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DEVELOPMENT OF MINIMUM INFORMATION GUIDELINES AND STANDARDIZED *IN VITRO* METHODS FOR BIOFILM EXPERIMENTS

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Laboratory for Process Engineering,
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Development of minimum information guidelines and standardized *in vitro* methods for biofilm experiments

A Dissertation presented to the University of Porto for the degree of Doctor in Chemical and Biological Engineering by

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“Every adventure requires a first step.”

Lewis Carroll

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Abstract

Biofilms are complex communities of microorganisms encased in a self-produced matrix, often attached to an abiotic or biotic surface. They impact various aspects of our lives and are known to create problems in industry and healthcare. In the latter, biofilms are responsible for a large array of infections e.g., Catheter Associated Urinary Tract Infections (CAUTIs). In biofilm research, the more widely used methods rely on microplate reactors. Like many other scientific areas, biofilm research suffers from a lack of reproducibility in published data. Different factors can contribute to this phenomenon such as selective reporting and unreliable methods. This thesis explores the idea of a new classification system for defining different levels of reproducibility and the use of different approaches to address the reproducibility issue, focusing on microplate-based methods.

First, we addressed the issue of selective reporting through the application of a minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates. These guidelines aim to improve reporting and as a result improve reproducibility. The guideline was built following recommendations by international groups working with these methods. It instructs authors and reviewers on the necessary information that a manuscript should include for the experiments in a study to be clearly interpreted and independently reproduced. The guideline and all its accompanying documents were made available online on the MIABiE website (http://miabie.org/introduction.php).

Subsequently, we evaluated the reproducibility of three microplate-based biofilm quantification methods for single species biofilms: plate counts, resazurin, and crystal violet. This was carried out through a 5-lab ring trial study. Experiments were separated into control and treatment. For treatment experiments, the efficacy of sodium hypochlorite (NaOCl) killing *Staphylococcus aureus* biofilms was evaluated. Control experiments showed that crystal violet was the most reproducible method with the lowest standard deviation (SD). In the treatment experiments, plate counts had the best reproducibility with respect to responsiveness (SD/slope), making it the more reliable method to use in a disinfectant efficacy test.

As biofilms are often multispecies, we evaluated the repeatability of the plate count method for different multispecies biofilms. CAUTI was used a model and four species of microorganisms were selected: *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis*

and *Candida albicans*. Eleven different combinations of biofilms (1-4 species) were grown on silicone coupons in artificial urine medium (AUM). The repeatability SDs values for each species within each combination were generally low and comparable to the numbers observed in the ring trial with a few exceptions. The least repeatable plate counts were observed for *C. albicans* in the three species biofilm with *E. coli* and *E. faecalis* (2.82±1.18 Log CFU/cm^2). Antagonistic relationships were observed between *E. coli, C. albicans* and *P. mirabilis*, whereas *E. faecalis* seemed to have a cooperative relationship when present. To further characterise the biofilms, fluorescence *in situ* hybridisation (FISH) was applied and visualised using confocal laser scanning microscopy (CSLM). The imaging provided an insight into the spatial arrangement of the different species, as well as a look at the 3D structures of all these different biofilms.

In conclusion, biofilms are complex structures that affect major aspects of our lives. Microplate-based methods are among the most used methods to study biofilms hence, it is important that we understand their limitations, and their reproducibility. The latter could be improved through the implementation of a minimum information guideline. The methods show potential for standardisation as demonstrated by their good reproducibility in the ring trial. The plate count method also showed good repeatability in assessing multispecies biofilms. Moreover, the application of the FISH method showed the potential of microscopy and the wealth of information we can obtain from it. As such, future work related to microscopy may expand our concept of reproducibility in biofilms beyond what it currently is.

Resumo

Biofilmes são comunidades complexas de microrganismos envolvidos por uma matriz polimérica e frequentemente aderidos a uma superfície biótica ou abiótica. Tem sido largamente descrito que os biofilmes impactam negativamente na indústria e saúde. Em particular, os biofilmes são responsáveis por uma vasta quantidade de infeções, incluindo infeções urinárias associadas a cateteres (CAUTIs). A investigação em biofilmes baseia-se, predominantemente, em reatores de microplaca e, tal como acontece noutras áreas científicas, sofre de falta de reprodutibilidade. Isto deve-se a diferentes fatores, como a publicação de selectiva de resultados e a utilização de métodos pouco fidedignos. Esta tese desenvolve o conceito de um novo sistema de classificação, para definir diferentes níveis de reprodutibilidade e abordagens, para responder ao problema da reprodutibilidade, focando-se em métodos baseados em microplaca.

Inicialmente, para fazer face ao problema da publicação seletiva de resultados, optámos pela aplicação de uma diretriz de informação mínima, no que concerne a avaliação da formação de biofilmes em microplaca, por métodos espectrofotométricos e fluorométricos. Esta diretriz visa aumentar a qualidade do que é publicado e, assim, melhorar a reprodutibilidade. Foi elaborada segundo recomendações de grupos internacionais que trabalham com os referidos métodos, instruindo autores e revisores sobre a informação necessária que um manuscrito deve incluir, de forma a assegurar a clara interpretação e reprodutibilidade das experiências reportadas. A diretriz, e todos os documentos associados, foram disponibilizados online no website MIABiE (http://miabie.org/introduction.php).

Seguidamente, avaliámos a reprodutibilidade de três métodos baseados em microplaca para quantificação de biofilme, em biofilme mono-espécie: contagem em placa, resazurina e cristal violeta. Isto foi feito através de um estudo '*ring trial'* que envolveu 5 laboratórios. As experiências foram divididas entre controlo e tratamento. Para tratamento, a eficácia de hipoclorito de sódio (NaOCl) na erradicação de biofilmes de *Staphylococcus aureus* foi avaliada. As experiências-controlo demonstraram que o cristal violeta foi o método mais reprodutível, com o desvio-padrão (DP) mais baixo. Nas experiências-tratamento, a contagem em placa apresentou a melhor reprodutibilidade, no que respeita à sensibilidade (DP/declive), demonstrando, assim, ser o método mais fidedigno para avaliação da eficácia de desinfeção.

Na verdade, os biofilmes são frequentemente multi-espécie. Portanto, de seguida, decidimos avaliar também a repetibilidade do método de contagem em placa para diferentes biofilmes multi-espécie. As CAUTI foram usadas como modelo e quatro espécies foram selecionadas: *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis* e *Candida albicans*. Onze combinações diferentes de biofilmes (1 a 4 espécies) foram desenvolvidas em cupões de silicone, em meio artificial de urina (MAU). Genericamente, e salvo raras exceções, o DP da repetibilidade para cada espécie foi baixa e comparável com os números observados no '*ring trial'*. A contagem em placa com menor repetibilidade foi obtida para *C. albicans* no biofilme tri-espécie com *E. coli* e *E. faecalis* (2.82±1.18 Log UFC/cm^2). Relações de antagonismo foram observadas entre *E. coli, C. albicans* and *P. mirabilis*, enquanto *E. faecalis*, quando presente, pareceu apresentar uma relação de cooperação. Com o intuito de caracterizar a estrutura 3D de todos os diferentes biofilmes e a organização espacial das diferentes espécies, aplicou-se hibridação *in situ* fluorescente (FISH) combinado com visualização por microscopia confocal.

Em conclusão, os biofilmes são estruturas complexas com efeitos profundos na sociedade. Métodos com base em microplaca encontram-se entre os mais usados para estudar biofilmes e, assim, é essencial entender as suas limitações e reprodutibilidade. Este trabalho mostrou que a implementação de uma diretriz de informação mínima melhorou a reprodutibilidade. Os métodos apresentam potencial de padronização, como verificado pela sua boa reprodutibilidade no '*ring trial'*. O método de contagem em placa demonstrou boa repetibilidade, para biofilmes multi-espécie. Além disso, a aplicação de FISH, combinado com microscopia, revelou a quantidade significativa de informação adicional que este método fornece. Desta forma, trabalho futuro com base em microscopia pode expandir o conceito de reprodutibilidade em biofilmes, desenvolvido nesta tese.

Structure and Aims

The main aim of this thesis is to investigate different methods of addressing the reproducibility issue within the biofilm field and improve upon it. The lack of reproducibility among published studies is one of the major issues we are facing in science today. There are many factors contributing to this problem, hence different strategies are necessary to tackle it.

The thesis is divided into 6 chapters. **Chapter 1** provides an overview of biofilms in general which highlights the various ways they impact us, and the importance of studying them. First it focuses on the process of biofilm formation, their behaviour, and social interactions, especially in multispecies biofilms. Their impact in industry and human infections is discussed, focusing on catheter associated urinary tract infections (CAUTI) as an example of the latter. Finally, a review of *in vitro* biofilm growth reactors, characterisation methods and data analysis, is provided. The section covers more extensively microplate-based reactors and methods, as well as the application of fluorescence *in situ* hybridisation (FISH) to study the spatial organisation of biofilms due to their extensive use in the field, as well as their relevance to later chapters.

Chapter 2 delves into the reproducibility problem and the difficulties we face in the biofilm area specifically. It offers a new perspective on the issue by expanding on the analogy of biofilms as cities and providing a sense of scale to the study of biofilms that illustrates its complexity. The chapter also explores the idea of a new classification system for defining different levels of reproducibility. Level 1 represents a non-reproducible system, Level 2 represents standard reproducibility where plate counts are similar between experiments, Level 3 represents potential standard reproducibility where the 3D structures of the biofilm are similar between experiments, and Level 4 represents a totally reproducible system where all the elements are the same between experiments. It also provides insights into different methods we can use to improve on reproducibility and a pathway to potentially reaching level 3 and level 4.

Chapter 1 and **Chapter 2** emphasise the fact that microplate-based methods are by far the most widely used methods in the field, however little is known on their reproducibility. Thus, our efforts in later chapters focus on these methods. **Chapter 3** introduces our first

approach towards more reproducible biofilm experiments through a minimum information guideline applicable to microplate-based spectrophotometric and fluorometric biofilm assessment methods. This guideline aims to improve reporting of these experiments in the amount of detail provided in the methodology section of an article and data presentation.

Another approach to tackling reproducibility is to evaluate and improve the methods used to study them. In **Chapter 4**, the first interlaboratory study for microplate-based methods was performed. The study includes 5 different laboratories and evaluates the reproducibility of three different biofilm methods for single species *Staphylococcus aureus* biofilms: plate counts, crystal violet and resazurin. These methods were evaluated both for a control study and for a disinfectant efficacy test. The study also evaluates whether different protocols for the assessment part of the experiment would majorly affect the reproducibility of the methods, by having the participants perform their in-house protocols in parallel to the protocol developed for the trial.

Additionally, since biofilms are often multispecies rather than single species, **Chapter 5** investigates the repeatability of the plate count method for different multispecies biofilms. The study uses the urinary catheter as model system. Biofilms were grown on silicone coupons placed in 24-well plates, in artificial urine medium (AUM). Four species of microorganisms often associated with CAUTI were selected: *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis* and *Candida albicans*. Combinations of single species, dual species, three species and four species biofilms were grown, and selective media was used to obtain plate counts per species for each combination. To further characterise the biofilms and investigate the possibility of transitioning to level 3 reproducibility, the FISH method was applied to these biofilms. Different probes were designed to specifically target each species and the biofilms were visualised using confocal laser scanning microscopy (CSLM).

The last chapter, **Chapter 6,** presents the general conclusions of the thesis and discusses future works that could build upon what was presented in this thesis.

This thesis reports on the work performed at the BEL (Biofilm Engineering Lab) group at LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy), Faculty of Engineering (University of Porto) and the SBML (Standard Biofilm Methods Lab) group at the CBE (Center for Biofilm Engineering), Montana State University, USA.

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Nomenclature

A. Glossary

Mixed effects ANOVA – statistical process for separating the variability of a group of observations into assignable sources and performing significance tests.

Error – represents variability attributable to well-to-well differences.

Day – represents variability attributable to day-to-day differences e.g., different inoculum, temperature, humidity, etc.

Lab – represents variability attributable to lab-to-lab differences e.g., different technicians, equipment, climate, etc.

Repeatability – represents the variability within each lab (error + day).

Reproducibility – represents the variability among labs (total = error + day + lab).

Responsiveness - sensitivity of a method; ability of a method to detect important changes in an antimicrobial's efficacy (e.g., due to changes in concentration or contact time).

B. Abbreviations

%	Percent
°C	Degrees Celsius
λ	Wavelength
µL	Microliter
µm	Micrometre
2´OMe	2´-O-methyl RNA
3D	Three dimensional
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
AUM	Artificial urine medium
bp	base pair
C. albicans	*Candida albicans*
CAUTIs	Catheter-associated urinary tract infections
CECT	Spanish Type Culture Collection
CFU	Colony-forming units

CI	Confidence intervals
CLED	Cystine Lactose Electrolyte Deficient
CLSM	Confocal laser scanning microscopy
cm	Centimetre
cm^2	Centimetre square
DAPI	4'-6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EPS	Extracellular polymeric substance
FISH	Fluorescence *in situ* hybridisation
g	Centrifugal force
g	Gram
GFP	Green fluorescence protein
hr	Hour
IHP	In-house protocol
ILP	Inter-lab protocol
ISO	International Organization for Standardization
km	Kilometre
L	Litre
LD	Log density
LNA	Locked nucleic acid
Log	Logarithm with base 10
LR	Log reduction
MBEC	Minimum biofilm eradication concentration
mg	Milligram
MIABiE	Minimum information about a biofilm experiment
mins	Minutes
mL	Millilitre
mm	Millimetre
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
nm	Nanometre
OD	Optical density

P. mirabilis	*Proteus mirabilis*
pH	Potential hydrogen
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SDA	Seaboard's Dextrose Agar
SE	Standard Error
sec	Second
SGSC	Salmonella Genetic Stock Centre - Canadian culture collection
S_r	Repeatability SD
S_R	Reproducibility SD
STM	Standard test method
Tris-HCl	Tris-aminomethane hydrochloride
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
v/v	Volume/volume percent
YPD	Yeast-extract peptone dextrose

Scientific outputs

Papers in peer reviewed journals

Nuno F. Azevedo, Jontana Allkja, and Darla M. Goeres. "Biofilms vs. cities and humans vs. aliens–a tale of reproducibility in biofilms." *Trends in Microbiology* (2021). doi:10.1016/j.tim.2021.05.003. **(Chapter 2)**

Jontana Allkja, Thomas Bjarnsholt, Tom Coenye, Paul Cos, Adyary Fallarero, Joe J. Harrison, Susana P. Lopes *et al.* "Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates." *Biofilm* 2 (2020): 100010. **(Chapter 3)**

Jontana Allkja, Frits van Charante, Juliana Aizawa, Inés Reigada, Clara Guarch-Perez, Jesus Augusto Vazquez-Rodriguez, Paul Cos *et al.* "Interlaboratory study for the evaluation of three microtiter plate-based biofilm quantification methods." Sci Rep 11, 13779 (2021). https://doi.org/10.1038/s41598-021-93115-w **(Chapter 4)**

Book chapters:

Jontana Allkja and Andreia S. Azevedo. "Characterization of Social Interactions and Spatial Arrangement of Individual Bacteria in MultiStrain or Multispecies Biofilm Systems Using Nucleic Acid Mimics-Fluorescence In Situ Hybridization." In *Fluorescence In-Situ Hybridization (FISH) for Microbial Cells*, pp. 97-109. Humana, New York, NY, 2021. **(Chapter 5)**

Communications in international conferences

Oral communications

Jontana Allkja, Albert E. Parker, Nuno F. Azevedo, Darla M. Goeres "Principles of method standardisation". eCM XIX: Orthopaedic Infection Conference. Davos, Switzerland, June 2019.

Jontana Allkja "Why we need minimum information guidelines" 6th European Congress on Biofilms, EUROBIOFILMS 2019. Glasgow, Scotland, September 2019.

Jontana Allkja, Frits van Charante, Juliana Aizawa, Inés Reigada, Clara Guarch-Perez, Jesus Augusto Vazquez-Rodriguez, Paul Cos *et al* "Interlaboratory study for the evaluation of three methods used for the quantification of biofilms in microtiter plates" 6th European Congress on Biofilms, EUROBIOFILMS 2019. Glasgow, Scotland, September 2019.

Jontana Allkja, Nuno F. Azevedo, Darla M. Goeres "Towards more reproducible biofilm experiments." Print-aid Outreach conference. Online, December 2020.

Jontana Allkja, Albert E. Parker, Nuno F. Azevedo, Darla M. Goeres “Towards more reproducible biofilm experiments” SurfSafe Scientific Workshop 1. Online, June 2021.

Poster communications

Jontana Allkja, Nuno F. Azevedo, Darla M. Goeres “Development of minimum information guidelines and standardized methods for biofilm experiments.” Montana Biofilm Science and Technology Meeting. Bozeman, Montana, USA, July 2018.

Jontana Allkja, Thomas Bjarnsholt, Tom Coenye, Paul Cos, Adyary Fallarero, Joe J. Harrison, Susana P. Lopes *et al.* "Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates." 6th European Congress on Biofilms, EUROBIOFILMS 2019. Glasgow, Scotland, September 2019.

Jontana Allkja, Albert E. Parker, Nuno F. Azevedo, Darla M. Goeres and Print-aid Ring trial group “Evaluation of the reproducibility of microtiter plate-based biofilm quantification methods.” CBE 2021 Regulatory Biofilm Meeting, Online, February 2021.

Jontana Allkja, Albert E. Parker, Nuno F. Azevedo, Darla M. Goeres “Down the rabbit hole: The journey to reproducible biofilm experiments.” Scandinavian Society for Biomaterials Conference (ScSB 2021), Online, June 2021.

Chapter 1.

General introduction

Abstract

This chapter provides a general overview on biofilms, how they are formed, their structure and their behaviour, with particular focus on the behaviour of multispecies biofilms and their social interactions. Moreover, the overall impact of biofilms in various industries as well as infections, particularly device associated infections will the reviewed. Lastly, a review of various biofilm *in vitro* reactors and assessment methods will be provided, with a particular focus on microplate-based methods and fluorescence *in situ* hybridisation due to their extensive use and relevance to other chapters.

Keywords

Biofilms, multispecies, reactors, assessment methods

1.1 Biofilms

Biofilms can be defined as communities of microorganisms encased in a self-produced matrix attached to a biotic or abiotic surface. While the most commonly studied organisms in association with biofilms are bacteria, fungal biofilms are also well-known. [1,2]

The matrix is composed of water and extracellular polymeric substances (EPS) such as lipids, proteins, extracellular DNA (eDNA), polysaccharides, filamentous phages and more. [3] It provides structural stability, efficient diffusion of nutrients and oxygen throughout the biofilm, as well as protection from toxins, disinfectants, antimicrobial compounds, and harsh environmental conditions. [4]

The environment within the matrix is ideal for communication between the different individuals through mechanisms such as quorum sensing. This involves the exchange of small chemical signalling molecules between bacteria. When recognized by surface receptors, they provide information on the environment, nutrient availability, presence of toxins and antimicrobials. [5] This form of communication can serve as a tool to direct biofilm formation, as well as induce genotypic and phenotypic changes to individual cells within the biofilm leading to changes in behaviour and properties. [6,7] An example of this system is the *las* system in *Pseudomonas aeruginosa* composed of LasI and LasR, which plays an important role in biofilm formation. [7] Another common phenomenon in biofilms is horizontal gene transfer due to the presence of free DNA in the matrix and close proximity of microorganisms within the biofilm. [8]

Biofilms are mostly heterogenous meaning they can differ in height, volume, porosity, cell density, matrix composition, spatial organisation and more. They can also have more than one species or strain present at the same time, or even kingdoms for example, fungi and bacteria together. [9] Despite this heterogeneity the biofilm formation process can be very similar. There are generally four main stages: attachment, early biofilm formation, maturation, and dispersal (Figure 1.1). In the attachment phase, microorganisms attach to a surface through adhesion proteins present on their cell membrane. These can be highly adaptable to the surface topography and range from pili and flagella to species-specific proteins such as USPA1 in *Moraxella catarrhalis.* [10,11] In the early biofilm formation phase, the microorganisms start to produce the biofilm matrix. This requires changes in gene expression as they move from a proliferation driven state to a more static state. Two-component signalling systems such as cAMP-CRP (Cyclic adenosine monophosphate-catabolite activator protein) are often associated with this stage. [12,13] The microorganisms

can also start to aggregate, for instance, in *Bacillus subtilis* biofilms the rod-shaped bacteria start to elongate and form chains. These chains are then stacked on top of each other as the biofilm grows. [14] In the maturation phase, microorganisms in the biofilm produce a variety of EPS products such as lipids and proteins. They also release eDNA and based on the signals and the environment within the biofilm they undergo a phenotypic variation. [15] Finally, when certain signals are released, individual cells or clumps of cells start to disperse and leave the biofilm. [16]

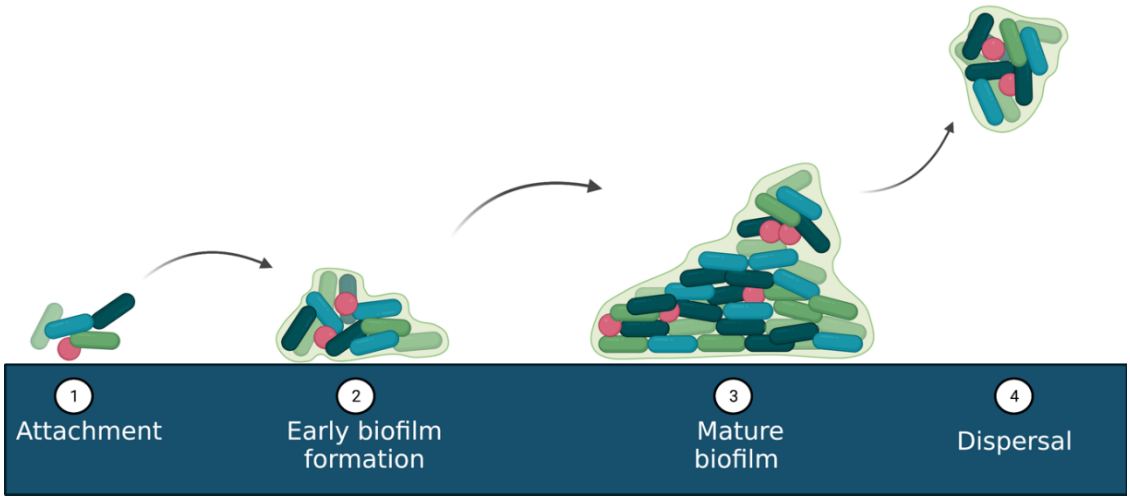

Figure 1.1. Stages of biofilm formation. 1. Attachment **2.** Early biofilm **3.** Mature biofilm **4.** Dispersal. Created with BioRender.com

1.1.1 Multispecies biofilms

Biofilms are commonly polymicrobial, containing multiple species of microorganisms. This means that, how different species of microorganisms interact with one another i.e., their social phenotypes play an important role in biofilm formation, structure, properties, and behaviour. Hence, many different types of relationships can exist between microorganisms. Three of the more common arrangements observed can be seen in Figure 1.2.

