998.

[20] Wim Teughels, Nele Van Assche, Isabelle Sliepen, and Marc Quirynen. Effect of material characteristics and/or surface topography on biofilm development. *Clin. Oral Imp. Res*, 17(S2):68–81, 10 2006.

[21] M. C. M. van Loosdrecht, D. Eikelboom, A. Gjaltema, A. Mulder, L. Tijhuis, and J. J. Heijnen. Biofilm structures. *Water Science and Technology*, 32(8):35–43, 10 1995.

[22] Kelli Buckingham-Meyer, Darla M. Goeres, and Martin A. Hamilton. Comparative evaluation of biofilm disinfectant efficacy tests. *Journal of Microbiological Methods*, 70(2):236–244, 8 2007.

[23] Evgenya S. Shelobolina, Diane K. Walker, Albert E. Parker, Dorian V. Lust, Johanna M. Schultz, and Grace E. Dickerman. Inactivation of Pseudomonas aeruginosa biofilms formed under high shear stress on various hydrophilic and hydrophobic surfaces by a continuous flow of ozonated water. *Biofouling*, 34(7):826–834, 8 2018.

[24] Sara Bas, Mateja Kramer, and David Stopar. Biofilm surface density determines biocide effectiveness. *Frontiers in Microbiology*, 8:2443, 12 2017.

[25] Simone Schulte, Jost Wingender, and Hans-Curt Flemming. Efficacy of biocides against biofilms. In Wilfried Paulus, editor, *Directory of Microbicides for the Protection of Materials*, chapter 5, pages 93–120. Springer Netherlands, Dordrecht, 1 edition, 2004.

[26] Claudia Esperanza Torres, Giles Lenon, Delphine Craperi, Reinhard Wilting, and Ángeles Blanco. Enzymatic treatment for preventing biofilm formation in the paper industry. *Applied Microbiology and Biotechnology*, 92(1):95–103, 10 2011.

[27] Suvi Manner, Darla M. Goeres, Malena Skogman, Pia Vuorela, and Adyary Fallarero. Prevention of Staphylococcus aureus biofilm formation by antibiotics in 96-Microtiter Well Plates and Drip Flow Reactors: Critical factors influencing outcomes. *Scientific Reports*, 7(1):1–10, 3 2017.

[28] Pernille Ommen, Natalia Zobek, and Rikke Louise Meyer. Quantification of biofilm biomass by staining: Non-toxic safranin can replace the popular crystal violet. *Journal of Microbiological Methods*, 141:87–89, 10 2017.

[29] Philipp Stiefel, Urs Rosenberg, Jana Schneider, Stefan Mauerhofer, Katharina Maniura-Weber, and Qun Ren. Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Applied Microbiology and Biotechnology*, 100(9):4135–4145, 5 2016.

[30] Joe J. Harrison, Howard Ceri, Jerome Yerly, Carol A. Stremick, Yaoping Hu, Robert Martinuzzi, and Raymond J. Turner. The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the Calgary biofilm device. *Biological Procedures Online*, 8(1):194–213, 12 2006.

[31] Sharon A Waller, Aaron I Packman, and Martina Hausner. Comparison of biofilm cell quantification methods for drinking water distribution systems. *Journal of Microbiological Methods*, 144:8–21, 2018.

[32] Ya Shen, Sonja Stojicic, and Markus Haapasalo. Bacterial viability in starved and revitalized biofilms: Comparison of viability staining and direct culture. *Journal of Endodontics*, 36(11):1820–1823, 11 2010.