For example, if an antagonistic species is present it tends to favour its own and produce proteins which can kill or inhibit any other species present in the biofilm, thus becoming the dominating species in the biofilm (Figure 1.2A).[17] In other cases, antagonistic relationships can produce biofilms where distinct microcolonies or aggregates of each species can be seen, with no apparently dominant species.[18] On the other hand, species often behave in the interest of the "common good" and cooperate with each other in a

mutualistic type relationship leading to a very well mixed heterogenous biofilm (Figure 1.2B). [19,20] There are also public good species which will secrete products that benefit all species in the biofilm, but still show a preference to its own clones. Hence, the public good species tends to be dominant and more abundant, but other species are also present leading to a heterogenous biofilm (Figure 1.2C). [21] This often results in a layered biofilm for example, in an *Acinetobacter sp.* and *Pseudomonas putida* biofilm; the *Acinetobacter sp.* found in the upper layer assists the growth of the lower layer *P. putida* by metabolising different substrates into compounds to be used by them. [22] Competitive interactions between different species are also known to form biofilms that resemble the well mixed and layered biofilms. [23,24]

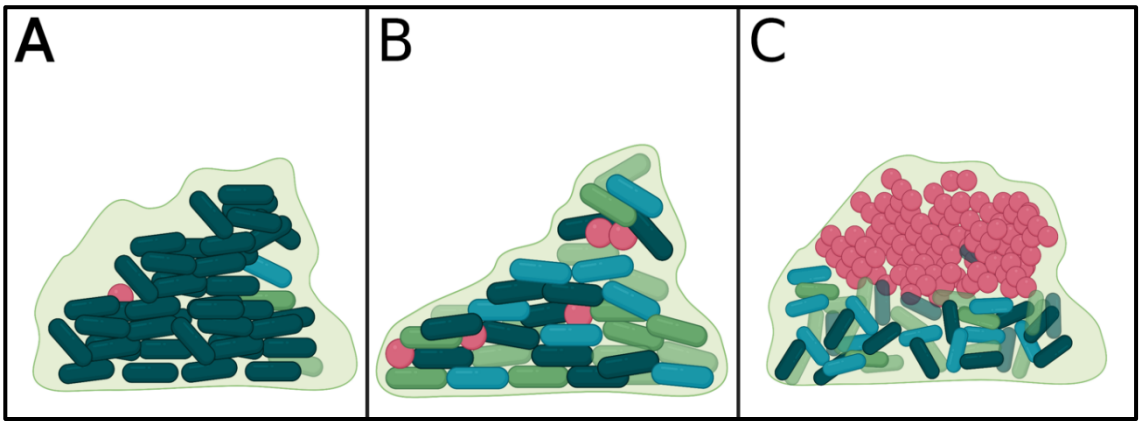

Figure 1.2. Common biofilm social phenotypes. A. Antagonistic – one dominating species **B.** Mutualistic – well mixed biofilm **C.** Public good layered – one species is more abundant but assists others within the biofilm as well. Created with BioRender.com

These mixed communities can result in an overall increase in biomass and even enhance certain functions of the biofilm such as its physical protection and virulence. Production of some compounds within the matrix can also increase in multispecies biofilms, when compared to single species ones. Interestingly, these effects have also been observed in biofilms where antagonistic phenotypes are present.[25] Many of these interactions have been observed in well-defined model systems, where specific strains and species are selected and introduced into an engineered system. [26] They have also been observed in microbial ecology studies which use undefined models i.e., the species and strains within the biofilm are directly taken from the naturally-occurring system rather than selecting specific ones. [27] An example of this are works modelling naturally occurring biofilms in drinking water systems. In this case, water samples are taken from either the drinking water supply or

groundwater sources and feeding them into a continuous flow reactor e.g., the CDC reactor, and monitoring biofilm growth over time.[28,29]

1.1.2 Biofilms in industry and healthcare

Biofilms are an important part of many ecosystems. They can often be found growing in places such as hot springs and freshwater rivers, which gives us an insight on their adaptability and survival strategies. [1,8] Biofilms can be engineered to assist us in many industrial processes, for example water purification in wastewater treatment plants.[30,31] Another useful application of biofilms is as a means of production for various proteins of commercial interest, such as lactic acid. [32,33] Bacteria known for their abundant biofilm production, such as *Escherichia coli*[32] and *B. subtilis*[34], are the ones most commonly-used in these settings to maximise production. Another possible beneficial use for biofilms is their application for biofuel production.[35]

Unfortunately, there is also a negative side to biofilms. They can often be very detrimental to many industries. One of the most prevalent examples is the food industry where biofilms can form during the production process and either contaminate the food or cause issues with machinery function. [36,37] Biofilms are also known to cause problems in water drainage and pipe systems, where they can cause obstructions of the pipes and impede proper flow and water distribution.[38] Furthermore, energy production industries reliant on cooling tower reactors can also be affected by biofilm contamination.[39] Ships hulls can also easily be colonised by marine biofilms which can increase drag and affect how well the ship travels.[40,41]

Another area biofilms are known to cause major issues is in healthcare, where they play a major role in both chronic and acute infections. They are especieally prevalent in device associated infections where they attach to the surface of the device or implant. An example of this type of infection occurs in urinary catheters leading to catheter associated urinary tract infection (CAUTI). [42] This can occur both on the inside and outside of the catheter. When on the inside of the catheter, the biofilm can travel upwards towards the bladder or even cause blockage requiring re-catheterisation which increases the chances for re-infection. [43,44] In the case of biofilm formation on the outside of the catheter lumen, the microorganisms can infect the urethra epithelial layers and cause complications. [44,45] If left untreated CAUTIs can lead to chronic infections, kidney and bladder damage, bladder stones and septicaemia. [46] They are mostly caused by multispecies biofilms with organisms such as *E. coli, Enterobacter sp, P. aeruginosa, Proteus spp., Candida spp.* and *Enterococcus spp.* as some of the most common culprits. [47–49] Other uncommon and flora

associated microorganisms have also been detected within these biofilms. [47] Studies using dual or multi-species models with uncommon microorganisms such as, *Delftia tsuruhatensis* and *Achromobacter xyloxosidans* have been shown to interact with *E. coli* and create a well-organized biofilm with increased tolerance to antibiotics. [20] Studies using dual or multi-species models have also shown that despite an initial antagonism against each other, with time the presence of the catheter material allows the pathogens cohabitate and cooperate with each other. [50–52]

Other common biofilms associated infections include orthopaedic device infections[53], oral infections (e.g., periodontitis)[54], chronic wound infections[55] and chronic lung infections especially prevalent in cystic fibrosis patients. [56] Unfortunately, treatment of CAUTI and other biofilm associated infections with antibiotics or antimicrobial compounds often fails. [8] This phenomenon has also been observed for disinfectant efficacy.[57] Biofilms are inherently more tolerant towards antimicrobials as their matrix acts as a physical barrier to diffusion and in some cases the dormant state of cells within the biofilm can prevent antimicrobial up-take. [58] Quorum sensing and horizontal gene transfer can also aid the exchange of resistance genes further complicating the issue. [59] In the case of multispecies biofilms, finding a treatment strategy capable of targeting a complex consortia of organisms is essential. [59] As a result, alternatives to conventional treatments have been suggested over the years such as preventing biofilm formation. An example of these are coatings of catheter surfaces with different compounds e.g., coating with silver hydrogel, quorum sensing inhibitors such as acylase and α-amylase, [60] or antimicrobial peptides. [43]

1.2 Biofilm models

As previously discussed, biofilms are complex structures that can positively and negatively impact many aspects of our life. Much of our understanding of biofilms is based on *in vitro* experimental models, which is why it is important for the experimental setup to mimic the real-life scenario as closely as possible. These experiments involve two parts, the reactor used to grow the biofilm and the characterisation method used to assess it. Both the reactor and the assessment method can impact the repeatability (within lab variability) and reproducibility (among lab variability) of the experiment.

1.2.1 Biofilm growth reactors

The simplest and most widely used biofilm reactors are multi-well reactors or microplates, where the biofilm is grown in a batch system, meaning there is no flow of nutrients into the system. Microplates offer a lot of advantages due to their ease of use, flexibility, and

potential for high-throughput screening. They are extensively used in novel antimicrobial compound screening, [61] as well as studies investigating basic biofilm behaviour. [62] Their repeatability was assessed for different characterisation methods in two separate studies and while some methods performed better, the overall observation was that the microplate was not robust. They found that minor variations in the biofilm growth conditions, as well as protocol performance from well-to-well and from day-to-day (even by experienced personnel) affected the repeatability of the biofilm. [63,64]

Another example of a biofilm reactor is the flow cell system, which is a continuous flow type reactor where biofilm grows under continuous flow conditions. In this case, nutrients are constantly fed into the system and their flow rate is controlled to mimic the desired real-life system. [32] The flow cell consists of a semi-circular Perspex duct of varying sizes, with multiple apertures on its flat wall to fit removable rectangular coupons (most common 2 × 1 cm growth surface area).[65] Bacterial suspension and fresh broth are constantly circulating through the system at a desired flow rate and the biofilm forms on the upper surface of the coupon. This method can be adapted for continuous assessment of the biofilm over-time through microscopy. [65] Its repeatability has been investigated by Heydorn *et al.*[66] They looked at the repeatability of biofilm thickness for different *P. aeruginosa* strains across different coupons in different experimental days. The measurements were performed using microscopy and their results showed that the method was repeatable within each strain. [66]

Standardised biofilm reactors are also used. These need to fulfil certain criteria to be classified as such. Firstly, the protocol needs to be reasonable i.e., not too expensive, relatively easy to learn and preferably use conventional laboratory materials and equipment. Additionally, it needs to be valid, meaning the method is relevant to its application and mimics the real-life situation as closely as it can while remaining unbiased. [67] Secondly, the ruggedness, responsiveness and repeatability of the method need to be evaluated in intra-laboratory studies. Ruggedness refers to the sensitivity of the outcome to minor differences in environmental or operational factors. Ideally in a standard method these minor differences should not affect the final results significantly. [68] Responsiveness refers to the sensitivity of the method itself. Hence, limits of detection need to be evaluated and different factors such as starting inoculum concentration and contact time need to be tested and compared. Repeatability looks at the day-to-day between independent repeats of the experiment, conducted in the same laboratory. [68,69] Lastly, the reproducibility of the method needs to be evaluated and validated by societies like the American Society for

Testing and Materials (ASTM). This means looking at the variability of results between experiments run in different laboratories, also called a ring trial or inter-laboratory study. [68]

The simplest of these is an alternative reactor to the microtiter plate system, the Calgary or MBEC reactor. [70] This is a modified microplate reactor, in which the lid contains a series of pegs where the biofilm forms. The reproducibility of the MBEC system has been evaluated for disinfectant efficacy testing on *P. aeruginosa* biofilm using the Colony Forming Unit (CFU) count method, through an eight-lab collaborative (ring trial) study. [68] Other standard reactors are continuous flow reactors which include the drip flow and CDC reactors. Both these reactors have been approved by ASTM for standard biofilm test methods (STMs). [71,72] The CDC reactor is a high shear continuous stir tank reactor composed of 24 rods each able to contain 3 coupons with a 4.05 cm^2/coupon growth surface area. [73] Ring trials have been performed to evaluate the reproducibility of *Staphylococcus aureus* (reproducibility SD = 0.44) and *P. aeruginosa* (reproducibility SD = 0.24) growth for the CDC reactor. Alternatively, the drip flow reactor is a low shear continuous flow reactor composed of 4-6 chambers each containing one coupon with an 18.75 cm^2/coupon growth surface area. [74] Data generated in a 10-laboratory ring trail using this reactor resulted in a reproducibility SD = 0.27 for the growth of *P. aeruginosa* biofilms. [73]

While all the above reactors enable the characterisation of biofilms at a large scale, microfluidics reactors can provide a way of studying smaller biofilms in a more controlled environment. [75,76] These are low-volume flow reactors (10^{-2}-10^{11} µl) with a very small adhesion surface area (10 mm^2-10^{-6} mm^2). [77] This makes it possible to investigate the early stages of biofilm formation and the effect of fluid dynamics in a more controllable manner, as experimental conditions (temperature, flow rate, pressure, pH, O_2 level) can be better regulated compared to existing techniques.

Furthermore, microfluidics reactors can be used in combination with various techniques such as confocal laser microscopy to visualise single individuals in the biofilm in a non-invasive way.[78] They can also be combined with nanochannels (<100 nm) to enable single cell analysis. [79] The possibility for automation of the system, could further improve reproducibility by removing the technician as a factor contributing to variability. [80] Commercial microfluidics systems such as Bioflux™ have been developed, however it is worth noting that no reports on the reproducibility of microfluidics reactors or validated methods are available yet. [81] This could be due to the fact that this is a fairly new approach to biofilm growth and not as widely used as some of the other reactors. It can also be a

result of the customisable design process offered by many microfluidics fabricating companies.

A common concern with biofilm reactors is that surface topography can affect the nature and development of biofilms, so nano-surface technology could allow us to model the topography (roughness, porosity) of the surface we want to use or study, to the micro and nano-scale. [82] This could allow us to control where biofilms grow on a surface. Surfaces could be modified or modelled to inhibit or stimulate adhesion, [83] or to direct cell growth in a specific direction. [84] To go a step further droplet printing of micro-patterned bacterial communities was recently achieved Kumar *et al.* Their work demonstrated the impact micro scale spatial location and structural differences can have on biofilm structure and interactions within it. [85]

1.2.2 Biofilm assessment

As microplates are the most widely used biofilm growth reactors, microplate based assessment methods are also very prevalent in the field. [86] The simplest and most widely used characterisation methods rely on dyes, most often crystal violet. This dye is used to quantify total biofilm mass as it binds to negatively changes polysaccharides and molecules in the matrix and cell surface. [87–89] However, it does not allow for differentiation between active and inactive cells, and sedimentation can influence the results which can be problematic when trying to assess the effect of antibiotics. [90,91] Thus, metabolism-based dyes such as resazurin, also known as Alamar Blue, have been used. Once the resazurin, originally blue in colour, gets into viable (live) cells it is metabolised by the bacteria and reduced to the fluorescent resorufin (pink). [92,93] This is mainly used to assess the effect of antimicrobials that affect metabolic pathways or various proteins rather than the cell membrane. [94,95] Another metabolic activity assay used for testing antimicrobial sensitivity is 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT). This is based on the reduction of tetrazolium salt into an fluorescent orange coloured formazan derivate. [61,96–98] Other tetrazolium salt based methods also exist such as, 2,3,5-triphenyl-tetrazolium chloride (TTC) and 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT), which are metabolized into red and purple derivates respectively. [99–102] Furthermore, FDA (Flourescein diacetate) is an enzymatic activity assay where the non-fluorescent, non-coloured fluorescein diacetate is hydrolysed and converted into a yellow fluorescent fluorescein when metabolised by the microorganism. [91,103] Spectrophotometric methods based on staining specific components such as, Syto 9 Green

Fluorescent Nucleic Acid Stain which passively diffuses into the cells and stains their DNA and Wheat Germ Agglutinin (WGA) which stains biofilm EPS are also widely used. [103–106] One of the major advantages of these characterisation methods is their potential for high-throughput screening i.e., range of several hundred samples per run. Sanberg *et al.*[107] showed how this could be achieved by automating their 96-well plate model for *S. aureus* to screen natural antimicrobial compound efficacy. They used a crystal violet assay and compared the performance of the automated system to manual runs of the protocol. [107] Another advantage is the ability to combine these different stains and measure different parameters of the same biofilm. For example, in the study by Skogman *et al.*[105] they combined resazurin, crystal violet and WGA staining. This assay was performed in sequential steps, where one staining method was used and measured then followed by a different method. This resulted in a quantification of the metabolic activity of the biofilm, the matrix and the total biomass, which gives a fuller picture of the biofilm as a whole. [105] The most widely used characterisation method associated with validated STMs is the plate count method, also referred to as CFU counts. It quantifies the amount of culturable bacteria/fungi within the biofilm and it can be applied independently of the growth reactor. The plate count method is a reproducible method for biofilm assessment and has been validated for many applications. [70,71,108] More recently, a new protocol "The single tube method" was approved as a standard method by ASTM. This protocol relies on plate counts for evaluating disinfectant efficacy on *P. aeruginosa* biofilms grown in the CDC reactor. [109,110]

Omics-based characterisation methods have risen in popularity in recent years, as the technology is becoming cheaper and easier to use. Through next generation sequencing techniques, it is possible to run metagenomic, metabolomic, proteomic, transcriptomic and lipidomic analyses on biofilm samples. [48,111] These methods provide information on the ecology of biofilms and the behaviour of different species within it. Combining two or more of them i.e. multi-omics analysis, can provide more in-depth information on an ecological system. [112] The use of omics techniques simultaneously eliminates the need for separate samples thereby minimising possible sources of variation. Additionally, high-throughput applications in combination with fluorescence-activated cell sorting (FACS) and next generation sequencing can be designed to allow single cell differentiation and more in-depth data about individuals in the biofilm community. [113] Single cell omics techniques have recently been coupled with microfluidics systems. As the cells go from one chamber to the other, they go through the different methodological steps for the omics

method which helps reduce the amount of reagent used, as well as sources of contamination as it is a closed system. [114,115] Some of these reactors can even be used to perform single cell multi-omics. [116]

The ability to investigate the location and spatial organisation of the biofilm and its components is crucial. Microscopy can be especially useful. Microscopy images can provide information on the spatial organisation of biofilms and the individual bacteria within them. [21] They can also provide information on the behaviour of the biofilm and the social interactions between different bacterial species in mixed biofilms. [20] Additionally, microscopy can be used to quantify biofilm structures by measuring areal porosity, fractal dimensions and average diffusion distances. [117]

Epifluorescence and Confocal Scanning Electron Microscopy (CSLM) are the most used light-based imaging techniques for biofilms. They have provided insight on the properties and structures of various biofilms with high-quality images within the optical diffraction limit (<100nm). [52,118] Various electron microscopy techniques such as, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) have been used over the years as an alternative to CSLM, to visualise biofilms at a greater detail. [119,120] These methods can reach resolutions as low as 1 nm, however they require more labour-intensive and stringent sample preparation. [121] Atomic Force Microscopy (AFM) is probe based scanning microscopy able to image samples past the optical diffraction limit and often used to obtain information on the topological and mechanical/interaction features of a sample. [122] AFM has been used to investigate biofilm adhesion forces to various surfaces and materials [123] as well as investigating structural components and mechanical properties of biofilms. [124] It can also provide an analysis of the topological features of biofilms. [125]

In later years, efforts on improving microscopy methods have been made to improve resolution, achieving high quality imaging past the limit of diffraction and minimising compromission of biofilm features. For example, the Mesolens is a novel microscope with epifluorescence and CSLM image acquisition capabilities at the subcellular level. It has a uniquely large aperture which allows for better resolution images of tissues and organisms without the need for special preparation or specimen dissection and destruction. Recently, the Mesolens was used to image *E. coli* biofilms and it made possible the imaging of intra-colony microchannels and tracking of the uptake of fluorescently labelled microspheres hinting at the possible role of these microchannels in biofilm formation and stability. [127]

Super-resolution microscopy techniques are another advancement in the field of light-based microscopy and allow imaging at resolutions past the optical diffraction limit. [128]

Some of these super-resolution techniques are Structured Illumination Microscopy (SIM), Stimulated Emission Depletion (STED) microscopy and Single Molecule Localisation Microscopy (SMLM). [119] SIM uses a patterned illumination system to excite the sample in different positions and orientations, and extracts the information obtained over multiple fluorescence images. This enables it to increase the resolution (~ 50 nm of less). [129,130] In STED a fluorophore in the sample is excited using a specific wavelength while simultaneously using a second beam to deplete all the surrounding fluorophores. This is continuously performed at random locations throughout the entire sample and can reach resolutions of ~ 40 nm of less. [131] Innovation is still on-going in the field and multi-imaging by combining different microscopy methods is becoming more frequent. [132] Stochastic optical reconstruction microscopy (STORM) is another imaging technique, often used in conjunction with PALM, that allows for single molecule visualisation. In STORM several images are acquired in cycles. In each of these cycles individual fluorophores are switched on and off to avoid overlapping and allow for accurate location and imaging of each fluorophore. Using STORM, resolutions of approximately 20 nm have been achieved which can enable us to view complex biological processes and image specific molecules below the optical diffraction limit. [133,134]

Additionally, microscopy techniques mentioned above require a labelling method to be able to image the biofilm and its various elements. One such labelling method is fluorescence *in-situ* hybridisation (FISH) which uses fluorescently labelled oligonucleotide probes to target a unique sequence of DNA or RNA within the microorganism, so it emits fluorescence and can be imaged through a microscope (Figure 1.3). [86] This method relies on several steps: sample fixation and permeabilization using paraformaldehyde and ethanol, to ensure that the probes can enter the biofilm and cells within it; hybridisation where optimal temperature and hybridisation solutions are necessary to ensure that the probe hybridises with its target; washing to remove any unattached probes; and finally imaging. [135]

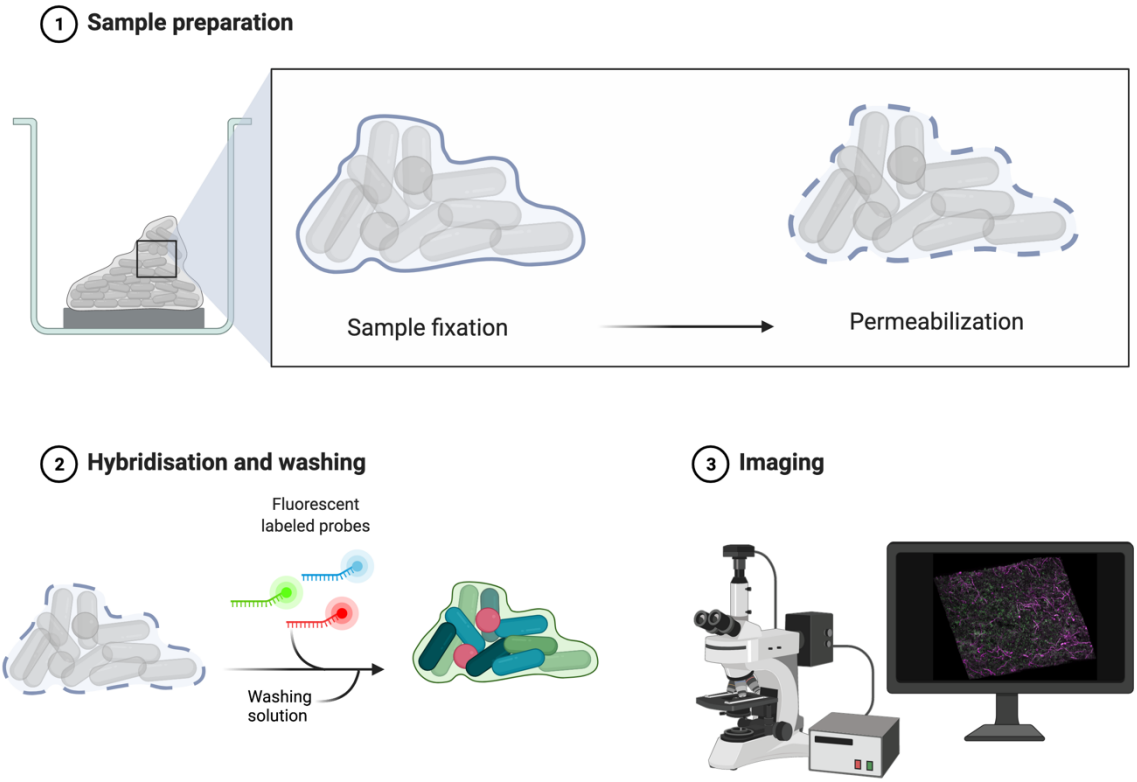

Figure 1.3. Illustration of the fluorescence *in situ* hybridisation process in biofilms. 1. Sample preparation – fixation and permeabilization. **2.** Hybridisation and washing **3.** Imaging. Created with BioRender.com

To improve the stability and binding of FISH probes, Nucleic Acid Mimics (NAMs) such as, peptide nucleic acid (PNA), locked nucleic acids (LNA) and 2'-O-methyl-RNA (2'OMe) have been used to substitutes DNA and RNA. NAM-FISH has enabled the visualisation of the 3D structure of biofilms and the spatial arrangement of 2-3 species of microorganisms within the same biofilm. [20] For example, its use in combination with confocal scanning laser microscopy (CSLM) to visualize the structure and spatial arrangement of single and dual species biofilms in urinary catheter model systems. [52,118] Recently, NAM-FISH has also been employed to study multi-strain biofilms of *E. coli*, in urinary catheter systems. [136]

Another variation of FISH developed by Valm *et al.,* is CLASI-FISH (Combinatorial Labelling and Spectral Imaging-FISH). [137] This method works by targeting one strain with two or more probes targeting different sections of the rRNA. Each fluorophore has a distinct colour and combinations of different fluorophores together produce different colours. Depending on the number of probes available to you there are many different

possible combinations. For example, if you were to start with 6 fluorophores and combine them 2 by 2, there are 15 possible combinations. [138] Recent work by Schlundt *et al*,[139] applied CLASI-FISH to study marine biofilm communities and their interaction with plastic materials commonly found in the ocean. Their work provided novel insights on the different stages of marine biofilm formation as well as, the spatial distribution and interaction of 7 common marine taxonomic groups of bacteria within marine biofilms formed on plastic debris over the course of 5 weeks in different geographical locations. [139] CLASI-FISH has also been used to study dental biofilms. For example, the study by Welch *et al*., where CLASI-FISH in combination with CSLM were used to study the spatial structure and overall interactions of dental plaque by using probes targeting 9 common oral microbiome taxa. [140]

Another version of the FISH method can be seen in the recent work by Shi *et al.*,[141] where they used high-phylogenetic-resolution microbiome mapping by FISH (HiPR-FISH) to differentiate between 1,023 *E. coli* isolates. This technique uses machine learning to combine the labelling of up to 10 different fluorophores and create a binary barcode for each species/strain and can form up to 1023 unique combinations. The images can then be used to create micrometre-scale maps of the locations and identities of microbial species in complex communities. [141]

GFP expression is another widely used labelling method. The bacteria are modified to carry the GFP or eGFP gene and express the GFP protein which emits fluorescence. There are many different types of GFP proteins which emit fluorescence at different wavelengths, and this can allow us to distinguish between different species of bacteria in the same biofilm without the need for fixation. [142,143] Other labelling methods include Live/Dead staining, which relies on the use of SYTO9 and Proprium Iodine (PI) staining to identify live (SYTO9-green) and dead (PI-red) cells within the biofilm. Recently, however, it was shown that this method is affected by the presence of eDNA in the EPS structure of the biofilm and therefore can provide unreliable results. [144]

Furthermore, single-molecule labelling coupled with super resolution microscopy, can enable the interrogation of complex biological processes which was not possible until very recently. One such labelling system is CRISPR-Cas9 based imaging. Different genetic loci can be targeted through CRISPR and a fluorescence tag on the Cas9 protein emits the signal. [145] Other single molecule labelling systems include BiFC-PALM (Photoactivated Localization Microscopy with Bimolecular Fluorescence Complementation) used to study protein-protein interactions which could potentially be applied to biofilms and provide

more information on the dynamics in the community. [146] Additionally, advances in fluorescence nanoscopy could enable us to visualise specific molecules or proteins in unprecedented detail both *in vitro* and *in vivo.* [147]

Alternatively, label free imaging techniques such as Raman and Mass Spectrometry Imaging (MS-I) can also be used to image biofilms. [148,149] Raman imaging can be used to map the chemical composition and molecular structure of a sample on the Raman spectrum at ~300 nm resolutions. [150] MSI relies on a similar principle. It uses mass spectrometry to map the spatial localisation of different molecules (lipids, protein, metabolites) within a sample. [151] Furthermore, these techniques can be combined with CSLM or super-resolution microscopy to obtain high resolution images of biofilm structures and their chemical composition. [152]

Another promising potential technology for use in biofilms is X-ray imaging, reaching resolutions as low as 0.01 nm. X-ray microprobes and nanoprobes are used in scanning X-ray microscopy to scan the sample and produce images on a pixel by pixel basis. [153] These techniques could be very useful for the mapping of nutrients and chemical components within the EPS structure of biofilms.

1.2.3 Data analysis methods

As new technologies are being developed, it is particularly important to keep in mind the value of reliable downstream data analysis. This is essential for techniques such as omics and microscopy where the outputs need to be analysed through different software. For image analysis, with each new application and acquisition method, new image analysis protocols need to be validated and developed. [117] There are many commercial software analysis packages available such as COMSTAT and Imaris, which can process mainly epifluorescence and CSLM based images for qualitative and quantitative image analysis. [154,155] Open-source tools are also available, such as ImageJ and more recently BiofilmQ. They contain readily available packages for qualitative and quantitative image analysis as well as the option for designing and implementing novel image analysis packages based on your specific needs. [156,157] In recent work by Gallelo-Hernandez *et al.,* BiofilmQ was used to quantify *Vibrio cholerae* biofilm and planktonic abundance and position based on virulence gene expressions in fluorescence microscopy images obtained from the mouse gut. [158] On the other hand, bioinformatics tools can also be used to develop algorithms personalized for your system. For example, in the previously mentioned work by Shi et al.,[141] their algorithm allowed them to calculate association matrices for the biofilm by extracting directional information on each cell within the biofilm in relation to

neighbouring ones. This enabled them to quantify the biofilms spatial organization and perform statistical assessments in 3D. [141]

Similarly to microscopy, with the improvement of omics technologies and development of novel approaches, the data analysis issue cannot be ignored. Different commercial and open source software packages exist for the quality assessment, assembly, qualitative and quantitative analysis of single omics techniques, such as MEGA-X [159], CLC Genomics Workbench [160], MetaboAnalyst [161] and many others. These range from user friendly graphical interface packages to code-based packages. Novel software packages are also being developed to analyse multi-omics data sets, such as various code-based R packages [162] e.g. mixOmics (http://mixomics.org/) and more user friendly visual packages e.g. PaintOmics 3. [163] Moreover, issues with primers bias and lack of complete and accurate gene libraries for biofilms need to be addressed in order to improve reproducibility and data analysis. [164]

1.3 References

1. Flemming, H.-C. *et al.* Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **14**, 563–575 (2016).
2. Ramage, G., Mowat, E., Jones, B., Williams, C. & Lopez-Ribot, J. Our Current Understanding of Fungal Biofilms. *Crit. Rev. Microbiol.* **35**, 340–355 (2009).
3. Flemming, H.-C. & Wingender, J. The biofilm matrix. *Nat. Rev. Microbiol.* (2010) doi:10.1038/nrmicro2415.
4. Flemming, H.-C., Neu, T. R. & Wozniak, D. J. The EPS Matrix: The "House of Biofilm Cells". *J. Bacteriol.* **189**, 7945–7947 (2007).
5. Li, Y.-H., Tian, X., Li, Y.-H. & Tian, X. Quorum Sensing and Bacterial Social Interactions in Biofilms. *Sensors* **12**, 2519–2538 (2012).
6. Sikdar, R. & Elias, M. Quorum quenching enzymes and their effects on virulence, biofilm, and microbiomes: a review of recent advances. *Expert Rev. Anti Infect. Ther.* **18**, 1221–1233 (2020).
7. Parsek, M. R. & Greenberg, E. P. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol.* **13**, 27–33 (2005).
8. Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2**, 95–108 (2004).
9. Nadell, C. D., Xavier, J. B. & Foster, K. R. The sociobiology of biofilms. *FEMS Microbiol. Rev.* **33**, 206–224 (2009).
10. Pearson, M. M., Laurence, C. A., Guinn, S. E. & Hansen, E. J. Biofilm Formation by Moraxella catarrhalis In Vitro: Roles of the UspA1 Adhesin and the Hag Hemagglutinin. *Infect. Immun.* **74**, 1588–1596 (2006).
11. Lee, S. W., Phillips, K. S., Gu, H., Kazemzadeh-Narbat, M. & Ren, D. How microbes read the map: Effects of implant topography on bacterial adhesion and biofilm formation. *Biomaterials* **268**, 120595 (2021).
12. Liu, C., Sun, D., Zhu, J. & Liu, W. Two-Component Signal Transduction Systems: A Major Strategy for Connecting Input Stimuli to Biofilm Formation. *Front. Microbiol.* **9**, 3279 (2019).
13. Liu, C., Sun, D., Zhu, J., Liu, J. & Liu, W. The Regulation of Bacterial Biofilm Formation by cAMP-CRP: A Mini-Review. *Front. Microbiol.* **11**, 802 (2020).
14. Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. & Kolter, R. Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev. Microbiol.* **11**, 157 (2013).
15. Serra, D. O. & Hengge, R. Bacterial Multicellularity: The Biology of Escherichia coli Building Large-Scale Biofilm Communities. *Annu. Rev. Microbiol.* **75**, null (2021).
16. O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm Formation as Microbial Development. *Annu. Rev. Microbiol.* **54**, 49–79 (2000).

17. Khan, F. *et al.* Mixed biofilms of pathogenic Candida-bacteria: regulation mechanisms and treatment strategies. *Crit. Rev. Microbiol.* **0**, 1–29 (2021).
18. Nielsen, A. T., Tolker-Nielsen, T., Barken, K. B. & Molin, S. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environ. Microbiol.* **2**, 59–68 (2000).
19. Mitri, S., Xavier, J. B. & Foster, K. R. Social evolution in multispecies biofilms. *Proc. Natl. Acad. Sci.* **108**, 10839–10846 (2011).
20. Azevedo, A. S., Almeida, C., Pereira, B., Melo, L. F. & Azevedo, N. F. Impact of Delftia tsuruhatensis and Achromobacter xylosoxidans on Escherichia coli dual-species biofilms treated with antibiotic agents. *Biofouling* **32**, 227–241 (2016).
21. Nadell, C. D., Drescher, K. & Foster, K. R. Spatial structure, cooperation and competition in biofilms. *Nat. Rev. Microbiol.* **14**, 589 (2016).
22. Christensen, B. B., Haagensen, J. A. J., Heydorn, A. & Molin, S. Metabolic Commensalism and Competition in a Two-Species Microbial Consortium. *Appl. Environ. Microbiol.* **68**, 2495–2502 (2002).
23. Fourie, R. & Pohl, C. H. Beyond Antagonism: The Interaction Between Candida Species and Pseudomonas aeruginosa. *J. Fungi* **5**, 34 (2019).
24. An, D., Danhorn, T., Fuqua, C. & Parsek, M. R. Quorum sensing and motility mediate interactions between Pseudomonas aeruginosa and Agrobacterium tumefaciens in biofilm cocultures. *Proc. Natl. Acad. Sci.* **103**, 3828–3833 (2006).
25. Burmølle, M., Ren, D., Bjarnsholt, T. & Sørensen, S. J. Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* **22**, 84–91 (2014).
26. Røder, H. L., Sørensen, S. J. & Burmølle, M. Studying Bacterial Multispecies Biofilms: Where to Start? *Trends Microbiol.* **24**, 503–513 (2016).
27. Tan, C. H., Lee, K. W. K., Burmølle, M., Kjelleberg, S. & Rice, S. A. All together now: experimental multispecies biofilm model systems. *Environ. Microbiol.* **19**, 42–53 (2017).
28. Beale, D. J. *et al.* Application of metabolomics to understanding biofilms in water distribution systems: a pilot study. *Biofouling* **29**, 283–294 (2013).
29. Shen, Y. *et al.* Effect of divalent ions and a polyphosphate on composition, structure, and stiffness of simulated drinking water biofilms. *Npj Biofilms Microbiomes* **4**, 1–9 (2018).
30. Huang, H. *et al.* Towards the biofilm characterization and regulation in biological wastewater treatment. *Appl. Microbiol. Biotechnol.* **103**, 1115–1129 (2019).
31. di Biase, A., Kowalski, M. S., Devlin, T. R. & Oleszkiewicz, J. A. Moving bed biofilm reactor technology in municipal wastewater treatment: A review. *J. Environ. Manage.* **247**, 849–866 (2019).
32. Soares, A., Azevedo, A., Gomes, L. C. & Mergulhão, F. J. Recombinant protein expression in biofilms. *AIMS Microbiol.* **5**, 232–250 (2019).

33. Cuny, L. *et al.* Evaluation of productive biofilms for continuous lactic acid production. *Biotechnol. Bioeng.* **116**, 2687–2697 (2019).
34. Arnaouteli, S., Bamford, N. C., Stanley-Wall, N. R. & Kovács, Á. T. Bacillus subtilis biofilm formation and social interactions. *Nat. Rev. Microbiol.* **19**, 600–614 (2021).
35. Roostaei, J., Zhang, Y., Gopalakrishnan, K. & Ochocki, A. J. Mixotrophic Microalgae Biofilm: A Novel Algae Cultivation Strategy for Improved Productivity and Cost-efficiency of Biofuel Feedstock Production. *Sci. Rep.* **8**, 12528 (2018).
36. Yuan, L. *et al.* Mixed-species biofilms in the food industry: Current knowledge and novel control strategies. *Crit. Rev. Food Sci. Nutr.* **60**, 2277–2293 (2020).
37. Coughlan, L. M., Cotter, P. D., Hill, C. & Alvarez-Ordóñez, A. New Weapons to Fight Old Enemies: Novel Strategies for the (Bio)control of Bacterial Biofilms in the Food Industry. *Front. Microbiol.* **7**, 1641 (2016).
38. Nisar, M. A., Ross, K., Brown, M., Bentham, R. & Whiley, H. Water Stagnation and Flow Obstruction Reduces the Quality of Potable Water and Increases the Risk of Legionelloses. *Front. Environ. Sci.* **8**, (2020).
39. Di Gregorio, L., Tandoi, V., Congestri, R., Rossetti, S. & Di Pippo, F. Unravelling the core microbiome of biofilms in cooling tower systems. *Biofouling* **33**, 793–806 (2017).
40. Hunsucker, K. Z. *et al.* Biofilm community structure and the associated drag penalties of a groomed fouling release ship hull coating. *Biofouling* **34**, 162–172 (2018).
41. Schultz, M. P. & Swain, G. W. The influence of biofilms on skin friction drag. *Biofouling* **15**, 129–139 (2000).
42. Donlan, R. M. Biofilms and device-associated infections. *Emerg. Infect. Dis.* **7**, 277–281 (2001).
43. Chua, R. Y. R., Lim, K., Leong, S. S. J., Tambyah, P. A. & Ho, B. An in-vitro urinary catheterization model that approximates clinical conditions for evaluation of innovations to prevent catheter-associated urinary tract infections. *J. Hosp. Infect.* **97**, 66–73 (2017).
44. Jacobsen, S. M., Stickler, D. J., Mobley, H. L. T. & Shirtliff, M. E. Complicated Catheter-Associated Urinary Tract Infections Due to Escherichia coli and Proteus mirabilis. *Clin. Microbiol. Rev.* **21**, 26–59 (2008).
45. Azevedo, A. S., Almeida, C., Melo, L. F. & Azevedo, N. F. Impact of polymicrobial biofilms in catheter-associated urinary tract infections. *Crit. Rev. Microbiol.* **43**, 423–439 (2017).
46. Feneley, R. C. L., Hopley, I. B. & Wells, P. N. T. Urinary catheters: history, current status, adverse events and research agenda. *J. Med. Eng. Technol.* **39**, 459–470 (2015).
47. Choe, H.-S. *et al.* Analysis of the distribution of bacteria within urinary catheter biofilms using four different molecular techniques. *Am. J. Infect. Control* **40**, e249–e254 (2012).

48. Lassek, C. *et al.* A Metaproteomics Approach to Elucidate Host and Pathogen Protein Expression during Catheter-Associated Urinary Tract Infections (CAUTIs). *Mol. Cell. Proteomics MCP* **14**, 989–1008 (2015).
49. Moryl, M., Torzewska, A., Jałmuzna, P. & Rózalski, A. Analysis of Proteus mirabilis distribution in multi-species biofilms on urinary catheters and determination of bacteria resistance to antimicrobial agents. *Pol. J. Microbiol.* **62**, 377–384 (2013).
50. Macleod, S. M. & Stickler, D. J. Species interactions in mixed-community crystalline biofilms on urinary catheters. *J. Med. Microbiol.* **56**, 1549–1557 (2007).
51. Galván, E. M., Mateyca, C. & Ielpi, L. Role of interspecies interactions in dual-species biofilms developed in vitro by uropathogens isolated from polymicrobial urinary catheter-associated bacteriuria. *Biofouling* **32**, 1067–1077 (2016).
52. Cerqueira, L., Oliveira, J. A., Nicolau, A., Azevedo, N. F. & Vieira, M. J. Biofilm formation with mixed cultures of Pseudomonas aeruginosa/Escherichia coli on silicone using artificial urine to mimic urinary catheters. *Biofouling* **29**, 829–840 (2013).
53. Moriarty, T. F. *et al.* Orthopaedic device-related infection: current and future interventions for improved prevention and treatment. *EFORT Open Rev.* **1**, 89–99 (2016).
54. Hajishengallis, G. & Lamont, R. J. Polymicrobial communities in periodontal disease: Their quasi-organismal nature and dialogue with the host. *Periodontol. 2000* **86**, 210–230 (2021).
55. Malone, M. *et al.* The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. *J. Wound Care* **26**, 20–25 (2017).
56. Smith, W. D. *et al.* Current and future therapies for Pseudomonas aeruginosa infection in patients with cystic fibrosis. *FEMS Microbiol. Lett.* **364**, (2017).
57. Stewart, P. S. Antimicrobial Tolerance in Biofilms. *Microbiol. Spectr.* **3**, (2015).
58. Harrison, J. J., Ceri, H. & Turner, R. J. Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol.* **5**, 928–938 (2007).
59. Burmolle, M. *et al.* Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. *Appl. Environ. Microbiol.* **72**, 3916–3923 (2006).
60. Ivanova, K. *et al.* Quorum-Quenching and Matrix-Degrading Enzymes in Multilayer Coatings Synergistically Prevent Bacterial Biofilm Formation on Urinary Catheters. *ACS Appl. Mater. Interfaces* **7**, 27066–27077 (2015).
61. Pierce, C. G. *et al.* A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat. Protoc.* **3**, 1494–1500 (2008).
62. Standar, K. *et al.* Setup of an in vitro test system for basic studies on biofilm behavior of mixed-species cultures with dental and periodontal pathogens. *PloS One* **5**, (2010).

63. Jorge, P., Lourenço, A. & Pereira, M. O. Data Quality in Biofilm High-Throughput Routine Analysis: Intralaboratory Protocol Adaptation and Experiment Reproducibility. *J. AOAC Int.* **98**, 1721–1727 (2015).
64. Kragh, K. N., Alhede, M., Kvich, L. & Bjarnsholt, T. Into the well—A close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm* **1**, 100006 (2019).
65. Gomes, L. C., Carvalho, D., Briandet, R. & Mergulhão, F. J. Temporal variation of recombinant protein expression in Escherichia coli biofilms analysed at single-cell level. *Process Biochem.* **51**, 1155–1161 (2016).
66. Heydorn, A. *et al.* Experimental reproducibility in flow-chamber biofilms. *Microbiology,* **146**, 2409–2415 (2000).
67. Goeres, D. M. Statistical assessment of a laboratory method for growing biofilms. *Microbiology* **151**, 757–762 (2005).
68. Parker, A. E. *et al.* Ruggedness and reproducibility of the MBEC biofilm disinfectant efficacy test. *J. Microbiol. Methods* **102**, 55–64 (2014).
69. Hamilton, M. *Testing Surface Disinfectants: Desirable Attributes of a Standardized Method*. (2010).
70. ASTM E2799-17. Standard Test Method for Testing Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm using the MBEC Assay. *ASTM Int. West Conshohocken PA* (2017).
71. ASTM. Standard Practice for Preparing a Pseudomonas aeruginosa or Staphylococcus aureus Biofilm Using the CDC Biofilm Reactor. (2018).
72. ASTM E2647-20. Standard Test Method for Quantification of a Pseudomonas aeruginosa Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. *ASTM Int. West Conshohocken PA* (2020).
73. Goeres, D. M. *et al.* Drip flow reactor method exhibits excellent reproducibility based on a 10-laboratory collaborative study. *J. Microbiol. Methods* **174**, 105963 (2020).
74. Goeres, D. M. *et al.* A method for growing a biofilm under low shear at the air–liquid interface using the drip flow biofilm reactor. *Nat. Protoc.* **4**, 783 (2009).
75. Liu, N. *et al.* Microfluidic study of effects of flow velocity and nutrient concentration on biofilm accumulation and adhesive strength in the flowing and no-flowing microchannels. *J. Ind. Microbiol. Biotechnol.* **46**, 855–868 (2019).
76. Aufrecht, J. A. *et al.* Pore-scale hydrodynamics influence the spatial evolution of bacterial biofilms in a microfluidic porous network. *PLoS ONE* **14**, (2019).
77. Tetala, K. K. R. & Vijayalakshmi, M. A. A review on recent developments for biomolecule separation at analytical scale using microfluidic devices. *Anal. Chim. Acta* **906**, 7–21 (2016).

78. Subramanian, S., Huiszoon, R. C., Chu, S., Bentley, W. E. & Ghodssi, R. Microsystems for biofilm characterization and sensing – A review. *Biofilm* **2**, 100015 (2020).
79. Petruk, A. A., Allen, C., Rivas, N., Pichugin, K. & Sciaini, G. High flow rate nanofluidics for in-liquid electron microscopy and diffraction. *Nanotechnology* **30**, 395703 (2019).
80. Hansen, S. H. *et al.* Machine-assisted cultivation and analysis of biofilms. *Sci. Rep.* **9**, 1–10 (2019).
81. Naudin, B., Heins, A., Pinhal, S., Dé, E. & Nicol, M. BioFlux^{TM} 200 Microfluidic System to Study A. baumannii Biofilm Formation in a Dynamic Mode of Growth. in *Acinetobacter baumannii* (eds. Biswas, I. & Rather, P. N.) vol. 1946 167–176 (Springer New York, 2019).
82. L Zhou & K Cheng. Dynamic cutting process modelling and its impact on the generation of surface topography and texture in nano/micro cutting. *Proc. Inst. Mech. Eng. Part B J. Eng. Manuf.* **223**, 247–266 (2009).
83. Thakral, G., Thakral, R., Sharma, N., Seth, J. & Vashisht, P. Nanosurface – The Future of Implants. *J. Clin. Diagn. Res. JCDR* **8**, ZE07-ZE10 (2014).
84. Hu, Y., You, J.-O. & Aizenberg, J. Micropatterned Hydrogel Surface with High-Aspect-Ratio Features for Cell Guidance and Tissue Growth. *ACS Appl. Mater. Interfaces* **8**, 21939–21945 (2016).
85. Krishna Kumar, R. *et al.* Droplet printing reveals the importance of micron-scale structure for bacterial ecology. *Nat. Commun.* **12**, 857 (2021).
86. Azeredo, J. *et al.* Critical review on biofilm methods. *Crit. Rev. Microbiol.* **43**, 313–351 (2017).
87. Coffey, B. M. & Anderson, G. G. Biofilm formation in the 96-well microtiter plate. *Methods Mol. Biol. Clifton NJ* **1149**, 631–641 (2014).
88. Stepanović, S., Vuković, D., Dakić, I., Savić, B. & Švabić-Vlahović, M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J. Microbiol. Methods* **40**, 175–179 (2000).
89. Stepanović, S. *et al.* Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* **115**, 891–899 (2007).
90. Elkhatib, W. F., Khairalla, A. S. & Ashour, H. M. Evaluation of different microtiter plate-based methods for the quantitative assessment of Staphylococcus aureus biofilms. *Future Microbiol.* **9**, 725–735 (2014).
91. Peeters, E., Nelis, H. J. & Coenye, T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods* **72**, 157–165 (2008).
92. Van den Driessche, F., Rigole, P., Brackman, G. & Coenye, T. Optimization of resazurin-based viability staining for quantification of microbial biofilms. *J. Microbiol. Methods* **98**, 31–34 (2014).

93. Foerster, S., Desilvestro, V., Hathaway, L. J., Althaus, C. L. & Unemo, M. A new rapid resazurin-based microdilution assay for antimicrobial susceptibility testing of Neisseria gonorrhoeae. *J. Antimicrob. Chemother.* **72**, 1961–1968 (2017).
94. Alonso, B., Cruces, R., Pérez, A., Sánchez-Carrillo, C. & Guembe, M. Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm. *J. Microbiol. Methods* **139**, 135–137 (2017).
95. Rampersad, S. N. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. *Sensors* **12**, 12347–12360 (2012).
96. Xu, Z. *et al.* Crystal Violet and XTT Assays on Staphylococcus aureus Biofilm Quantification. *Curr. Microbiol.* **73**, 474–482 (2016).
97. Ramage, G. Comparing apples and oranges: considerations for quantifying candidal biofilms with XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] and the need for standardized testing. *J. Med. Microbiol.* **65**, 259–260 (2016).
98. Tunney, M. M., Ramage, G., Field, T. R., Moriarty, T. F. & Storey, D. G. Rapid Colorimetric Assay for Antimicrobial Susceptibility Testing of Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **48**, 1879–1881 (2004).
99. Trafny, E. A., Lewandowski, R., Zawistowska-Marciniak, I. & Stepinska, M. Use of MTT assay for determination of the biofilm formation capacity of microorganisms in metalworking fluids. *World J. Microbiol. Biotechnol.* **29**, 1635–1643 (2013).
100. Sabaeifard, P., Abdi-Ali, A., Soudi, M. R. & Dinarvand, R. Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. *J. Microbiol. Methods* **105**, 134–140 (2014).
101. Brown, H. L., van Vliet, A. H. M., Betts, R. P. & Reuter, M. Tetrazolium reduction allows assessment of biofilm formation by Campylobacter jejuni in a food matrix model. *J. Appl. Microbiol.* **115**, 1212–1221 (2013).
102. Junka, A. F. *et al.* A.D.A.M. test (Antibiofilm Dressing's Activity Measurement) — Simple method for evaluating anti-biofilm activity of drug-saturated dressings against wound pathogens. *J. Microbiol. Methods* (2017) doi:10.1016/j.mimet.2017.09.014.
103. Honraet, K., Goetghebeur, E. & Nelis, H. J. Comparison of three assays for the quantification of Candida biomass in suspension and CDC reactor grown biofilms. *J. Microbiol. Methods* **63**, 287–295 (2005).
104. Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. & Ren, Q. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiol.* **15**, 36 (2015).
105. Skogman, M. E., Vuorela, P. M. & Fallarero, A. Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against Staphylococcus aureus biofilms. *J. Antibiot. (Tokyo)* **65**, 453 (2012).

106. Burton, E., Yakandawala, N., LoVetri, K. & Madhyastha, M. S. A microplate spectrofluorometric assay for bacterial biofilms. *J. Ind. Microbiol. Biotechnol.* **34**, 1–4 (2006).

107. Sandberg, M., Määttänen, A., Peltonen, J., Vuorela, P. M. & Fallarero, A. Automating a 96-well microtitre plate model for Staphylococcus aureus biofilms: an approach to screening of natural antimicrobial compounds. *Int. J. Antimicrob. Agents* **32**, 233–240 (2008).

108. ASTM E2562-17. Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor. *ASTM Int. West Conshohocken PA* (2017).

109. ASTM E2562. Standard Test Method for Evaluating Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method. (2019).

110. Goeres, D. M. *et al.* Development, standardization, and validation of a biofilm efficacy test: The single tube method. *J. Microbiol. Methods* **165**, 105694 (2019).

111. Hu, T. & Zhang, J.-L. Mass-spectrometry-based lipidomics. *J. Sep. Sci.* (2017) doi:10.1002/jssc.201700709.

112. Ramos, P. I. P. *et al.* An integrative, multi-omics approach towards the prioritization of Klebsiella pneumoniae drug targets. *Sci. Rep.* **8**, 1–19 (2018).

113. Wessel, A. K., Hmelo, L., Parsek, M. R. & Whiteley, M. Going local: technologies for exploring bacterial microenvironments. *Nat. Rev. Microbiol.* **11**, 337–348 (2013).

114. Hu, Y. *et al.* Single Cell Multi-Omics Technology: Methodology and Application. *Front. Cell Dev. Biol.* **6**, (2018).

115. Zhu, C., Preissl, S. & Ren, B. Single-cell multimodal omics: the power of many. *Nat. Methods* **17**, 11–14 (2020).

116. Prakadan, S. M., Shalek, A. K. & Weitz, D. A. Scaling by shrinking: empowering single-cell 'omics' with microfluidic devices. *Nat. Rev. Genet.* **18**, 345–361 (2017).

117. Beyenal, H., Lewandowski, Z. & Harkin, G. Quantifying Biofilm Structure: Facts and Fiction. *Biofouling* **20**, 1–23 (2004).

118. Almeida, C. *et al.* Rapid detection of urinary tract infections caused by Proteus spp. using PNA-FISH. *Eur. J. Clin. Microbiol. Infect. Dis.* **32**, 781–786 (2013).

119. Sen, M. & Yadav, P. Modern Methods in Microscopy for the Assessment of Biofilms. in *Biofilms in Human Diseases: Treatment and Control* (eds. Kumar, S., Chandra, N., Singh, L., Hashmi, M. Z. & Varma, A.) 59–69 (Springer International Publishing, 2019). doi:10.1007/978-3-030-30757-8_5.

120. Weiner, A. & Enninga, J. The Pathogen–Host Interface in Three Dimensions: Correlative FIB/SEM Applications. *Trends Microbiol.* **27**, 426–439 (2019).

121. Techniques for Oral Microbiology. in *Atlas of Oral Microbiology* 15–40 (Elsevier, 2015). doi:10.1016/B978-0-12-802234-4.00002-1.

122. Zhang, P., Chen, Y.-P., Qiu, J.-H., Dai, Y.-Z. & Feng, B. Imaging the Microprocesses in Biofilm Matrices. *Trends Biotechnol.* **37**, 214–226 (2019).

123. Wu, S., Huang, F., Zhang, H. & Lei, L. Staphylococcus aureus biofilm organization modulated by YycFG two-component regulatory pathway. *J. Orthop. Surg.* **14**, 10 (2019).

124. Bessa, L. J. *et al.* Intragenic Antimicrobial Peptide Hs02 Hampers the Proliferation of Single- and Dual-Species Biofilms of P. aeruginosa and S. aureus: A Promising Agent for Mitigation of Biofilm-Associated Infections. *Int. J. Mol. Sci.* **20**, 3604 (2019).

125. Wright, C. J., Shah, M. K., Powell, L. C. & Armstrong, I. Application of AFM from microbial cell to biofilm. *Scanning* **32**, 134–149 (2010).

126. McConnell, G. *et al.* A novel optical microscope for imaging large embryos and tissue volumes with sub-cellular resolution throughout. *eLife* **5**, e18659 (2016).

127. Rooney, L. M., McCann, L., Hoskisson, P. A. & McConnell, G. Applying the Mesolens to microbiology: investigating the structural organisation of bacterial biofilms. in (2018).

128. Wegel, E. *et al.* Imaging cellular structures in super-resolution with SIM, STED and Localisation Microscopy: A practical comparison. *Sci. Rep.* **6**, 1–13 (2016).

129. Ding, Y. *et al.* Molecular evidence of a toxic effect on a biofilm and its matrix. *Analyst* **144**, 2498–2503 (2019).

130. Ingerman, E. A., London, R. A., Heintzmann, R. & Gustafsson, M. G. L. Signal, noise and resolution in linear and nonlinear structured-illumination microscopy. *J. Microsc.* **273**, 3–25 (2019).

131. Keren-Paz, A. *et al.* A novel calcium-concentrating compartment drives biofilm formation and persistent infections. *bioRxiv* 2020.01.08.898569 (2020) doi:10.1101/2020.01.08.898569.

132. Lybrand, R. A. *et al.* A coupled microscopy approach to assess the nano-landscape of weathering. *Sci. Rep.* **9**, 1–14 (2019).

133. Xu, K., Babcock, H. P. & Zhuang, X. Dual-objective STORM reveals three-dimensional filament organization in the actin cytoskeleton. *Nat. Methods* **9**, 185–188 (2012).

134. Rust, M. J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **3**, 793–796 (2006).

135. Allkja, J. & Azevedo, A. S. Characterization of Social Interactions and Spatial Arrangement of Individual Bacteria in MultiStrain or Multispecies Biofilm Systems Using Nucleic Acid Mimics-Fluorescence In Situ Hybridization. in *Fluorescence In-Situ Hybridization (FISH) for Microbial Cells: Methods and Concepts* (eds. Azevedo, N. F. & Almeida, C.) 97–109 (Springer US, 2021). doi:10.1007/978-1-0716-1115-9_7.

136. Azevedo, A. S. *et al.* Increased Intraspecies Diversity in Escherichia coli Biofilms Promotes Cellular Growth at the Expense of Matrix Production. *Antibiotics* **9**, 818 (2020).

137. Valm, A. M. *et al.* Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 4152–4157 (2011).

138. Valm, A. M., Welch, J. L. M. & Borisy, G. G. CLASI-FISH: Principles of Combinatorial Labeling and Spectral Imaging. *Syst. Appl. Microbiol.* **35**, 496–502 (2012).

139. Schlundt, C., Mark Welch, J. L., Knochel, A. M., Zettler, E. R. & Amaral-Zettler, L. A. Spatial structure in the "Plastisphere": Molecular resources for imaging microscopic communities on plastic marine debris. *Mol. Ecol. Resour.* (2019) doi:10.1111/1755-0998.13119.

140. Mark Welch, J. L., Ramírez-Puebla, S. T. & Borisy, G. G. Oral Microbiome Geography: Micron-Scale Habitat and Niche. *Cell Host Microbe* **28**, 160–168 (2020).

141. Shi, H. *et al.* Highly multiplexed spatial mapping of microbial communities. *Nature* (2020) doi:10.1038/s41586-020-2983-4.

142. Røder, H. L., Liu, W., Sørensen, S. J., Madsen, J. S. & Burmølle, M. Interspecies interactions reduce selection for a biofilm-optimized variant in a four-species biofilm model. *Environ. Microbiol. Rep.* **11**, 835–839 (2019).

143. Hansen, M. F., Torp, A. M., Madsen, J. S., Røder, H. L. & Burmølle, M. Fluidic resistance control enables high-throughput establishment of mixed-species biofilms. *BioTechniques* **66**, 235–239 (2019).

144. Rosenberg, M., Azevedo, N. F. & Ivask, A. Propidium iodide staining underestimates viability of adherent bacterial cells. *Sci. Rep.* **9**, 1–12 (2019).

145. Dreissig, S. *et al.* Live-cell CRISPR imaging in plants reveals dynamic telomere movements. *Plant J.* **91**, 565–573 (2017).

146. Nickerson, A., Huang, T., Lin, L.-J. & Nan, X. Photoactivated Localization Microscopy with Bimolecular Fluorescence Complementation (BiFC-PALM) for Nanoscale Imaging of Protein-Protein Interactions in Cells. *PLoS ONE* **9**, (2014).

147. Sahl, S. J., Hell, S. W. & Jakobs, S. Fluorescence nanoscopy in cell biology. *Nat. Rev. Mol. Cell Biol.* **18**, nrm.2017.71 (2017).

148. Hu, F., Shi, L. & Min, W. Biological imaging of chemical bonds by stimulated Raman scattering microscopy. *Nat. Methods* **16**, 830–842 (2019).

149. Brockmann, E. U., Steil, D., Bauwens, A., Soltwisch, J. & Dreisewerd, K. Advanced Methods for MALDI-MS Imaging of the Chemical Communication in Microbial Communities. *Anal. Chem.* **91**, 15081–15089 (2019).

150. Stewart, S., Priore, R. J., Nelson, M. P. & Treado, P. J. Raman Imaging. *Annu. Rev. Anal. Chem.* **5**, 337–360 (2012).

151. Rae Buchberger, A., DeLaney, K., Johnson, J. & Li, L. Mass Spectrometry Imaging: A Review of Emerging Advancements and Future Insights. *Anal. Chem.* **90**, 240–265 (2018).

152. Wagner, M., Ivleva, N. P., Haisch, C., Niessner, R. & Horn, H. Combined use of confocal laser scanning microscopy (CLSM) and Raman microscopy (RM): Investigations on EPS – Matrix. *Water Res.* **43**, 63–76 (2009).

153. Mino, L. *et al.* Materials characterization by synchrotron x-ray microprobes and nanoprobes. *Rev. Mod. Phys.* **90**, 025007 (2018).

154. Biofilms In Imaris Tutorial | Imaris. *Oxford Instruments* https://imaris.oxinst.com/learning/view/article/biofilms.

155. Heydorn, A. *et al.* Quantification of biofilm structures by the novel computer program comstat. *Microbiology* **146**, 2395–2407 (2000).

156. Rueden, C. T. *et al.* ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* **18**, 529 (2017).

157. Hartmann, R. *et al.* Quantitative image analysis of microbial communities with BiofilmQ. *Nat. Microbiol.* **6**, 151–156 (2021).

158. Gallego-Hernandez, A. L. *et al.* Upregulation of virulence genes promotes *Vibrio cholerae* biofilm hyperinfectivity. *Proc. Natl. Acad. Sci.* **117**, 11010–11017 (2020).

159. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).

160. Chacon, J. & Cuajungco, M. P. Comparative De Novo Transcriptome Assembly of Notophthalmus viridescens RNA-seq Data using Two Commercial Software Programs. *Californian J. Health Promot.* **16**, 46–53 (2018).

161. Chong, J. *et al.* MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* **46**, W486–W494 (2018).

162. Rajasundaram, D. & Selbig, J. More effort — more results: recent advances in integrative 'omics' data analysis. *Curr. Opin. Plant Biol.* **30**, 57–61 (2016).

163. Hernández-de-Diego, R. *et al.* PaintOmics 3: a web resource for the pathway analysis and visualization of multi-omics data. *Nucleic Acids Res.* **46**, W503–W509 (2018).

164. Ogawa, A., Celikkol-Aydin, S., Gaylarde, C., Baptista-Neto, J. A. & Beech, I. Microbiomes of Biofilms on Decorative Siliceous Stone: Drawbacks and Advantages of Next Generation Sequencing. *Curr. Microbiol.* **74**, 848–853 (2017).\

Chapter 2.

Reproducibility in biofilms

Parts of this chapter are included in the following publication: Azevedo, N.F., Allkja, J. & Goeres, D. "Biofilms vs. cities and humans vs. aliens – a tale of reproducibility in biofilms." *Trends in Microbiology* (2021). doi:10.1016/j.tim.2021.05.003.

Abstract

Biofilms are complex and dynamic structures that include many more components than just viable cells. Therefore, growing reproducible biofilms is often elusive. One of the challenges in defining reproducibility for biofilm research is that different research fields use a spectrum of parameters to define reproducibility for their application. For instance, is the researcher interested in achieving a similar population density, height of biofilm structures, or function of the biofilm in a certain ecosystem/industrial context? Within this chapter we categorize reproducibility into four different levels: level 1- No reproducibility, level 2- Standard reproducibility, level 3- Potential standard reproducibility and level 4- Total reproducibility. To better understand the need for these different levels of reproducibility, we will expand on the "cities of microbes" analogy for biofilms by imagining that a new civilization has reached the Earth outskirts and starts studying Earth's cities. This will provide a better sense of scale and illustrate how small details can impact profoundly the growth and behavior of a biofilm and our understanding of reproducibility.

Keywords

Biofilm, reproducibility, methods

2.1 Biofilms and cities

Due to their complexity and organization, biofilms have been likened to cities of microbes. [1,2] Under this analogy, microbial cells represent city dwellers, the matrix represents the buildings where people live, and the land surface/atmosphere ecosystem is the interface where biofilms form. This analogy will provide context for a discussion on the reproducibility of biofilm experiments.

The issues of experimental reproducibility have been the focus of numerous debates in the scientific community (e.g., Baker, 2016[3] and Schloss, P. D., 2018 [4]). The lack of reproducibility in scientific studies represents a significant economic burden. While estimates for biofilm science alone are unknown, we can look at other areas of biological science as a representation of the cost to the biofilm field. For example, it was estimated that 50% of the preclinical research in the US, at a cost of approximately US$28B/year, is not reproducible. [5] There is also a scientific cost as subsequent research is often based on conclusions from preclinical studies, the lack of reproducibility could result in dead-end studies. [6,7] Moreover, lack of reproducibility can have an impact on public trust in science leading to major consequences. [8] One area where we can see the impact of this mistrust is the current rise in anti-vaccination campaigns, which has led to outbreaks of preventable diseases such as measles and mumps. [9]

Biofilms are complex and dynamic structures, and the apparently simple goal of growing reproducible biofilms is often elusive. Furthermore, reproducible biofilms will have different meanings for different scientists and/or applications. Is the goal to have a similar population density, height of the biofilm structures, or function of the biofilm in a certain ecosystem/industrial context? For example, if the aim of the experiment is to evaluate the efficacy of an antimicrobial compound at eradicating biofilms, then it is important to achieve similar log densities each time. Hence, the level of reproducibility achieved with the different ASTM approved methods is appropriate. [10,11] On the other hand, in the case of biofilms involved in wastewater treatment processes, the most important feature is its metabolism which is needed to keep the water parameters at the outlet of the reactor below a certain threshold. [12] Hence, it is important to define reproducibility in the context of the biofilm experiment.

In this chapter categorize reproducibility into four different levels (Figure 2.1). Level 1, represents no reproducibility, meaning the biofilm varies significantly in the number of cells, structure, and EPS components between experiments and among different labs. This level of reproducibility is often associated with biofilm growth reactors that do not

currently have any standardized protocols available for them. Methods used only in a single laboratory, untested in other laboratories, may also fit in this category. Level 2 captures standard reproducibility, which is currently defined by regulatory agencies and standard setting bodies and requires consistent numbers of viable cells per surface area within the biofilm. This level of reproducibility is validated using ring trial studies, where the same experiment is performed across different laboratories and the reproducibility standard deviation is calculated. If this standard deviation is deemed acceptable by organizations like ASTM and ISO, then the method is approved as a Standard Test Method (STM). Regulatory agencies reference STMs in their guidance documents to define the pathway companies must follow to get one of the following label claims on their product: kills, removes, or prevents biofilm. Level 3 represents potential standard reproducibility and encompasses similarity in not only viable cell numbers, but also the concentration of EPS components and overall architecture of the biofilm (shape, structure). When verifying level 3 reproducibility, not only does the biofilm need to comply with the specifications set for level 2, but the biofilm structure, architecture, and EPS components need to be deemed statistically equivalent among experimental repeats within a single laboratory and among repeats across different laboratories. While this level of reproducibility has not yet been achieved, this concept has been described before in the literature by Jackson *et al*., where they discuss the possibility of growing biofilms (referred as identical biofilms) that are reproducible in both cell number and structure. [13] Level 4 refers to total reproducibility which from a deterministic perspective, means that all biofilm elements (cells, architecture, EPS components, etc.) are present at exactly the same number and location within the biofilm when the experiment is repeated within a single laboratory and in different laboratories. This level of reproducibility includes the location i.e., overlapping coordinates of biofilm components, as an extra requirement when compared to level 3. Hence, the biofilms not only need to have a similar shape and architecture, but their components must also have the same 3-D spatial location within the biofilm each time. As with level 3 reproducibility, this has not been reached experimentally.

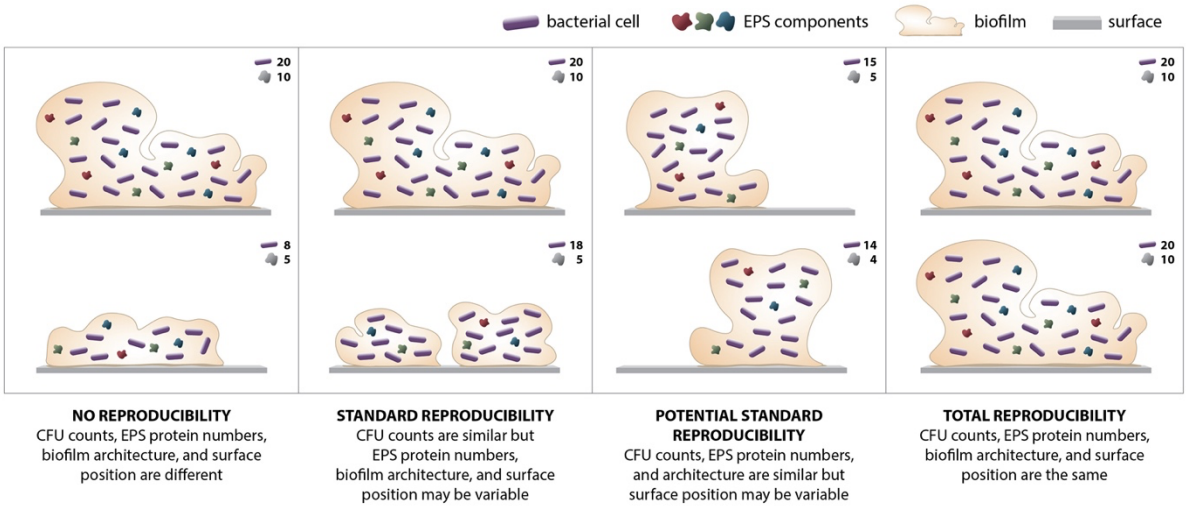

Figure 2.1. Levels of reproducibility: Level 1- No reproducibility. **Level 2**- Standard reproducibility. **Level 3** - Potential standard reproducibility. **Level 4** - Total reproducibility. Each box represents one level of reproducibility. The represented molecules are explained in the figure legend (with the sizes having been enlarged for easier visualization). Illustration created by Jill Story (Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA).

Achieving reproducible biofilms depends on the reproducibility of the methods used to grow and study those biofilms. For example, if the observed variability is due to the growth protocol used, even a more advanced assessment method would fail to provide reproducible results. The other side of the coin comprises of variability in the assessment method used. There is also an inherent conviction within the biofilm community that due to the complex nature of biofilms, there is only so much that we can do to control biofilm heterogeneity. This belief impacts progress towards more reproducible research.

To be able to grow totally reproducible biofilms is no doubt a tremendous task. To better explain what is necessary in order to achieve this and to provide a sense of scale, we will go beyond the analogy of biofilms and cities and ask the help friends from outer space.

2.2 Cities and aliens

Let us imagine that a new civilization has reached the outskirts of Earth and the members of this civilization start looking at the surface of our planet using a magnifying equipment for visualization. For the sake of argument, that magnifying equipment would allow them to have a resolution of roughly 0.1 meters, i.e., only structures/features over 0.1 meters would be discriminated (Figure 2.2). This would allow aliens to identify individual humans

(complex 3D structures), but no individual features. Facial traits, race, height, and weight would pass mostly unnoticed by them, and so would smaller animals. That civilization would look down to Earth and find that complex 3D structures have been formed in its surface.

Under these study conditions, the aliens would no doubt be able to very quickly characterize a large number of features in a city such as, the number of human inhabitants, the volume of buildings and parks, and the average height of the city skyline. Based on these observations, they would soon conclude that water is an essential nutrient, and that roads provide a means of communication for individuals and other goods. [14] Nonetheless, aliens would find it hard to understand why cities grow at different rates and why they form at certain locations. They would also struggle to understand the impact that each subpopulation (i.e., type of worker) would have on the overall functioning of a city, and if and how communication between different individuals occurs.

2.3 Biofilms and humans

The choice for the resolution of alien telescopes was not random. It was selected with respect to the resolution that we scientists use to visualize biofilms. In fact, when we observe a biofilm using an optical or epifluorescence microscope, we are aware that we can get a sense of the general structure of the biofilm but only at the 0.1 µm resolution (Figure 2.2). This can be improved using electron microscopy or super-resolution imaging,

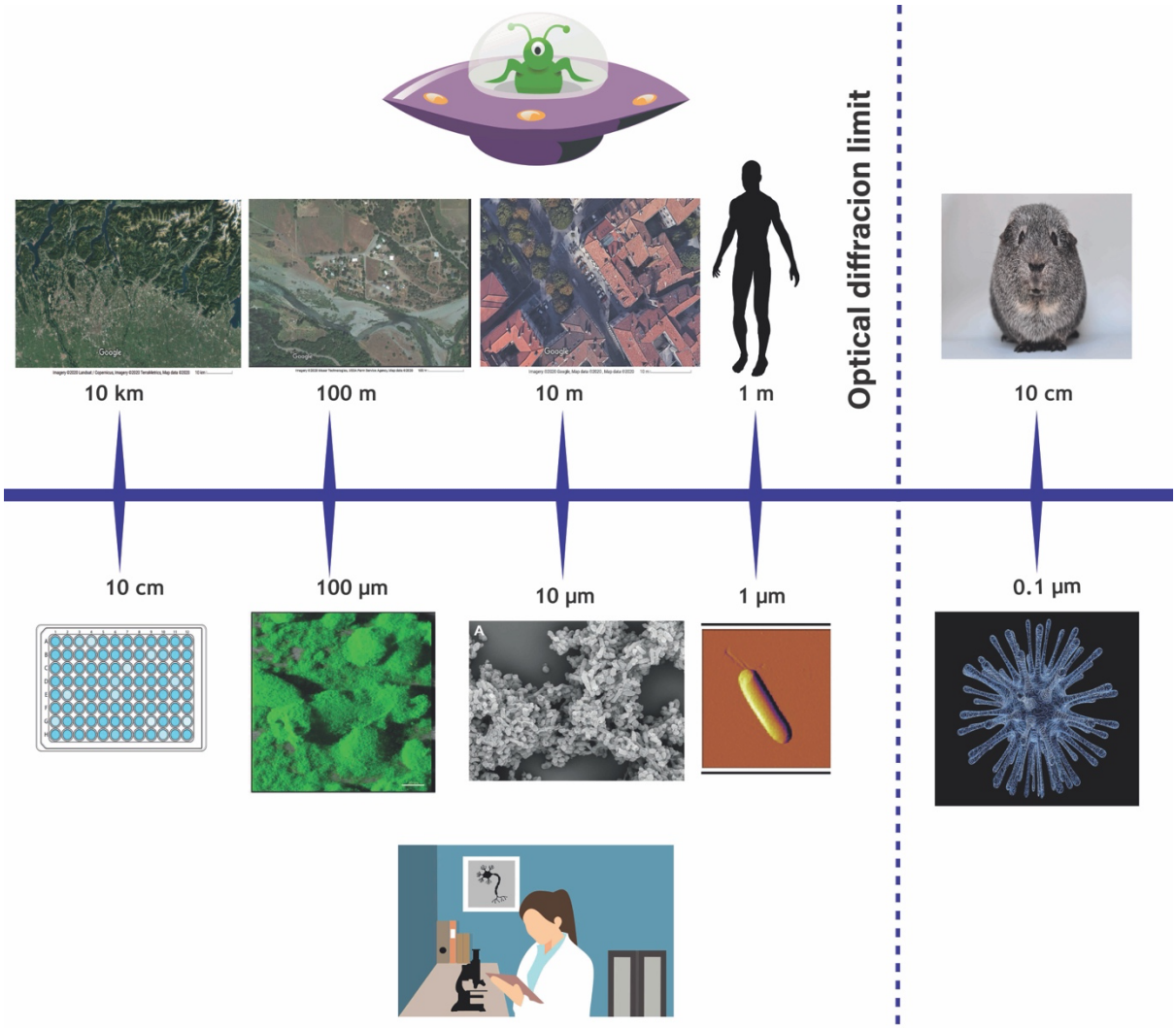

Figure 2.2 – Comparison between different visualisation scales for biofilms and earth. The scale for visualising biofilms is based on current microscopy technologies and the known optical diffraction limit. Examples of the level of detail visible for each scale are shown for the visualisation of both the earth and the biofilm. At **10 cm/10 km** – no details are visible; **100 µm/ 100 m** – general shape, architecture can be assessed; **10 µm/ 10 m** – internal structure/organization can be assessed; **1 µm/ 1 m** – individual cells/ humans can be assessed; **0.1 µm/ 10 cm** – minute components can be visualised. Microscopy images were repurposed under the creative commons attribution license: SEM [15]; AFM[16]. CLSM image courtesy: Kelli Buckingham-Meyer (Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA). Map images obtained from Google Maps.

but generally at the expense of some biofilm features. [15] Regardless, we know that biofilms are commonly formed at interfaces. Depending on environmental conditions such as liquid flow rates, they can form common structures e.g., fronds and mushrooms which provide shelter and channels for a better transport of nutrients. These structures can be self-produced by the microorganisms or based on already existing structures. [16] A microscope quickly characterizes a large number of features in a biofilm, such as the number of microbial cells, the volume of biomass and the average biofilm thickness. [17] Other methods can measure the most relevant substrates utilized by the microorganisms for growth. Nonetheless, we find it challenging to predict why biofilms exposed to the same conditions grow at different rates and in various configurations. We are only starting to understand the effects that each subpopulation (i.e. type of microorganisms) have on the overall functioning of a multispecies biofilm [18], and how communication between different cells occurs. [19] Hence in terms of reproducibility, it is important that we not only assess the log density of biofilms, but also other structural and behavioural characteristics.

2.4. Humans and aliens

If aliens arrived in 1931, they would be puzzled with the existence of a city in the middle of the desert – Las Vegas. If water was indeed essential, what could explain the presence of a city in such a dire location? Those familiar with history will reply that the creation of Las Vegas was a one-off event, led by the desire of an individual to create a city where gambling regulations did not apply. Expectedly, aliens are not groomed to learn US history, and as such they would probably just look at Las Vegas as an oddity without a clear explanation.

While we are not advocating that complex written rules govern the way microbes interact in biofilms, it is clear to us that even when every single parameter of a biofilm experiment seems to be the same, technical replicates will at times provide significantly different results e.g., ± 1 log difference in cell counts or different architecture. [20] These results are not (solely) due to experimental errors, but also to minute but unavoidable differences in the initial cellular contents, physiology, and location, as well variations in the substrate/coupon. While generally these differences are considered for log density numbers in level 2 devices during ruggedness testing[21], their effect on other aspects of biofilm growth is not. This might explain why a colony is not precisely located in a specific section of a coupon, and why these structures are not composed of the same compounds or have the same geometry, for apparently the same initial starting conditions.

As a conclusion to the story of aliens, let us consider that they had the technology to create an Earth-like planet to study the process of city building on Earth. After understanding, that humans are responsible for the creation of structures, they would take a sample of a human population and place it in the surface of the Earth-like planet. To increase reproducibility, they would select the same number of humans to start a city, and the land provided would have same characteristics. Soon, humans would pick a spot to settle and start exploring the neighbouring natural resources in order to start building their city. While apparently aliens started with what was a homogeneous human population and surrounding environment, it would soon be apparent that cities would diverge in location and structure, sometimes in spectacular ways. Hence, it is not just selecting the same number of humans that is critical, but also selecting the humans with the right skill set to build a city.

When we are trying to reproduce a biofilm, we are hoping that the structures will be forming themselves repeatedly in a similar fashion. Considering level 3 reproducibility, achieving a reproducible biofilm would mean that cells would form similar structures. In order to achieve this, we control for different experimental conditions such as flow rate, temperature, microbe strains and type of material used. [22] If we consider one of these factors, say, type of material, what are perceived as small differences during fabrication, could lead to different outcomes. For instance, a city built on a flat surface will be completely different from one built on a hillslope. Considering relative sizes, a 100m hill for us corresponds roughly to a 0.1 mm surface irregularity for a bacterium. While we typically pay little attention to these details, common materials e.g., polystyrene, can have a different surface roughness coefficient as a result of variations in manufacturing. Among manufacturers of polystyrene microtiter plates, the RMS (Root Mean Square of measured microscopic peaks and valleys of a surface) can range from 1 to 6 μm. [23] These differences in roughness can affect bacterial adhesion which in turn affects biofilm formation and leads to different experimental outcomes. [24]

Moreover, “external” or global factors may contribute to the mix. Dust blown from the Sahara is known to help fertilize the Amazon, located more than 3000 km away. In the case of biofilms, usually overlooked factors such as light and sound exposure or uneven (albeit minute) distributions of oxygen and temperature in multi-well plates may lead to variability. [25] While standard laboratory practices are obviously followed, researchers should consider recording more variables and employing additional analytical methods to ensure variability is kept to a minimum.

2.5. The road to total reproducibility

The analogy with aliens observing Earth was meant to illustrate how daunting the task to obtain totally reproducible biofilms is. Even at the planktonic state where cells supposedly face identical conditions (unlike biofilms), they are known to have bias towards each other, i.e., cellular noise. [26] Clearly, aiming for absolute control and prediction of biofilm formation is distant, but new strategies are being developed that might have the potential to generate more reproducible results.

One approach to improve reproducibility is to focus on biofilm growth reactors. Most biofilm reactors only achieve level 2 reproducibility as shown in Figure 2.3. Many of them already have been validated for STMs by ASTM for specific microorganisms and applications, such as the CDC and drip flow reactors described in the previous chapter. [10,27] However, in order to aim for level 3 reproducibility, the overall structure and architecture of the biofilm needs to be considered. As this is not a current criterion for STM validation by ASTM and other standard setting organizations, no tests have been performed to investigate the reproducibility of the structure and architecture of biofilms in any reactors. Hence, we do not have a current reproducibility standard for these criteria. Nonetheless, new reactors such as microfluidics devices, which allow for better control of the growth environment, conditions and surface, could potentially help reach level 3. [28] Recent advances in surface modification technologies have also allowed for improved control on the initial site of cell attachment and can also potentially improve reproducibility. [29]

Another approach is to focus on biofilm characterisation methods. While some of these methods enable quantification of the biofilm as a single entity e.g., the plate count method, others look at one specific element of the biofilm. The former can only reach level 2 reproducibility, as they provide no information on the composition and spatial location or arrangement of components within the biofilm. On the other hand, being able to characterise and quantify specific elements of the biofilm, coupled with microscopy, could help us get closer to level 3 reproducibility. Recent breakthroughs using these techniques in other fields provide insights on how they may be applied to biofilm research. For example, combining advanced microscopy techniques such as cryo-SMLM (Single-molecule localization microscopy) and cryo-SEM (Scanning Electron Microscopy) has made it possible to image whole mammalian cells in 3D.[30] This has allowed for the visualisation of cell ultrastructure to unprecedented details, down to the visualisation of individual proteins, their location within this structure and how they interact with the different organelles and each other. [30,31]

Furthermore, we must not neglect the importance of data analysis. Regardless of how detailed or sophisticated a method is, if the data are not processed and interpreted correctly, we cannot evaluate 'true' reproducibility. While advances in microscopy have allowed us to visualise samples in greater detail, quantitative data analysis protocols are needed to fully take advantage of these new technologies. If we consider the requirements for level 3 reproducibility, then the application of spatial statistics becomes paramount to biofilm reproducibility in 3D. For example, in the work described in chapter 1 by *Shi et al*.,[32] an algorithm was developed which could extract each cell's directional information, within the biofilm, in relation to neighbouring ones. This allowed them to calculate association matrices, a form of quantification for the spatial organisation of the biofilm that can be compared across different biofilms. [32]

Currently, none of the laboratory method described in chapter 1 can reach level 3 or 4 reproducibility. The application of mathematical and computer modelling could be the way to achieve this seemingly impossible task. These models allow for the control of every aspect of the experimental set-up such as number of cells, area of growth, environmental conditions, nutrient molecules and many more. [33] While, at present, no models that are able to fully mimic what occurs in nature exist, data from totally reproducible models have been used to start understanding how altering one seemingly insignificant factor could affect biofilm growth and behaviour. The data obtained from these models can in turn be used to help us design better laboratory experiments with improved reproducibility. However, as most models rely on experimental data as a reference, they are dependent on the information available and its quality. [34] Moreover, since these models are computationally demanding and time-consuming, it is not feasible to model large scale systems. Hence, most models analyse a chosen subset of the biofilm which doesn't account for everything in the system. [33]

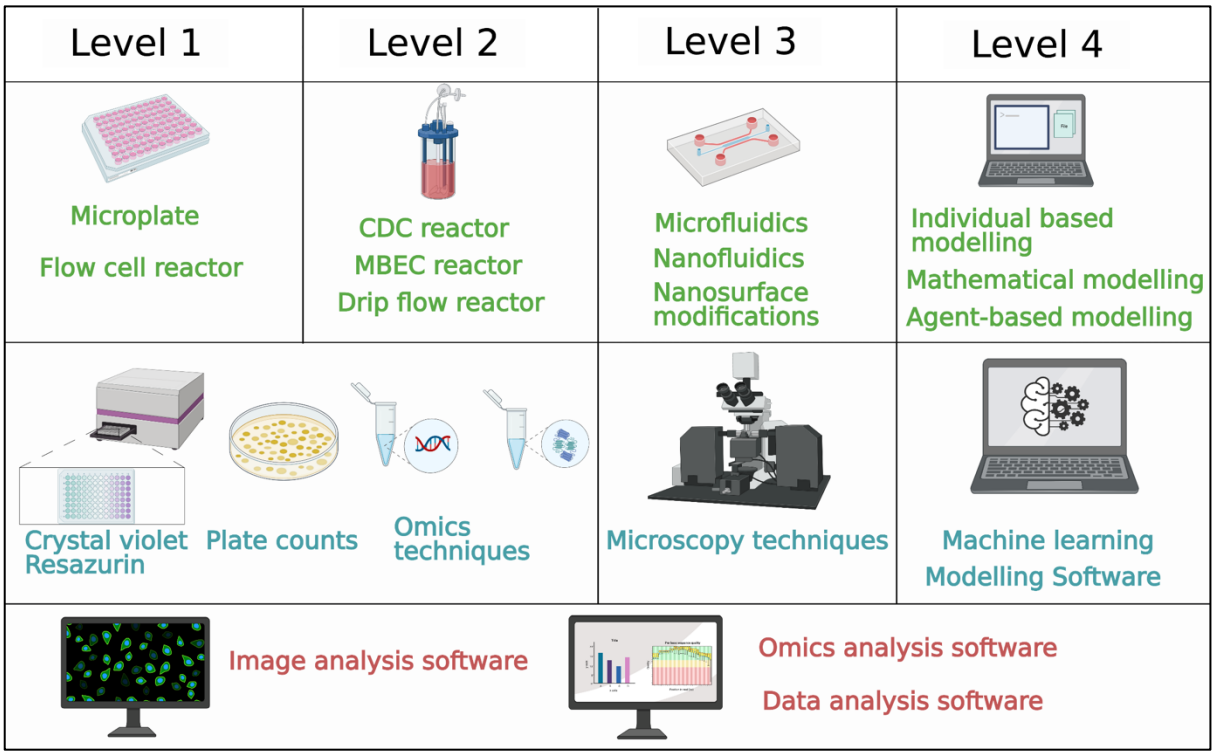

Figure 2.3. Summary of various biofilm research tools in use based on their reproducibility level. Green – biofilm growth reactors. Blue – biofilm assessment methods. Red – Data analysis strategies. Created with BioRender.com

2.5.1. Alternative approaches for tackling reproducibility

Advances in the methodologies mentioned above are not the only solution to the problem of reproducibility. Alternatives are available to address other issues pertaining reproducibility, such as poor reporting and unavailability of raw (untreated) experimental data. [3] For instance, existing initiatives such as the BiofOmics database can be used to store raw data from various biofilm studies, thereby improving accessibility. [35]

Furthermore, initiatives such as the minimum information about a biofilm experiment (MIABiE) guidelines can help improve reporting of biofilm experiments in the literature. [36] To improve compliance with the guidelines, the information that MIABiE asks researchers to report is easily obtained by most labs working in this area. For instance, regarding the surface where the biofilm is grown, only the type of material and the manufacturer of the surface must be reported. However, to achieve level 3 reproducibility, a map of the surface showing both its topography and composition should be provided. While this is not achievable for most research groups, some publications already provide a

general term (e.g., roughness) as a topography indication. For example in the work by Borch, T *et al.,* material composition and synthesis descriptions are provided in detail. [37] Additionally, the new ontology initiative by the National Biofilms Innovation Centre (NBIC) (https://www.biofilms.ac.uk/biofilm-ontology/) can help in standardising the terminology used across different biofilm-related publications. This will help avoid ambiguity in the vocabulary used, thus increasing the effectiveness of minimum information guidelines.

2.6. Concluding remarks

Biofilms exhibit a chaotic behaviour popularly known as "the butterfly effect." Small changes in initial conditions can lead to significantly different outcomes, affecting scientific conclusions. Hence, the ability to perform reproducible biofilm experiments is crucial. One can ask if it is necessary that biofilms achieve total reproducibility. The answer to this question depends on the aim of a study. For many applications, achieving level 2 or level 3 reproducibility will be sufficient. Nonetheless, achieving total reproducibility would enable us to study effects caused by miniscule changes in the system. The development of more reliable and reproducible biofilm methods is necessary to reach this goal. In the meantime, we can strive to minimize sources of variation and rely on statistical methods that enable us to quantify the chaotic behaviour of biofilms.

2.7. References

1. Watnick, P. & Kolter, R. Biofilm, City of Microbes. *J. Bacteriol.* **182**, 2675–2679 (2000).
2. Paula, A. J., Hwang, G. & Koo, H. Dynamics of bacterial population growth in biofilms resemble spatial and structural aspects of urbanization. *Nat. Commun.* **11**, 1354 (2020).
3. Baker, M. Reproducibility Crisis? *Nature* **533**, 26 (2016).
4. Schloss, P. D. Identifying and Overcoming Threats to Reproducibility, Replicability, Robustness, and Generalizability in Microbiome Research. *mBio* **9**, (2018).
5. Freedman, L. P., Cockburn, I. M. & Simcoe, T. S. The Economics of Reproducibility in Preclinical Research. *PLoS Biol.* **13**, (2015).
6. Goodman, S. N., Fanelli, D. & Ioannidis, J. P. What does research reproducibility mean? *Sci. Transl. Med.* **8**, 341ps12-341ps12 (2016).
7. Van Acker, H. & Coenye, T. The Role of Reactive Oxygen Species in Antibiotic-Mediated Killing of Bacteria. *Trends Microbiol.* **25**, 456–466 (2017).
8. Grimes, D. R., Bauch, C. T. & Ioannidis, J. P. Modelling science trustworthiness under publish or perish pressure. *R. Soc. Open Sci.* **5**, 171511 (2018).
9. Rosenau, J. Science denial: a guide for scientists. *Trends Microbiol.* **20**, 567–569 (2012).
10. ASTM E2647-20. Standard Test Method for Quantification of a Pseudomonas aeruginosa Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. *ASTM Int. West Conshohocken PA* (2020).
11. ASTM. Standard Practice for Preparing a Pseudomonas aeruginosa or Staphylococcus aureus Biofilm Using the CDC Biofilm Reactor. (2018).
12. Pérez, J., Laureni, M., van Loosdrecht, M. C. M., Persson, F. & Gustavsson, D. J. I. The role of the external mass transfer resistance in nitrite oxidizing bacteria repression in biofilm-based partial nitritation/anammox reactors. *Water Res.* **186**, 116348 (2020).
13. Jackson, G., Beyenal, H., Rees, W. M. & Lewandowski, Z. Growing reproducible biofilms with respect to structure and viable cell counts. *J. Microbiol. Methods* **47**, 1–10 (2001).
14. Andersson, E. Urban Landscapes and Sustainable Cities. *Ecol. Soc.* **11**, (2006).
15. Hufnagel, L. & Pepperkok, R. Let there be light—at the right place. *Nat. Biotechnol.* **34**, 1246 (2016).
16. Teodósio, J. S., Simões, M., Melo, L. F. & Mergulhão, F. J. Flow cell hydrodynamics and their effects on E. coli biofilm formation under different nutrient conditions and turbulent flow. *Biofouling* **27**, 1–11 (2011).
17. Harrison, J. J. *et al.* The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the calgary biofilm device. *Biol. Proced. Online* **8**, 194–215 (2006).

18. Nadell, C. D., Drescher, K. & Foster, K. R. Spatial structure, cooperation and competition in biofilms. *Nat. Rev. Microbiol.* **14**, 589 (2016).
19. McDougald, D., Rice, S. A., Barraud, N., Steinberg, P. D. & Kjelleberg, S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat. Rev. Microbiol.* **10**, 39 (2012).
20. Hamilton, M. *Testing Surface Disinfectants: Desirable Attributes of a Standardized Method*. (2010).
21. Hamilton, M. A. KSA-SM-03—Testing surface disinfectants: Desirable attributes of a standardized method. (2010).
22. Kragh, K. N., Alhede, M., Kvich, L. & Bjarnsholt, T. Into the well—A close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm* **1**, 100006 (2019).
23. Zeiger, A. S., Hinton, B. & Van Vliet, K. J. Why the dish makes a difference: Quantitative comparison of polystyrene culture surfaces. *Acta Biomater.* **9**, 7354–7361 (2013).
24. Encinas, N. *et al.* Submicrometer-Sized Roughness Suppresses Bacteria Adhesion. *ACS Appl. Mater. Interfaces* **12**, 21192–21200 (2020).
25. Grosch, J.-H. *et al.* Enzyme activity deviates due to spatial and temporal temperature profiles in commercial microtiter plate readers. *Biotechnol. J.* **11**, 519–529 (2016).
26. Eldar, A. & Elowitz, M. B. Functional roles for noise in genetic circuits. *Nature* **467**, 167–173 (2010).
27. Goeres, D. M. *et al.* Drip flow reactor method exhibits excellent reproducibility based on a 10-laboratory collaborative study. *J. Microbiol. Methods* **174**, 105963 (2020).
28. Aryasomayajula, A., Bayat, P., Rezai, P. & Selvaganapathy, P. R. Microfluidic Devices and Their Applications. in *Springer Handbook of Nanotechnology* (ed. Bhushan, B.) 487–536 (Springer, 2017). doi:10.1007/978-3-662-54357-3_16.
29. Hu, Y., You, J.-O. & Aizenberg, J. Micropatterned Hydrogel Surface with High-Aspect-Ratio Features for Cell Guidance and Tissue Growth. *ACS Appl. Mater. Interfaces* **8**, 21939–21945 (2016).
30. Libberton, B. Cryo-EM and super-resolution microscopy on the same cell - when will we see this in biofilm? *Nature Research Microbiology Community* https://naturemicrobiologycommunity.nature.com/users/7272-ben-libberton/posts/58437-cryo-em-and-super-resolution-microscopy-on-the-same-sample-when-will-we-see-this-in-biofilm (2020).
31. Hoffman, D. P. *et al.* Correlative three-dimensional super-resolution and block-face electron microscopy of whole vitreously frozen cells. *Science* **367**, eaaz5357 (2020).
32. Shi, H. *et al.* Highly multiplexed spatial mapping of microbial communities. *Nature* (2020) doi:10.1038/s41586-020-2983-4.

33. Hellweger, F. L., Clegg, R. J., Clark, J. R., Plugge, C. M. & Kreft, J.-U. Advancing microbial sciences by individual-based modelling. *Nat. Rev. Microbiol.* **14**, nrmicro.2016.62 (2016).
34. Yan, J., Monaco, H. & Xavier, J. B. The Ultimate Guide to Bacterial Swarming: An Experimental Model to Study the Evolution of Cooperative Behavior. *Annu. Rev. Microbiol.* **73**, 293–312 (2019).
35. Lourenço, A. *et al.* BiofOmics: A Web Platform for the Systematic and Standardized Collection of High-Throughput Biofilm Data. *PLoS ONE* **7**, e39960 (2012).
36. Lourenço, A. *et al.* Minimum information about a biofilm experiment (MIABiE): standards for reporting experiments and data on sessile microbial communities living at interfaces. *Pathog. Dis.* **70**, 250–256 (2014).
37. Borch, T. *et al.* Evaluation of Characterization Techniques for Iron Pipe Corrosion Products and Iron Oxide Thin Films. *J. Environ. Eng.* **134**, 835–844 (2008).

Chapter 3.

Minimum information guideline development

Parts of this chapter are included in the following publication: J Allkja, *et al.* "Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates". Biofilm 2 (2020).
https://doi.org/10.1016/j.bioflm.2019.100010.

Abstract

One of the major issues facing the scientific community today is the lack of reproducibility in published studies. One effective strategy against this complex problem is the use of minimum information guidelines. These provide a guide for authors and reviewers on the necessary information that a manuscript should include for the experiments in a study to be clearly interpreted and independently reproduced. The guideline on the spectrophotometric and fluorometric assessment of biofilm formation in microplates was built following several discussions between international groups working in the area of biofilms. This guideline has been divided into 5 main sections, each containing a comprehensive set of recommendations. The intention of the minimum information guideline is to improve the quality of scientific communication and thus enhance interlaboratory reproducibility in biofilm microplate assays.

Keywords

Biofilm, reproducibility, guidelines, microplate, spectrophotometry, fluorometry

3.1. Introduction

A major challenge facing science today is the lack of reproducibility between published studies.[1,2] Many factors contribute to this phenomenon, including the selective or insufficient reporting of experimental details in the published literature, either in the methodology or data processing sections, which are essential for conducting the experiment.[3] Furthermore, due to the rapid development of science, new terms are often introduced, or existing terminology is repurposed, which can create confusion when trying to understand a paper or reproduce a study.[4]

Minimum information guidelines are an effective strategy for addressing the reproducibility crisis.[5] These guidelines instruct authors and reviewers on the minimum information required for the experiments to be reproducible and the data to be comparable. They also allow the scientific community to standardise terminology leading to the development of ontology databases. However, they do not offer any information on whether a method is appropriate for a certain study nor endorse any specific protocols. The Minimum Information for Biological and Biomedical Investigations (MIBBI) Project is a web based platform (www.mibbi.org) that gathers different minimum information guidelines in the biological and biomedical field, as well as any databases or standard ontologies related to them.[4]

Minimum information about a biofilm experiment (MIABiE) (www.miabie.org) is one of the guidelines presented in MIBBI.[6] It offers a broad view of the information necessary when conducting experiments related to biofilms. As discussed in chapter 1, biofilms are defined as a community of microorganisms embedded in an extracellular polymeric substance, often attached to a biotic or abiotic surface, which are essential in certain ecosystems but can also have detrimental effects in industry and healthcare.[7] MIABiE includes several modules, each addressing specific parts of a biofilm study, and presents an initiative for a biofilm ontology guide.

The present guideline will expand some of the MIABiE modules by focusing on spectrophotometric and fluorometric methods of biofilm assessment in microplate experiments. These are widely used biofilm assessment methods due to their versatile applications in medical, industrial and environmental biofilm research.[8] They can serve as a generic test, which does not require overly specialised or expensive equipment, or training, and can generate high-throughput data because they are microplate compatible.

Although several options for photometric or fluorescence-based methods in microplates exist, this guideline will focus on those methods most frequently used. This includes spectrophotometric methods used to quantify total biofilm mass based on the binding of dyes, such as crystal violet and safranin, to cells and negatively-charged molecules (such as polysaccharides) in the biofilm matrix.[9–11] Additionally, the guideline is applicable to fluorometric (or fluorescence-based) methods used to quantify the metabolic activity of cells within a biofilm, including those based on resazurin (also known as alamarBlue™), fluorescein diacetate (FDA) and various tetrazolium salts like 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT) and 2,3,5-triphenyl-tetrazolium chloride (TTC).[8,12–15] Furthermore, methods that stain specific biofilm components such as SYTO 9, which stains nucleic acids [8,16], and Wheat Germ Agglutinin (WGA), which stains the extracellular polymeric substances (EPS), are also compatible with the guideline.[17]

3.2. Methodology

To create the minimum information guideline, we conducted a literature review using three different databases: Pubmed, Google Scholar and Web of Science. The research was separated into literature related to the methods and literature related to biofilm properties and the various factors affecting them. For the former, very broad search terms such as, "Biofilm AND microtit* plate", "Biofilm AND Spectro*" and "Biofilm AND Fluor*" were used as a starting point. These resulted in thousands of hits from all three databases, and to further refine this output more specific terms such as "Crystal violet", "Resazurin Or Alamar Blue", "XTT", "TTC", "MTT", "FDA", "Syto9" and "WGA" were used. The results were ordered according to number of citations (most to least) and publishing date (newest to oldest). 180 papers were selected to be used as references to write the guideline. These were categorised into papers evaluating the methods and highlighting critical factors or steps, and papers that used the method in a specific investigation. The latter were used to create an understanding of what is commonly reported in scientific articles. A list of the refences used in the research phase but not included in the chapter references can be found in Appendix S3.1. Approximately 30 of the papers in this category were discarded from the literature review, as the only description of the method was a reference to a previously published paper.

When researching the literature on biofilm properties and what affects them, terms such as "impact", "influence", "effect or affect", "changes or differences" were used. These helped

in creating an understanding of the different parameters that should be reported for a biofilm experiment. Additionally, other minimum information guidelines were used as templates in the initial drafting process.

The final guidelines are the result of a dialog among biofilm experts familiar with microplate methods. These experts are included in the consortium list on the MIABiE website http://miabie.org/consortia.php as well as the authors list of the publication related to this chapter.

3.3 The Guideline

This guideline focuses on spectrophotometric and fluorometric measurement of biofilm grown in microplates and is divided into 5 different sections labelled 01-05 (Figure 3.1). Although there may be minor differences between staining reagents and techniques, this outline is designed to follow the chronological order in which the assays are typically performed and described. Section 01 pertains to the experimental design. Here the investigators determine the research question and how they may answer it. Once the experiment is mapped out, the next step is to grow the biofilm (section 02). This step includes inoculum preparation as well as biofilm growth in the microplate. Subsequently the biofilm is typically quantified or assessed using a specific stain and this biofilm assessment method is detailed in section 03. This process allows for minor variations depending on the target and the stain; however, the main steps are generally the same: washing, drying, staining, elution of stain and/or measuring absorbance or fluorescence (Figure 3.1). Once the reading is concluded and the data are collected, the next step is to analyse them (04). Moreover, in the interest of data sharing and communication we propose that data should be submitted to biofilm databases in the future (05).

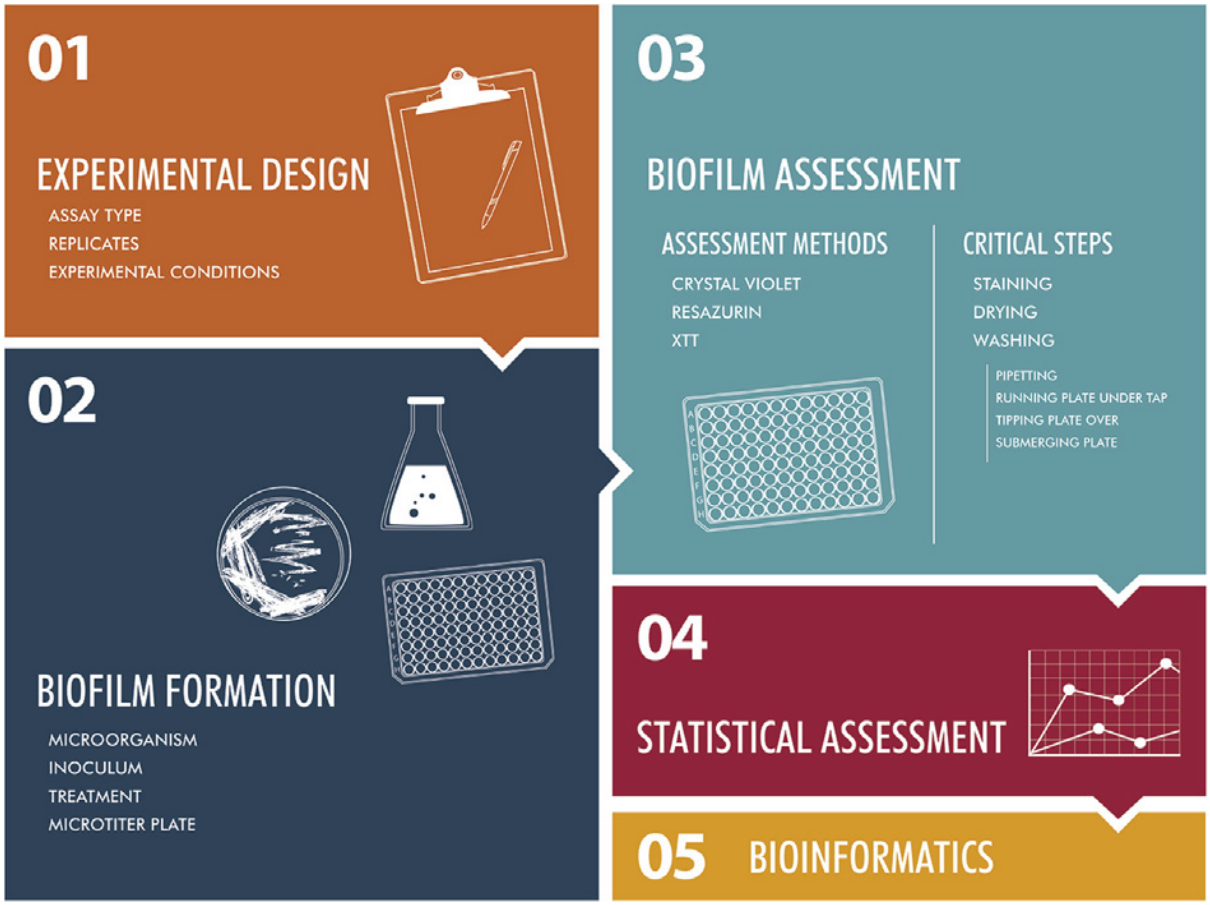

Fig. 3.1. Schematic diagram of the guideline and critical steps for spectrophotometric and fluorometric methods of biofilm assessment. Schematic diagram of the different sections of this guideline, highlighting the various critical steps that can increase variability in biofilm experiments. Different approaches to washing were illustrated to showcase how variable these can be in different protocols. (Illustration courtesy of Jill Story).

While developing the guideline, it became clear that methodological details that may be essential to achieve reproducibility of a biofilm experiment are often lacking critical information or omitted entirely. Therefore, in Table 3.1 we describe the most common omissions in reporting microplate methods and reflect on the potential impact of these omissions on the outcome of the experiment.

Table 3.1. Common omissions in reporting spectrophotometric and fluorometric methods of biofilm assessment

Omission	Impact
References	Often papers cited as containing the protocol followed in the study do not describe the full protocol and redirect you to another paper. This can create confusion when trying to understand the protocol that was followed.
Replicates	The number of replicates within one experiment is not reported in the published paper. Furthermore, there are inconsistencies in the terminology used when describing replicates. For example, biological and technical replicates vs day-to-day and within experiment.
Controls	While controls are mostly mentioned in the published articles, their values are usually not reported. This makes it difficult to understand the variability associated with the method and how the raw data was processed.
Inoculum preparation	Different culturing methods can affect the behaviour of microorganisms, their ability to attach to a surface, formation of aggregates, and response to different stimuli, chemicals, or other microorganisms.[18]
Environmental factors	In dry conditions, the microtiter plate wells easily dry out, which affects biofilm formation. Hence, investigators take certain measures to avoid the problem which are usually not addressed in the methodology section.
Position of samples in the wells	The layout of plates is often not reported, but the position of samples in the microtiter plate can affect the results. This is due to the fact that humidity and air distribution can differ in the inner wells compared to the outer ones, thus resulting in differences in biofilm formation.[19]

Orbital shaker settings	Most papers only refer to the rpm settings on their orbital shaker and omit other details such as the orbital diameter which can affect the shear stress exerted in the wells.[20]
Washing	Description of this step is often omitted or vague terms such as, "gently rinse" or "slowly tip over plate" are used, which leave it up to the reader to determine how to perform the step.[11,21]
Drying	This step is very often omitted altogether from the method description or contains very little detail on how it was performed.
Raw data	Most articles do not provide their raw data and omit information on how this was analysed.[22]
Outliers	Outliers are very often not included in the paper or, if reported, their exclusion is simply mentioned with little argumentation for it and how the final data analysis was affected by their removal.

At the end of the guideline requirements list, we provide examples of two hypothetical experiments related to biofilm formation using crystal violet (Table 3.2).

01. Experimental design

1. Describe the main question to be addressed in the study. This includes proposed main (and possibly secondary) hypothesis(es).
2. Explain the experimental design for the study, in other words, what type of experiment is being conducted to test the hypothesis (es)? For example, a comparison between different treatments or factors; different microbial bacterial strains (i.e., reference "type" strains, mutant constructs, or clinical isolates) or different concentrations and exposure times?
3. State the number of biological replicates, meaning independent repeats of the same experiment. Ideally, these should be day-to-day replicates to account for changes in humidity and room temperatures, for example. Include the number of technical replicates within the experiment, meaning the number of replicates for each sample group in the experiment. If applicable state whether the technical replicates are within one plate or in separate ones.

4. Include the number of replicates for the controls used in the experiment. Additionally, describe what these controls were and report their data to improve overall understanding of results. Depending on the test hypothesis they could be very straightforward, such as a growth check as a positive control and a sterility check as a negative control. On the other hand, they could be more complex. For example, if an antimicrobial agent is added and then rinsed off, an appropriate control is to use a mock carrier (e.g., saline) which accounts for the removal of microbes resulting from the exchange of fluids. Other appropriate controls for antimicrobial testing include solvent controls (e.g., DMSO) to verify that decrease in biofilm is due to the compound and not the solvent, and pre-treatment controls to verify that the effects observed are due to bactericidal not bacteriostatic activity. Furthermore, when biocide tests are performed, it is recommended to perform a neutralizer verification, as well as checking for interactions between the microplate material, biocide (e.g., bleach) and dye. It should be noted that controls are highly dependent on the experimental design, therefore it is important to report all the relevant controls.
5. When applicable, provide reference to all published protocols followed, ideally to the original articles containing all the necessary information. Additionally, if any changes were made to these published protocols they need to be described in detail.
6. Provide a link to any supplementary information or data not reported in the main body of the article, such as more detailed method descriptions, a metadata sheet containing raw data and layouts of microplate designs, etc.

02. Biofilm formation

1. Describe the microorganisms selected for the experiment. List the species and strain number, and if available the strain numbers assigned in international culture collections, e.g., ATCC, BCCM/LMG bacteria Collection, or DSMZ, or provide a reference in which the relevant details of the strains are reported. Alternatively, if clinical or environmental isolates are used, provide all available and relevant background and ethical information. Describe the stock preservation conditions, and any modifications made to the microorganism (plasmid insertions, gene knockouts, etc) using established genetic nomenclature.
2. Describe the inoculum preparation protocol. Include information on incubation conditions such as concentration, growth phase, temperature, time, shaking (rpm and orbital diameter or static conditions) and growth media (ingredients, concentration, origin). Depending on the microorganism, include other applicable incubation

conditions such as light, CO_2 concentration, humidity, etc. Additionally, if any washing steps were performed include detailed information on centrifugation conditions (*g* force, time, equipment) and the washing agent used (water, PBS, etc). Other important factors might be whether a culture was grown up then diluted to a specific concentration, and how this was measured, i.e., optical density is commonly used.

3. Describe the compounds or conditions being tested. In case of antimicrobials, describe their concentration (molarity, g/L, or any other appropriate SI units), origin (manufacturers if purchased and catalogue numbers if allowed by the journal of choice), and time point in the experiment when they were added, and whether an agent was used to neutralize the active ingredient. If applicable, describe pH, any solvents used, activity corrections and whether agents were filtered prior to use.
4. Provide information on microplates used. This includes type of plate (clear, white, or black), number of wells (6, 9, 24, 96 or 384 well), shape of the wells (flat, rounded, U-shaped or V-shaped), the material and the manufacturing company, including catalogue numbers if allowed by the journal of choice. Report any modifications made to the manufactured microplate such as pre-coating of the wells or addition of coupons.
5. Describe how the microplate was prepared. Provide information on the inoculum conditions at harvest such as growth phase, optical density (wavelength, zero solution, equipment) and concentration of microorganism (CFU/mL for bacteria or cells/mL for yeast) and growth media (if different from point 2). If a biofilm prevention experiment is being conducted provide information on the antibiofilm agent used (concentration in relevant SI units, preparation, and origin).

   If possible, provide a short description of the layout of the microplate showing the position of controls and samples. Additionally, if applicable mention any extra steps taken such as adding water to the outer wells to avoid “edge effects”.
6. Provide a description of incubation conditions for the microplate. Include information on temperature, time and shaking (rpm and orbital diameter or stationary). Similar to point 2 include a description of any other relevant conditions such as light, CO_2 concentration or humidity. Additionally, if applicable mention any extra steps such as sealing the plate with parafilm or other films or incubating within a humidified container.

03. Biofilm assessment method

1. Describe the method followed to discard the planktonic suspension, e.g., pipetting, suction manifold.
2. Describe all washing steps in detail. Provide information on the washing agent such as sterility, origin, concentration, and pH, if applicable. Additionally, describe the number of washes and method(s) used to add and remove the washing agent (immersion, rinsing or pipetting). When possible, avoid the sole use of vague terms such as "gently" which are subject to interpretation and include more detailed descriptions. For example, describing the angle and depth at which a pipette tip was inserted into the well or stating the number of times the plate was shaken to remove excess liquid when inverted. If automatic liquid handling devices are used provide information of equipment and settings.
3. Describe the staining process. This includes information on the stain: origin (manufacturer), stock and working solution concentrations, solvents used as well as information on the staining: time and incubation conditions (light, temperature, volumes, shaking etc.). If applicable, provide information on any standard curves performed with the experiment.
4. In cases where extra steps such as fixation, drying and elution are required, describe how these were performed and any solvents or chemicals (origin, concentration) used.
5. Describe how the spectrophotometric or fluorometric signal was measured. Provide information on the equipment (model number, company, software) used as well as its settings (excitation, emission and detection wavelengths, endpoint, or continuous read, shaking). When using fluorometric reading, provide information on the type of readout (top or bottom reading). If bottom reading is performed, provide information on the number and distribution of the points measured across each well.

   Furthermore, if imaging functions of the microplate reader were used, describe the settings (time, shaking, imaging mode, filters, camera).

04. Statistical assessment and data presentation

1. Describe how the raw data were processed and/or transformed. If possible, include raw data in the supplementary data section.
2. Present all outliers. Argumentation should be given if they were removed from the analysis in the results and ideally how their removal affected the data.

3. Test the data for normality. Report if the data has been transformed or normalised for example, using a standard curve, log transformation, square root, or any other appropriate normalisation method.
4. Describe statistical tests and rationale for use (i.e., parametric, non-parametric, small sample, paired etc.) performed and any post-hoc tests. Provide information on the test parameters, descriptive statistics such as significant differences, standard errors, standard deviation, variance, and confidence intervals. Additionally, include descriptive statistics for the controls used in the experiment. If a high-throughput screening assay is being reported, it is recommended to include the calculation of the screening windows coefficient, or Z'. [23]
5. Ensure that the appropriate graph types and data visualizations are used. Figures should provide all the essential and relevant information necessary for a full understanding of the results.[24] We suggest the use of scatter plots or box and whisker plots instead of line graphs or bar charts, which often do not portray all the necessary information in a dataset (Figure 3.2). For instance, many different normal, skewed or bimodal data distributions can lead to the same mean and standard deviation values. [24] Summarizing data as a mean with standard deviation can also conceal unequal sample sizes and outliers. [24,25] Plotting all measurements in tandem with means and standard deviations provides transparency and allows readers to evaluate data for themselves (Figure 3.2).
6. Provide details of the statistical package used and its version. If more than one was used, they all need to be mentioned. Additionally, if any open-source systems such as R packages were used, provide a reference or a link to it.

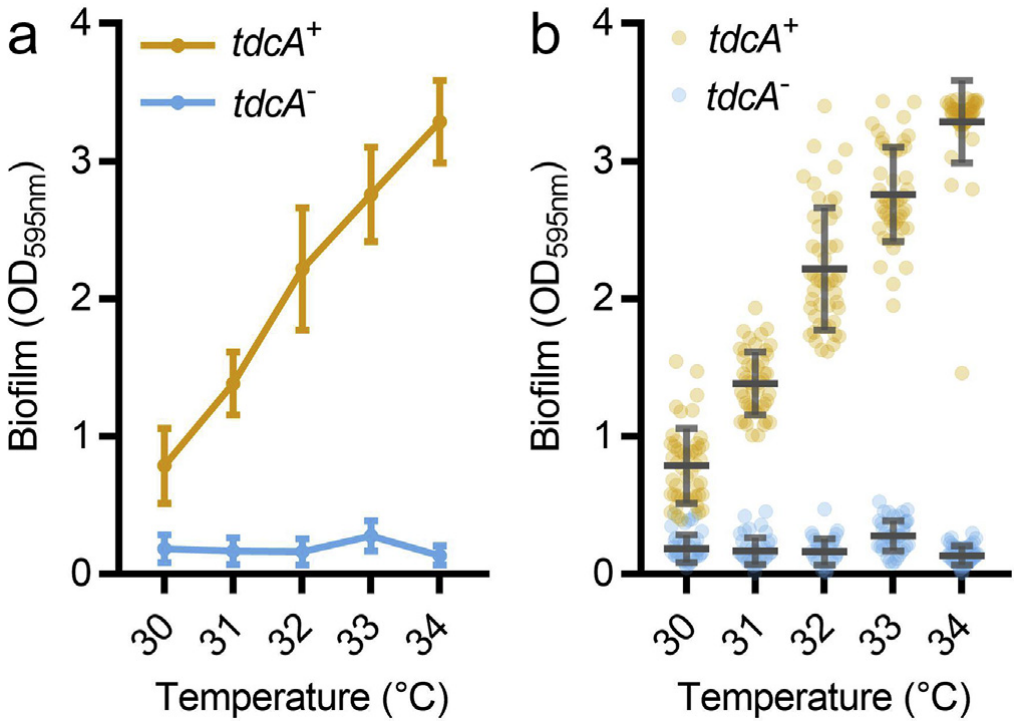

Figure. 3.2. Show the dots on plots: scatter graphs allow readers to evaluate data distributions for themselves. Biofilm formation was measured for *Pseudomonas aeruginosa* strains CF39S and CF39, which express functional and mutant alleles of the thermosensory diguanylate cyclase (*tdcA*$^{+}$ and *tdcA*$^{-}$), respectively. Each condition has 48 replicates, representing sixteen technical replicates from each of three independent biological replicates. (a) Line graph. Datum points represent means and standard deviations. (b) Scatter plot. Each point denotes a replicate datum point and lines and bars represent means and standard deviations, respectively. (Data courtesy of Joe J. Harrison).

05. Bioinformatics (Optional)

1. Use standard terminology. In the coming years ontology guidelines for biofilm terminology are expected to be developed. A starting guide can be found on the MIABiE website.[26]
2. The data should be formatted in a way that makes it easier to submit and extrapolate it to existing databases such as BiofOmics (http://www.biofomics.org/) or other databases currently in development.[27]

Table 3.2. Section by section description of two hypothetical experiments following the guideline requirements. Simple experiment on the study of the effect growth media on biofilm formation. Complex experiment on biofilm prevention efficacy testing.

Section	Simple experiment example	Complex experiment example
Experimental design	*This study investigated the effect of growth media concentration on Staphylococcus aureus biofilm formation in a microplate. Total biofilm mass formation after 24 hours for four different concentrations of Tryptic Soy Broth (TSB) was compared. Each experiment consisted of one plate that used 6 sample wells per TSB concentration, and 6 negative control wells containing only TSB for all four concentrations tested. Each experiment was repeated in three independent weeks. A more detailed description of the methodology together with a schematic illustration of the sample and negative control positions within the plate can be found in our supplementary data section [Link].*	*This study investigated the ability of tobramycin [Manufacturer, Catalogue nr.] to prevent biofilm formation on Pseudomonas aeruginosa biofilm. The metabolic activity of the biofilm was measured after 24 hours for seven different concentrations of tobramycin. Each experiment consisted of one plate that used 6 sample wells per tobramycin concentration. Furthermore, 6 sample wells were used for each of the following controls: sterility, solvent and growth. Each experiment was repeated in three independent weeks. A more detailed description of the methodology together with a schematic illustration of the sample and control positions within the plate can be found in our supplementary data section [Link].*
Biofilm formation	*Staphylococcus aureus strain ATCC 25923™ was used. To prepare the inoculum, -80 °C glycerol stocks were streaked out on Tryptic Soy Agar [Manufacturer] plates. One colony from the plate was transferred into 15 ml TSB and incubated at 37 °C, 125 rpm in a shaker incubator [Model number] with an orbital diameter of 1.9 cm. After 18 hours a 1:100 dilution of the inoculum was incubated at 37 °C, 125 rpm until it reached the exponential growth phase (OD=0.300 [595nm; Model number]). Four 2 ml aliquots of the suspension were made and washed by centrifugation (2000 g for 15 minutes [Model number]) and resuspending the pellets in PBS [pH 7.4; Manufacturer] twice. Subsequently, the pellets were resuspended in 4 different TSB broths (30 g/ml, 3 g/ml, 0.3 g/ml and 0.03 g/ml) and 200 µl per well of each of these suspensions was added to a flat bottom polystyrene 96 well plate [Manufacturer] according to the layout in the supplementary data. The plate was incubated at 37 °C under static*	*Pseudomonas aeruginosa strain ATCC 15442™ was used. Tobramycin [Manufacturer, Catalogue nr.] stocks of 1 g/L were prepared in sterile water and stored at -20 °C for a maximum of 4 months. Seven concentrations of tobramycin were prepared in sterile water for use in the experiment: 20 µg/mL, 10 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL and 0.5 µg/mL.* *To prepare the inoculum, -80 °C glycerol stocks were streaked out on R2-A agar [Manufacturer] plates. One colony from the plate was transferred into 15 ml TSB and incubated at 37 °C, 125 rpm in a shaker incubator [Model number] with an orbital diameter of 1.9 cm. After 18 hours a 1:100 dilution of the inoculum was incubated at 37 °C, 125 rpm until it reached the exponential growth phase (OD=0.300 [595nm; Model number]). 196 µl per well the suspension was added 48 wells of a flat bottom polystyrene 96 well plate [Manufacturer]. 4 µl per well of each tobramycin concentration were added to 42 of these wells and 4 µl per well of*

	conditions in a non-humidified incubator for 24 hours. To prevent excess drying the outer wells were filled with 200 µl/well of sterile water.	*sterile was added to the other 6 as solvent control. 200 µl per well of suspension was added to 6 wells as growth control and 200 µl per well of TSB broth was added to 6 wells as sterility control. The plate was incubated at 37 °C under static conditions in a non-humidified incubator for 24 hours. To prevent excess drying the outer wells were filled with 200 µl/well of sterile water.*
Biofilm assessment	*The planktonic suspension was carefully removed using a multichannel pipette [Model, Manufacturer] fitted with a 300 µL tip inserted slowly at a 45° angle while making sure to avoid touching the sides and bottom of the wells. The plate was washed twice with 250 µl/well of PBS using a multichannel pipette fitted with a 300 µL tip and left to air-dry for 15 min in under laminar flow at room temperature (RT, 20 ±5 °C). The biofilm was fixed for 15 min with 200 µl/well of 99% v/v ethanol [Manufacturer] and then allowed to air-dry until fully dry, between 5 and 10 minutes. The plate was stained with 200 µL of 0.1 % v/v Crystal violet [Manufacturer] for 15 min at RT, under static conditions. After staining the plates were washed twice with 250 µl/well of MilliQ water using a multichannel pipette and left to air-dry for 15 min in laminar flow. The stain was eluted with 200 µl/well of 99% v/v ethanol for 30 min at RT, no shaking. The eluted stain was mixed by pipetting up and down 4 times and 100 µl/well of it were transferred to an empty 96-well plate using a multichannel pipette. The absorbance was measured at 595 nm using a [Company; Model number] plate reader.*	*After 24 hours the planktonic suspension was carefully removed using a multichannel pipette [Model, Manufacturer] fitted with a 300 µL tip inserted slowly at a 45° angle while making sure to avoid touching the sides and bottom of the wells. Tips were changed between different controls and tobramycin concentration. The plate was washed twice with 250 µl/well of PBS using a multichannel pipette fitted with a 300 µL tip and left to air-dry for 15 min in under laminar flow at room temperature (RT, 20 ±5 °C). The plate was stained with 200 µL of 50 µg/mL v/v Resazurin [Manufacturer] prepared in sterile water, covered in foil for 45-60 minutes RT, under static conditions. Colour change was monitored due to day-to-day differences in time needed for the reaction to start. The fluorescence was measured using a [Company; Model number] plate reader at λexcitation = 560 nm; λemission = 590 nm.*
Statistical assessment	*Raw absorbance data can be found in our supplementary data section. To evaluate the within plate variability, the mean ± 1 standard deviation (STDEV) of all the technical replicates for each sample were calculated and are summarised in table [1]. The means of all the different samples were*	*Raw fluorescence data can be found in our supplementary data section. To evaluate the within plate variability, the mean ± 1 standard deviation (STDEV) of all the technical replicates for each sample were calculated and are summarised in table [1]. The means of all the different samples were corrected*

	corrected by subtracting the corresponding negative control (TSB only) values. The data from all three replicate experiments were analysed using a one-way ANOVA test with a Levene's post-hoc analysis to compare the absorbance values. These results were represented in Figure 1 and a more detailed description can be found in the figure legend. [Statistical Programme; version] was used to perform all tests.	*by subtracting the sterility control (TSB only) values. Furthermore, percent reduction in metabolic activity for each of the seven tobramycin concentrations and the solvent control were calculated by dividing their fluorescence with the growth control fluorescence. The data from all three replicate experiments were analysed using a one-way ANOVA test with a Levene's post-hoc analysis to compare the absorbance values. These results were represented in Figure 1 and a more detailed description can be found in the figure legend. [Statistical Programme; version] was used to perform all tests.*

As illustrated above, the amount of information necessary to fully characterize a complex system such as a biofilm experiment is significant. Therefore, a simplified checklist of the guideline has been generated (Table 3.3). This checklist can assist authors during their writing process, as well as reviewers during the peer-review process. In fact, complementary fields such as ecology and evolution have very recently started to make checklists available in their field of knowledge.[28] Moreover, certain sections of this guideline can be applied to other biofilm assessment methods in microplate experiments, such as when viable plate counts are used to assess biofilm density and treatment efficacy.

Table 3.3. Simplified checklist for minimum information guideline spectrophotometric

methods of biofilm assessment.

01. Experimental design	
Aim of the experiment/hypothesis presented	✓
Type of experiment	
Biological and technical replicates	
Positive and negative control replicates and description	
Reference to original article containing protocol (If applicable)	
Supplementary information (If applicable)	
02. Biofilm forming method	
Microorganism description	
Inoculum preparation protocol	
Treatment description (If applicable)	
Microtiter plate description	
Plate layout i.e. sample distribution (Optional)	
Incubation conditions for microtiter plate	
03. Biofilm assessment method	
Planktonic suspension removal	
Washing description	
Staining description	
Additional steps: fixing, drying, buffer solutions (if applicable)	
Absorbance / Fluorescence measurement	
04. Statistical assessment and data interpretation	
Raw data handling	
Outliers	
Normality testing	
Appropriate data presentation	
Statistical test with post-hoc and descriptive stats	
Statistical programme used	
05. Bioinformatics (Optional)	
Standardised terminology	
Data formatting according to data submission guidelines	
Submission to online database	

3.4. Discussion

Microplate-based spectrophotometric and fluorometric methods of biofilm assessment have led to the generation of a vast amount of data throughout the years. However, while these data have provided essential information on biofilm biology and experimental therapeutic strategies to tackle biofilms, biofilm experiments have often been difficult to reproduce. Furthermore, most of the time it is not possible to compare data between studies, which means that attempting to draw conclusions by combining data from different studies is not feasible. To minimize this problem, we suggest that a minimum information guideline should be adopted by researchers.

Lack of data comparability can in part be attributed to the high variability of protocols used for these types of methods. Table 3.4 illustrates this phenomenon of variability in protocols of the crystal violet assay for three common organisms: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. It contains the range of parameters (low to high) for different conditions of inoculum preparation, biofilm growth and biofilm assessment for each microorganism. Major differences in the inoculum preparation and biofilm growth parameters, are expected as the parameters of these steps are largely dictated by the physiology of the microorganism being investigated and the type of experiment being performed. However, table 3.4 shows that large differences are also present among the biofilm assessment parameters such as dye concentrations and absorbance wavelengths. Taken together, this information means that comparing different datasets at this stage is not possible for different studies and that the guidelines can only facilitate reproducibility and comparison to a certain degree. On the other hand, it is important to note that the variability in protocols used in the biofilm area is often due to the differences in the subject of the investigations.

Table 3.4. Example of the variability in protocol conditions of crystal violet assays for three different example microorganisms

Condition	Organism		
	Staphylococcus aureus spp.	***Pseudomonas aeruginosa spp.***	***Candida albicans***
Inoculum preparation			
Media	TSB, TSB wS*, LBb**, Water [8,11,23,29–34]	TSB, TSB wS*, LBb**, LB**, BHI**, MHI**, T-broth**, AB* [8,35–42]	YNB**, YPD**, RPMI-1640**, SDB** [8,43–50]
Inoculum incubation temperature (°C)	35-37 [8,11,23,29–34]	25-37 [8,35–42]	30-37 [8,43–50]
Incubation time (hours)	0***-24 [8,11,23,29–34]	0***-24 [8,35–42]	12-24 [8,43–50]
Inoculum shaking conditions	0-200 rpm/min [8,11,23,29–34]	0-250 rpm/min [8,35–42]	0-200 rpm/min, Roller drum [8,43–50]
Inoculum concentration / OD / growth phase at harvest	10^3-10^8 CFU/mL, 0.5 McFarland, OD_{600nm}=0.1 [8,11,23,29–34]	10-10^8 CFU/mL, OD_{600nm}=0.0025, OD_{595}=1.5 [8,35–42]	10^4-10^8 CFU/mL, OD_{600nm}=1 [8,43–50]
Biofilm growth			
Media	TSB, LB**, BHI** [8,11,23,29–34]	TSB, T-broth**, AB**, BHI**, MHI** [8,35–42]	YNB**, YPD**, RPMI-1640**, ASM**, SDB**, PBS**** [8,43–50]
Incubation temperature (°C)	35-37 [8,11,23,29–34]	25-37 [8,35–42]	37 [8,43–50]
Incubation time (hours)	18-48 [8,11,23,29–34]	2-48 [8,35–42]	2-48 [8,43–50]
Shaking conditions	0-200 rpm/min [8,11,23,29–34]	0-180 rpm/min [8,35–42]	0-120 rpm/min [8,43–50]
Biofilm Assessment			
Washing agent	Water, Saline, PBS****, MilliQ water [8,11,23,29–34]	Saline, Water, PBS*** [8,35–42]	PBS****, Water, Saline [8,43–50]
Washing (x times)	1-3 [8,11,23,29–34]	1-3 [8,35–42]	1-3 [8,43–50]
Crystal violet concentration	0.01-2.3 % [8,11,23,29–34]	0.1-2 % [8,35–42]	0.02-1 % [8,43–50]
Staining time	1-20 min [8,11,23,29–34]	5-30 min [8,35–42]	5-45 min [8,43–50]
Solubilisation agent	33 % acetic acid, 95-100 % ethanol [8,11,23,29–34]	30-33 % acetic acid, 95-100 % ethanol, DMSO**** [8,35–42]	30-33% acetic acid, 95 % ethanol, 0.1% Triton-X [8,43–50]
Absorbance wavelength (nm)	540-595 [8,11,23,29–34]	550-595 [8,35–42]	540-595 [8,43–50]

***wS** – with Supplement i.e. added yeast and/or glucose
****TSB**- Tryptic Soy Broth; **LBb** – Luria Bertani broth; **BHI**- Brain Heart Infusion; **LB** – Lysogeny broth; **MHI** – Mueller-Hinton broth; **T-broth** – Terrific broth; **AB** – minimal growth media; **YNB** – Yeast Nitrogen Base; **YPD** – Yeast Peptone Dextrose; **SDB** – Sabauraund Dextrose Broth; **RPMI-1640** - Roswell Park Memorial Institute–1640 medium; **ASM** – Artificial Saliva Medium;
*****0** – Inoculum prepared directly from agar culture
******PBS**- Phosphate buffered saline; **DMSO** – Dimethyl sulfoxide

Hence, a consensus regarding certain aspects of the methodology is necessary to improve reproducibility. On this matter, there are already standardised biofilm methods approved by the ASTM which could serve as a starting point for this process, such as the E2647-08 Standard Test Method for Quantification of a *Pseudomonas aeruginosa* Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow[51], the E2562-17 Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor[52] and the E2799-17 Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay. [53] They can also provide an excellent example on how to identify critical steps and describe the methodology to ensure reproducibility.

In addition, many investigations aim at optimising and modifying spectrophotometric and fluorometric methods to increase their efficiency, reliability, and their applications. For example, Junka *et al*, developed a way to assess wound dressing effectiveness in 24-well plates using crystal violet and TTC analysis.[54] This means that with time, as new steps are introduced or more robust ways of performing certain steps are developed, the methods will evolve. Minimum information guidelines have the advantage of remaining applicable to the methods despite these changes.

Moreover, as science evolves, we will be able to measure new parameters and conditions which affect reproducibility. As discussed in chapter 2, even when manufactured from the same base polymer, microplates can have different surface properties depending on the production process, resulting in differences in cell adhesion. [55] As the methods used to characterise surface properties become more accessible, parameters such as surface roughness might be used in the future. Since guidelines are often part of an online database such as MIBBI, they can be updated when necessary and evolve together with the methods.

3.5. Guideline implementation and dissemination

As is the case with compliance to MIABiE and other guidelines, compliance to the new guideline presented here will be difficult as it needs to be endorsed by both authors and journals.[56,57] To improve compliance a balance needs to be obtained between the level of detail asked, and the ability of most labs to be able to provide such data. As an example, many studies have shown that oxygen availability influences biofilm formation and can lead to different physiological features being expressed.[58] Therefore, understanding the oxygen availability within a well and across different wells in a microplate might be useful.

However, most laboratories lack the kind of system needed to assess this environmental parameter and it would be very difficult to implement this reading routinely. Hence, the oxygen profile within the microplate is not a requirement in the guideline.

In order to increase awareness, various strategies were employed to disseminate the guideline and encourage members of the biofilm community to apply it to their research. The first step was ensuring the guideline availability on the MIABiE website http://miabie.org/introduction.php, including some accompanying documents like the terminology clarification text box included in Appendix S3.2.

The guideline was presented in several academic conferences, examples of these can be found on the MIABiE website (http://miabie.org/contacts.php) as well. Moreover, since its publication in November 2019 the guideline has been cited by 22 publications, a majority of which are research papers adopting the guideline requirements. A breakdown of these citations can be found on Table 3.5. Additionally, to further increase guideline implementation in the biofilm community, a future aim of the consortium is to include the guideline in the authors requirements of a biofilm journal, as well as continued dissemination in various conferences and gatherings.

Table 3.5. Implementation of the minimum information guideline in the literature

Publication type	**Number of citations***
Research paper	*17* [59–75]
Review or Opinion paper	*3* [76–78]
Thesis dissertation	*2* [79,80]
Total	**22**
***Number of citations as of September 2021*	

3.6. Concluding remarks

In conclusion, microplate-based methods of biofilm assessment remain among the more widely used methods in biofilm studies, and as such it is important for the field that we consider their reproducibility and try to improve it. We are convinced that the implementation of minimum information guidelines will contribute to solving the reproducibility crisis and thus improve the use that the research community makes of data and ultimately advance science.

3.7. References

1. Baker, M. Reproducibility Crisis? *Nature* **533**, 26 (2016).
2. Dirnagl, U. Rethinking research reproducibility. *EMBO J.* **38**, e101117 (2019).
3. Grimes, D. R., Bauch, C. T. & Ioannidis, J. P. Modelling science trustworthiness under publish or perish pressure. *R. Soc. Open Sci.* **5**, 171511 (2018).
4. Taylor, C. F. *et al.* Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nat. Biotechnol.* **26**, 889–896 (2008).
5. Anon. A checklist for our community. *Nat. Ecol. Evol.* **2**, 913 (2018).
6. Lourenço, A. *et al.* Minimum information about a biofilm experiment (MIABiE): standards for reporting experiments and data on sessile microbial communities living at interfaces. *Pathog. Dis.* **70**, 250–256 (2014).
7. Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2**, 95–108 (2004).
8. Peeters, E., Nelis, H. J. & Coenye, T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods* **72**, 157–165 (2008).
9. Lopes, S. P., Azevedo, N. F. & Pereira, M. O. Emergent Bacteria in Cystic Fibrosis: In Vitro Biofilm Formation and Resilience under Variable Oxygen Conditions. *BioMed Res. Int.* **2014**, (2014).
10. Machado, I., Graça, J., Sousa, A. M., Lopes, S. P. & Pereira, M. O. Effect of antimicrobial residues on early adhesion and biofilm formation by wild-type and benzalkonium chloride-adapted Pseudomonas aeruginosa. *Biofouling* **27**, 1151–1159 (2011).
11. Stepanović, S. *et al.* Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* **115**, 891–899 (2007).
12. Alonso, B., Cruces, R., Pérez, A., Sánchez-Carrillo, C. & Guembe, M. Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm. *J. Microbiol. Methods* **139**, 135–137 (2017).
13. van Hengel, I. A. J. *et al.* Selective laser melting porous metallic implants with immobilized silver nanoparticles kill and prevent biofilm formation by methicillin-resistant Staphylococcus aureus. *Biomaterials* **140**, 1–15 (2017).
14. Honraet, K., Goetghebeur, E. & Nelis, H. J. Comparison of three assays for the quantification of Candida biomass in suspension and CDC reactor grown biofilms. *J. Microbiol. Methods* **63**, 287–295 (2005).
15. Tunney, M. M., Ramage, G., Field, T. R., Moriarty, T. F. & Storey, D. G. Rapid Colorimetric Assay for Antimicrobial Susceptibility Testing of Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **48**, 1879–1881 (2004).

16. Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K. & Ren, Q. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiol.* **15**, 36 (2015).
17. Skogman, M. E., Vuorela, P. M. & Fallarero, A. Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against Staphylococcus aureus biofilms. *J. Antibiot. (Tokyo)* **65**, 453 (2012).
18. Kragh, K. N. *et al.* The Inoculation Method Could Impact the Outcome of Microbiological Experiments. *Appl. Environ. Microbiol.* **84**, e02264-17 (2018).
19. Kostenko, V., Salek, M. M., Sattari, P. & Martinuzzi, R. J. Staphylococcus aureus biofilm formation and tolerance to antibiotics in response to oscillatory shear stresses of physiological levels. *FEMS Immunol. Med. Microbiol.* **59**, 421–431 (2010).
20. Azevedo, N. F., Pinto, A. R., Reis, N. M., Vieira, M. J. & Keevil, C. W. Shear Stress, Temperature, and Inoculation Concentration Influence the Adhesion of Water-Stressed Helicobacter pylori to Stainless Steel 304 and Polypropylene. *Appl. Environ. Microbiol.* **72**, 2936–2941 (2006).
21. Erriu, M. *et al.* Microtiter spectrophotometric biofilm production assay analyzed with metrological methods and uncertainty evaluation. *Measurement* **45**, 1083–1088 (2012).
22. Anon. Standardizing data. *Nat. Cell Biol.* **10**, 1123–1124 (2008).
23. Sandberg, M., Määttänen, A., Peltonen, J., Vuorela, P. M. & Fallarero, A. Automating a 96-well microtitre plate model for Staphylococcus aureus biofilms: an approach to screening of natural antimicrobial compounds. *Int. J. Antimicrob. Agents* **32**, 233–240 (2008).
24. Weissgerber, T. L., Milic, N. M., Winham, S. J. & Garovic, V. D. Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. *PLOS Biol.* **13**, e1002128 (2015).
25. Anon. Show the dots in plots. *Nat. Biomed. Eng.* **1**, 0079 (2017).
26. Sousa, A. M., Pereira, M. O., Azevedo, N. F. & Lourenço, A. Designing an Ontology Tool for the Unification of Biofilms Data. in *8th International Conference on Practical Applications of Computational Biology & Bioinformatics (PACBB 2014)* 41–48 (Springer, Cham, 2014). doi:10.1007/978-3-319-07581-5_5.
27. Lourenço, A. *et al.* BiofOmics: A Web Platform for the Systematic and Standardized Collection of High-Throughput Biofilm Data. *PLoS ONE* **7**, e39960 (2012).
28. Parker, T. H. *et al.* Empowering peer reviewers with a checklist to improve transparency. *Nat. Ecol. Evol.* **2**, 929–935 (2018).
29. Stepanović, S., Vuković, D., Ježek, P., Pavlović, M. & Švabic-Vlahović, M. Influence of Dynamic Conditions on Biofilm Formation by Staphylococci. *Eur. J. Clin. Microbiol. Infect. Dis.* **20**, 0502–0504 (2001).

30. Izano, E. A., Amarante, M. A., Kher, W. B. & Kaplan, J. B. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Appl. Environ. Microbiol.* **74**, 470–476 (2008).
31. Zmantar, T., Kouidhi, B., Miladi, H., Mahdouani, K. & Bakhrouf, A. A Microtiter plate assay for Staphylococcus aureus biofilm quantification at various pH levels and hydrogen peroxide supplementation. 10.
32. Xu, Z. *et al.* Crystal Violet and XTT Assays on Staphylococcus aureus Biofilm Quantification. *Curr. Microbiol.* **73**, 474–482 (2016).
33. Mohanty, S. *et al.* An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. *Nanomedicine Nanotechnol. Biol. Med.* **8**, 916–924 (2012).
34. Kaplan, J. B. *et al.* Low Levels of beta-Lactam Antibiotics Induce Extracellular DNA Release and Biofilm Formation in Staphylococcus aureus. *Mbio* **3**, e00198-12 (2012).
35. Sanchez, C. J. *et al.* Biofilm formation by clinical isolates and the implications in chronic infections. *BMC Infect. Dis.* **13**, 47 (2013).
36. Kim, H.-S. & Park, H.-D. Ginger Extract Inhibits Biofilm Formation by Pseudomonas aeruginosa PA14. *PLOS ONE* **8**, e76106 (2013).
37. Palanisamy, N. K. *et al.* Antibiofilm properties of chemically synthesized silver nanoparticles found against Pseudomonas aeruginosa. *J. Nanobiotechnology* **12**, 2 (2014).
38. Friedman, L. & Kolter, R. Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the Pseudomonas aeruginosa Biofilm Matrix. *J. Bacteriol.* **186**, 4457–4465 (2004).
39. Sabaeifard, P., Abdi-Ali, A., Soudi, M. R. & Dinarvand, R. Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. *J. Microbiol. Methods* **105**, 134–140 (2014).
40. Jagani, S., Chelikani, R. & Kim, D.-S. Effects of phenol and natural phenolic compounds on biofilm formation by Pseudomonas aeruginosa. *Biofouling* **25**, 321–324 (2009).
41. O'Toole, G. A. Microtiter Dish Biofilm Formation Assay. *J. Vis. Exp. JoVE* (2011) doi:10.3791/2437.
42. Bauer, J., Siala, W., Tulkens, P. M. & Van Bambeke, F. A Combined Pharmacodynamic Quantitative and Qualitative Model Reveals the Potent Activity of Daptomycin and Delafloxacin against Staphylococcus aureus Biofilms. *Antimicrob. Agents Chemother.* **57**, 2726–2737 (2013).
43. Marcos-Zambrano, L. J., Escribano, P., Bouza, E. & Guinea, J. Production of biofilm by Candida and non-Candida spp. isolates causing fungemia: Comparison of biomass production and metabolic activity and development of cut-off points. *Int. J. Med. Microbiol.* **304**, 1192–1198 (2014).

44. Negri, M. *et al.* Crystal violet staining to quantify Candida adhesion to epithelial cells. *Br. J. Biomed. Sci.* **67**, 120–125 (2010).
45. Monteiro, D. R. *et al.* Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of Candida albicans and Candida glabrata. *Biofouling* **27**, 711–719 (2011).
46. Li, Z., Sun, J., Lan, J. & Qi, Q. Effect of a denture base acrylic resin containing silver nanoparticles on Candida albicans adhesion and biofilm formation. *Gerodontology* **33**, 209–216 (2016).
47. Deveau, A. & Hogan, D. A. Linking Quorum Sensing Regulation and Biofilm Formation by Candida albicans. in *Quorum Sensing: Methods and Protocols* (ed. Rumbaugh, K. P.) 219–233 (Humana Press, 2011). doi:10.1007/978-1-60761-971-0_16.
48. Dovigo, L. N. *et al.* Susceptibility of clinical isolates of Candida to photodynamic effects of curcumin. *Lasers Surg. Med.* **43**, 927–934 (2011).
49. Khan, S., Alam, F., Azam, A. & Khan, A. U. Gold nanoparticles enhance methylene blue–induced photodynamic therapy: a novel therapeutic approach to inhibit Candida albicans biofilm. *Int. J. Nanomedicine* **7**, 3245–3257 (2012).
50. Li, X., Yan, Z. & Xu, J. Quantitative variation of biofilms among strains in natural populations of Candida albicans. *Microbiology* **149**, 353–362 (2003).
51. ASTM E2647-08. Standard Test Method for Quantification of a Pseudomonas aeruginosa Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. *ASTM Int. West Conshohocken PA* (2008).
52. ASTM E2562-17. Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor. *ASTM Int. West Conshohocken PA* (2017).
53. ASTM E2799-17. Standard Test Method for Testing Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm using the MBEC Assay. *ASTM Int. West Conshohocken PA* (2017).
54. Junka, A. F. *et al.* A.D.A.M. test (Antibiofilm Dressing's Activity Measurement) — Simple method for evaluating anti-biofilm activity of drug-saturated dressings against wound pathogens. *J. Microbiol. Methods* (2017) doi:10.1016/j.mimet.2017.09.014.
55. Zeiger, A. S., Hinton, B. & Van Vliet, K. J. Why the dish makes a difference: Quantitative comparison of polystyrene culture surfaces. *Acta Biomater.* **9**, 7354–7361 (2013).
56. Deutsch, E. W. *et al.* Minimum information specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE). *Nat. Biotechnol.* **26**, 305–312 (2008).
57. Anon. Time for leadership. *Nat. Biotechnol.* **25**, 821 (2007).
58. Xu, K. D., Stewart, P. S., Xia, F., Huang, C.-T. & Mcfeters, G. A. Spatial Physiological Heterogeneity in Pseudomonas aeruginosa Biofilm Is Determined by Oxygen Availability. *APPL Env. MICROBIOL* **64**, 5 (1998).

59. Young, T. *et al.* Candida albicans as an Essential "Keystone" Component within Polymicrobial Oral Biofilm Models? *Microorganisms* **9**, 59 (2021).
60. Jevons, A. L. & Quain, D. E. Draught beer hygiene: use of microplates to assess biofilm formation, growth and removal. *J. Inst. Brew.* **127**, 176–188 (2021).
61. Kadam, S., Madhusoodhanan, V., Bandgar, A. & Kaushik, K. S. From Treatise to Test: Evaluating Traditional Remedies for Anti-Biofilm Potential. *Front. Pharmacol.* **11**, 566334 (2020).
62. Muras, A., Parga, A., Mayer, C. & Otero, A. Use of Quorum Sensing Inhibition Strategies to Control Microfouling. *Mar. Drugs* **19**, 74 (2021).
63. Diepoltová, A., Konečná, K., Janďourek, O. & Nachtigal, P. 2021. Study of the impact of cultivation conditions and peg surface modification on the in vitro biofilm formation of Staphylococcus aureus and Staphylococcus epidermidis in a system analogous to the Calgary biofilm device. *J. Med. Microbiol.* **70**, 001371.
64. Oliveira, V. de C. *et al.* Expression of virulence factors by Pseudomonas aeruginosa biofilm after bacteriophage infection. *Microb. Pathog.* **154**, 104834 (2021).
65. Abusrewil, S. *et al.* Filling the Void: An Optimized Polymicrobial Interkingdom Biofilm Model for Assessing Novel Antimicrobial Agents in Endodontic Infection. *Microorganisms* **8**, 1988 (2020).
66. Pipattanachat, S., Qin, J., Rokaya, D., Thanyasrisung, P. & Srimaneepong, V. Biofilm inhibition and bactericidal activity of NiTi alloy coated with graphene oxide/silver nanoparticles via electrophoretic deposition. *Sci. Rep.* **11**, 14008 (2021).
67. Gomaa, O., Kareem, H. A. E. E. & Selim, N. Nitrate modulation of Bacillus sp. biofilm components: a proposed model for sustainable bioremediation. (2021).
68. Vandana, M., Kadam, S., Bandgar, A. & Kaushik, K. S. Towards 'Ancientbiotics' for Biofilms: How can we bring traditional medicinal remedies out of treatise and into contemporary science? *bioRxiv* (2020).
69. Allkja, J. *et al.* Interlaboratory study for the evaluation of three microtiter plate-based biofilm quantification methods. *Sci. Rep.* **11**, 1–10 (2021).
70. Almblad, H. *et al.* Bacterial cyclic diguanylate signaling networks sense temperature. *Nat. Commun.* **12**, 1–14 (2021).
71. Arias, L. S. *et al.* A nanocarrier system that potentiates the effect of miconazole within different interkingdom biofilms. *J. Oral Microbiol.* **12**, 1771071 (2020).
72. Arias, L. S. *et al.* Chitosan ameliorates Candida auris virulence in a Galleria mellonella infection model. *Antimicrob. Agents Chemother.* **64**, e00476-20 (2020).
73. Coupri, D. *et al.* Inhibition of d-alanylation of teichoic acids overcomes resistance of methicillin-resistant Staphylococcus aureus. *J. Antimicrob. Chemother.* (2021) doi:10.1093/jac/dkab287.

74. Pinto, R. M. *et al.* N-Acetyl-l-cysteine-Loaded Nanosystems as a Promising Therapeutic Approach Toward the Eradication of Pseudomonas aeruginosa Biofilms. *ACS Appl. Mater. Interfaces* (2021) doi:10.1021/acsami.1c05124.
75. Ramírez-Granillo, A. *et al.* Microbial Warfare on Three Fronts: Mixed Biofilm of Aspergillus fumigatus and Staphylococcus aureus on Primary Cultures of Human Limbo-Corneal Fibroblasts. *Front. Cell. Infect. Microbiol.* **11**, 646054 (2021).
76. Dolan, S. K. Current knowledge and future directions in developing strategies to combat Pseudomonas aeruginosa infection. *J. Mol. Biol.* (2020).
77. Azevedo, N. F., Allkja, J. & Goeres, D. M. Biofilms vs. cities and humans vs. aliens – a tale of reproducibility in biofilms. *Trends Microbiol.* (2021) doi:10.1016/j.tim.2021.05.003.
78. Coenye, T. Do results obtained with RNA-sequencing require independent verification? *Biofilm* **3**, (2021).
79. Becerra, B. & Mar, M. D. Microorganismos asociados a periodontitis en sujetos atendidos en la clínica de la Facultad de Odontología de la Pontificia Universidad Javeriana para la formación de un modelo de biopelículas polimicrobianas in vitro. (2021).
80. Lyimo, G. V. Green synthesised Zinc Oxide Nanoparticles and their antifungal effect on Candida albicans Biofilms. (2020).

Chapter 4.

Evaluation of single species biofilm methods

Parts of this chapter are included in the following publication: Allkja, J., van Charante, F., Aizawa, J. *et al.* Interlaboratory study for the evaluation of three microtiter plate-based biofilm quantification methods. Sci Rep 11, 13779 (2021).
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Abstract

Microplate methods are commonly used for biofilm assessment. However, results obtained with these methods have often been difficult to reproduce. Hence, it is important to obtain a better understanding of the repeatability and reproducibility of these methods. An interlaboratory study was performed in five different laboratories to evaluate the reproducibility and responsiveness of three methods to quantify *Staphylococcus aureus* biofilm formation in 96-well microplates: crystal violet, resazurin, and plate counts. An inter-lab protocol was developed for the study. The protocol was separated into three steps: biofilm growth, biofilm challenge, biofilm assessment. For control experiments participants performed the growth and assessment steps only. For treatment experiments, all three steps were performed and the efficacy of sodium hypochlorite (NaOCl) in killing *S. aureus* biofilms was evaluated. In control experiments, on the log-scale, the reproducibility SD was 0.44 for crystal violet, 0.53 for resazurin, and 0.92 for the plate counts. In the treatment experiments, plate counts had the best responsiveness to different levels of efficacy and the best reproducibility with respect to responsiveness (Slope/S_R = 1.02), making it the more reliable method to use in an antimicrobial efficacy test. This study showed that the microplate is a versatile and easy-to-use biofilm reactor, which exhibits good repeatability and reproducibility for different types of assessment methods, if suitable experimental design and statistical analysis are applied.

Keywords

Biofilm, microplate, ring trial, plate count, crystal violet, resazurin

4.1. Introduction

The lack of reproducibility among published studies has become one of the bigger concerns across different scientific research areas and biofilm research is certainly no exception. [1,2] This is due to many different factors, including selective or incomplete reporting of the experiments performed and lack of robust methods. [3,4]

Microplate-based methods are some of the more widely used methods in biofilm research. They are inexpensive, easy to use, versatile and adaptable for high-throughput screening. [5,6] However, little is known about their repeatability (within lab variation) and there are currently no reports on their reproducibility (among lab variation).[7,8] The microplate is a batch system in which the biofilm is grown in a small volume and then assessed using a variety of methods. Most assessment methods rely on the use of dyes (e.g. crystal violet and resazurin) to stain the biofilm or some of its components; colony forming unit (CFU) counts are also used.[9] The variety of assessment methods available has led to many possible applications for microplate-based methods such as antimicrobial compound screening, assessing biofilm forming ability of different microorganisms, and evaluating the effect of various factors on biofilm formation.[10–13]

Considering the widespread use of microplate methods in the field, the evaluation of their reproducibility is of a clear interest to the biofilm community as it would provide an insight into the potential of microplates for standardisation, which could be useful for antimicrobial screening tests.[14] Additionally, it could serve as a starting point for making microplate data comparable across research groups and also help us better understand what factors might affect the performance of microplate experiments.

As outlined in the previous chapter, efforts to improve the reporting of microplate experiments, through the implementation of minimum information guidelines on reporting biofilm experiments (www.miabie.org) have started.[15,16] Moreover, the repeatability and reproducibility of methods using other biofilm reactors have been evaluated, which can serve as a reference for evaluating microplate methods. Devices such as the MBEC, the CDC Biofilm Reactor and drip-flow reactors were assessed through interlaboratory studies, where 6-8 different laboratories perform the same protocol. [17–19] As a result of these tests, several standard methods protocols for the use of these devices in antimicrobial testing have been approved.[20–22] In order for a protocol to be accepted as a standard method, the standard deviation (SD) of the reproducibility needs to be “small”, often determined by standard methods societies, e.g., ASTM.

In the present chapter, we aim to evaluate the reproducibility and responsiveness of three different biofilm assessment methods (crystal violet, resazurin and plate counts) applied to microplates through a ring trial, performed across five different laboratories located in the US and Europe. The study reports on the repeatability in each lab and the reproducibility across different labs of each of these three assessment methods. Additionally, it evaluates the responsiveness and reproducibility of these methods in assessing an antimicrobial efficacy test.

4.2. Materials and Methods

The description provided in this materials and methods section complies with the recently-published guideline on reporting spectrophotometric and fluorometric methods to assess biofilms in microplates (Chapter 3). [16]

4.2.1. Protocol optimisation

To design the inter-lab protocol (ILP) for the ring trial, we used protocols from all the participating labs together with the information acquired during the guideline development process on chapter 3. Our first challenge was deciding on the incubation method for the microplate. As the protocol was optimised in Montana, which has a very dry climate, the outside wells in the plate were drying out completely. Thus, we had to adapt the biofilm growth step to overcome this issue. Furthermore, considering that all participants were in very different climates we had to find the simplest incubation method that could work for all. For this purpose, we decided that shaking the plate would not be a suitable option, as in dry climates it would increase evaporation rates and standardizing the settings on orbital shakers would be difficult to achieve. Additionally, we noticed that if we sealed the plate with parafilm this prevented evaporation. However, due to humidity levels in Montana our plates would create a lot of condensation on the lid, which in turn led to cross contamination. Therefore, we decided to adjust the humidity in the incubator by adding a tray of water at the bottom. This part would only be applied in laboratories with dry climates.

Another challenge was deciding on the plate count protocol. Most of the protocols we received, as well as most of the literature use sonication to detach the biofilm from the wells. We tested the efficacy of sonication by staining the wells pre and post sonication and some of the biofilm was left behind in the wells (Figure S4.1.1). Furthermore, standardizing the settings on all the different sonicator baths used by the trial participants would be very difficult and time consuming. Thus, we decided to test an alternative method

and scrape the biofilm to remove it. We tried different scraping tools such as, a dental pick tool (Figure S4.1.2) and a wooden applicator stick (Figure S4.1.3). The wooden applicator sticks were very effective in removing the biofilm and the plate count test results showed good repeatability within experiment and among experiments (Table S4.1.1). Furthermore, we quantified the Log CFU/well left behind on the wooden applicator sticks post scraping and when we added those results to the plate count there was an average increase of 0.03 Log CFU/well to the final values (Table S4.1.2). Hence, we decided this method was appropriate to use for the study.

Moreover, we needed to consider the variability of plate readers used throughout the study. Each plate reader processes the information differently and will usually give different output values especially for fluorescence intensity readings. This makes comparing the results from different laboratories difficult, thus we decided to create a test for every participant to conduct so we can correlate all the outputs from the different plate readers. The complete optimised ILP and all the protocols collected from the participating labs can be found in Appendix S4.2.

4.2.2. Study design

The ring trial investigated three different biofilm assessment methods in flat bottom, untreated, polystyrene, 96-well microplates: plate counts, resazurin and crystal violet. Six laboratories initially took part and are identified by numerical Lab IDs between 1 and 6. One participant from each laboratory performed the experiments. The participants were provided with the optimised ILP and a list of supplies (Appendix S4.2). ILP was divided into three distinct steps: biofilm growth, biofilm challenge and biofilm assessment. For the latter, separate protocols for each assessment method were provided. Participants were instructed to find closest substitute/match for materials in the supply list (Appendix S4.2) when possible, hence there is a difference in supplier between labs.

The data collected from Lab 5 have been removed from all data analysis. An initial analysis identified their control data as severe outliers. Subsequent inquiries revealed that their treatment experiments data were also unusable due to deviations from performing the protocols and a lack of documentation. Results of control data analysis with Lab 5 data included can be found in Appendix S4.3.

4.2.2.1. Biofilm growth

Staphylococcus aureus subsp. aureus (ATCC 25923) was used for the ring trial. Briefly, stocks were streak plated on Tryptic Soy Agar (TSA) at 37 ± 2 °C for 24 hrs. One or two

colonies were transferred into 15 mL Tryptic Soy Broth (TSB) and incubated at 37 ± 2 °C, 125 rpm, overnight. An aliquot was sub-inoculated in fresh TSB at 37 ± 2 °C, 125 rpm until exponential growth phase [OD = 0.300 (595 nm) or 7.5 ± 0.5 Log CFU/mL] was achieved. This inoculum was diluted to a concentration of 5.5 ± 0.5 Log CFU/mL. 200 µL/well was transferred to a 96-well plate for biofilm formation at 37 ± 2 °C, no shaking for 24 hrs in a humidified incubator. Wells containing only TSB were used as negative controls to check for contamination. The full biofilm growth protocol can be found in Appendix S4.2

4.2.2.2. Plate reader tests

Crystal violet and resazurin require calibration curves were performed by each laboratory. For crystal violet different concentrations of the dye were added to the wells of a microplate (starting from 0.01 g/L) and their absorbance at λ=595 nm was measured. For resazurin, the participants chemically reduced resazurin into resorufin and added different concentrations to a microplate (starting from 5 µg/mL) and measured fluorescence at $\lambda_{excitation}$ = 560 nm; $\lambda_{emission}$ = 590 nm. The detailed protocol can be found in Appendix S4.2.

4.2.2.3. Control experiments

For the control experiments, only the biofilm growth and biofilm assessment parts of the protocol were performed. One 96-well plate was used per assessment method, per experimental day, two experimental days in total. Due to the nature of the methods for plate counts only 15 wells/plate were quantified, whereas for crystal violet and resazurin the entire plate (minus control wells) was assessed. Laboratories with their own in-house protocols (IHP) for the assessment methods prepared an extra plate and performed their own assessment protocol in parallel with the ILP used for the ring trial. For both plates the biofilm was prepared using the ILP biofilm growth protocol. The detailed protocols are found in Appendix S4.2.

4.2.2.4. Treatment experiments

For the treatment experiment, all three steps of the ILP were performed. For the biofilm challenge step, sodium hypochlorite (NaOCl), i.e., bleach, was used to challenge the biofilm. Half of one 96-well plate was used per method, per experimental day, with three experimental days in total. Titration tests were used to measure the total concentration of chlorine (Cl). The antimicrobial was then diluted to four different concentrations of Cl in NaOCl reported as NaOCl concentrations (1000 mg/L, 500 mg/L, 100 mg/L and 10 mg/L) which, were added to the biofilm (200 µl/well) for 10 minutes and neutralised by washing

twice with PBS. Ultra-pure water was added to the controls to account for the effect of the fluid exchange on biofilm removal. All concentrations were tested on the same day, 8 wells per treatment. For plate counts only 3 wells per concentration per plate were quantified, for crystal violet and resazurin 8 wells per concentration per plate were assessed. No IHP treatment data were generated; IHP data were generated only for control experiments. Detailed protocols are found in Appendix S4.2.

4.2.3. Statistical analysis

4.2.3.1. Plate count data treatment

The colonies formed were counted on each plate, averaged across plates, multiplied by the dilution factor 10^d, divided by the drop volume (10 µL), multiplied by the total volume (200 µL) and then converted to a log density (LD) per well using the following equation:

$LD = \log (CFU / well) = \log [(10^d \times (average\ CFU) / 10\ \mu L) \times 200\ \mu L]$

For the treatment experiments when no colonies were present on agar plates, a value of 0.5 CFU/well was assigned to a single plate at the lowest dilution counted in the previous formula. The antimicrobial's effect was quantified as a log reduction (LR). A single LR value was calculated for each chlorine concentration used, for each experimental day. The LR is equal to the mean of the treated log density (LD) for each treatment concentration subtracted from the mean of the control LD.

4.2.3.2. Crystal violet and resazurin data transformation

"Raw" data collected from the plate reader tests were used to generate calibration curves for each method and each lab. Equations were generated for these curves by linear regression analysis and they were used to transform the data. Based on the regression analysis, log fluorescence vs log resorufin was chosen as the calibration curve for the resazurin data as it better fit the initial calibration data and allowed for better prediction for resazurin/resorufin concentrations from the later part of the study that fell outside the range of the calibration curve. This extrapolation occurred because the resazurin was chemically reduced for the calibration phase of the study making it difficult to ensure that the fluorescence values used to generate the calibration curve contained the values measured during the later biofilm experimentation phase. This extrapolation is a potential weakness of our results. Hence, "raw" fluorescence data were transformed to log fluorescence and further to log resorufin concentration (µg/mL) based on the calibration curve equation for each lab.

For crystal violet, absorbance vs crystal violet concentration was chosen as the calibration curve. Hence, "raw" optical density data were transformed to crystal violet concentrations (µg/mL) based on the calibration curve equation for each lab. Although initial experiments were performed at a single lab to estimate the CFUs corresponding to crystal violet concentrations at that lab, each lab did not generate such a calibration curve, so it is not possible to transform the ODs or crystal violet concentrations to CFU densities.

For both methods, negative control values (wells containing TSB only, no bacteria) were not taken into consideration as, there were no negative controls in the plate reader test, so the calibration curves do not account for negative controls. Calibration curve plots and equations can be found in Appendix S4.4.

4.2.3.3. Repeatability and reproducibility assessment

To investigate the repeatability (day-to-day variability) and reproducibility (lab-to-lab variability) for the control data generated by the methods were first log-transformed: LD for plate counts, log resorufin or log crystal violet concentration. A mixed effects ANOVA model was then fit to the log-transformed responses for each of the 3 methods separately. 'Lab ID' and 'day' were assigned as random factors, with 'day' nested within 'Lab ID', meaning the levels of the factor 'day' are different for each lab. The model estimates the variability in the response for three different sources: error (well-to-well), day (day-to-day) and lab (lab-to-lab). The repeatability variance of the method is represented by the error + day variance. The repeatability standard deviation (S_r) is the square root of this variance. The reproducibility variance of the method is represented by the sum of all variance components and the reproducibility SD (S_R) is the square root of this variance.[23] It is common to report the variance components as percentages of the "total" reproducibility variance (see e.g. ASTM E691) calculated by dividing each variance component by the reproducibility variance.

For the treatment experiments, the reproducibility of LRs was estimated for each NaOCl concentration separately by fitting a mixed effects ANOVA to the LRs for each method separately with a random factor for Lab ID.

4.2.3.4. ILP versus IHP comparison

To evaluate the effect that the change of protocol (ILP vs IHP) had on the control data we fit a mixed effects ANOVA to the log responses for all 3 methods where Day and Lab ID were assigned as random factors and protocol was assigned as a fixed factor. The ANOVA was followed by Tukeys test (95% CI) to determine statistical equivalence at 97.5%

confidence for the median responses for ILP and IHP (CFU density for plate counts, resorufin concentration, and crystal violet). For the plate count method the equivalency margin of 0.5 logs was chosen based on previous examples in the literature.[24,25] On the other hand, no previous reference was found for establishing an equivalency margin for resazurin and crystal violet method, so the equivalency margin of 0.5 log was used for these methods as well. When comparing IHP vs ILP for each of the 3 methods, the equivalency margin of 0.5 applied on the log scale means that differences in means of the log-transformed responses up to 0.5 log are negligible and not of practical importance. When antilogging, the conclusion is that differences up to a 70% decrease ($1 - \underline{10^{-0.5}}$) or a 3-fold increase ($\underline{10^{0.5}}$) in the median response for the IHP compared to median response for the ILP are negligible and not of practical importance.

4.2.3.5. Responsiveness assessment

To assess the responsiveness of the methods to NaOCl treatment we constructed dose-response curves using the mean LR concentrations per NaOCl concentration, per day, per lab. A mixed effects ANOVA with a random factor for Lab ID and Log NaOCl as a covariate was fit to the LRs for all concentrations to assess the responsiveness of the method and to assess the reproducibility pooled across all concentrations.

Furthermore, mixed effects ANOVA analyses were performed for each NaOCl concentration separately and the resulting S_R values were fit to a parabola as a function of LR. To enforce our prior belief that the parabola should be concave, the leading term was constrained to be non-positive.[26] This constraint only had to be enforced for crystal violet data, where the leading term was set to zero.

4.2.3.6. Model assessment and Software

The choice to log-transform the responses (CFU density for the plate count data, resorufin concentration for the resazurin data, crystal violet concentration) is crucial. Initially, for control and efficacy responses each of the 3 methods, the log-transform was required to satisfy the ANOVA assumptions of normality and constant variance. Checking these assumptions was based on "normal probability" and "residual vs fits" plots of the residuals. Just as importantly, having all responses on the log-scale made method comparisons straightforward. Because SDs of log responses are invariant to a change in units, here we directly compare the SDs from the different methods. Furthermore, because a difference in log responses such as a LR is unitless, we directly compare slopes and the mean LR values when assessing the responsiveness of the methods.

All statistical assessments were performed using Minitab v.18® [Minitab 18 Statistical Software (2019). State College, PA: Minitab, Inc. (www.minitab.com)] Mixed Effects Models Functionality that allows the user to specify (as described above): the response (control or LR), fixed effects (e.g., ILP vs IHP), random effects (lab and day nested in lab) and covariate (e.g., time). From the full data set supplied via the link in the Supplementary Materials, the Minitab's Mixed Effects Model function, with the inputs just described, could be used to reproduce the results that we present here. Minitab was also used to generate all the figures. Horizontal jitter has been applied to the points in graphs to better visualise overlapping datapoints.

4.3. Results

4.3.1. Control

4.3.1.1. Interlaboratory protocol analysis

To investigate the repeatability and reproducibility of the control experiments for the ILP protocols, the data collected for each lab, across two experimental days, have been included in Figure 4.1. A summary of the results can be found in Table 4.1. From Figure 4.1A and Table 4.1 we can see that most of the variability is due to lab-to-lab differences. Similar to the plate count method, the variability of resazurin method (Figure 4.1B) and crystal violet method (Figure 4.1C) is mostly due to lab-to-lab differences. The trend of the data is similar across all three methods (Figure 4.1), meaning labs that respond lower for one method do so for the other two as well e.g., lab 3 has the lower values on average for all 3 methods

compared to the rest of the labs. Likewise, the labs with higher responses for one method, have higher responses for the other methods as well e.g., lab 6.

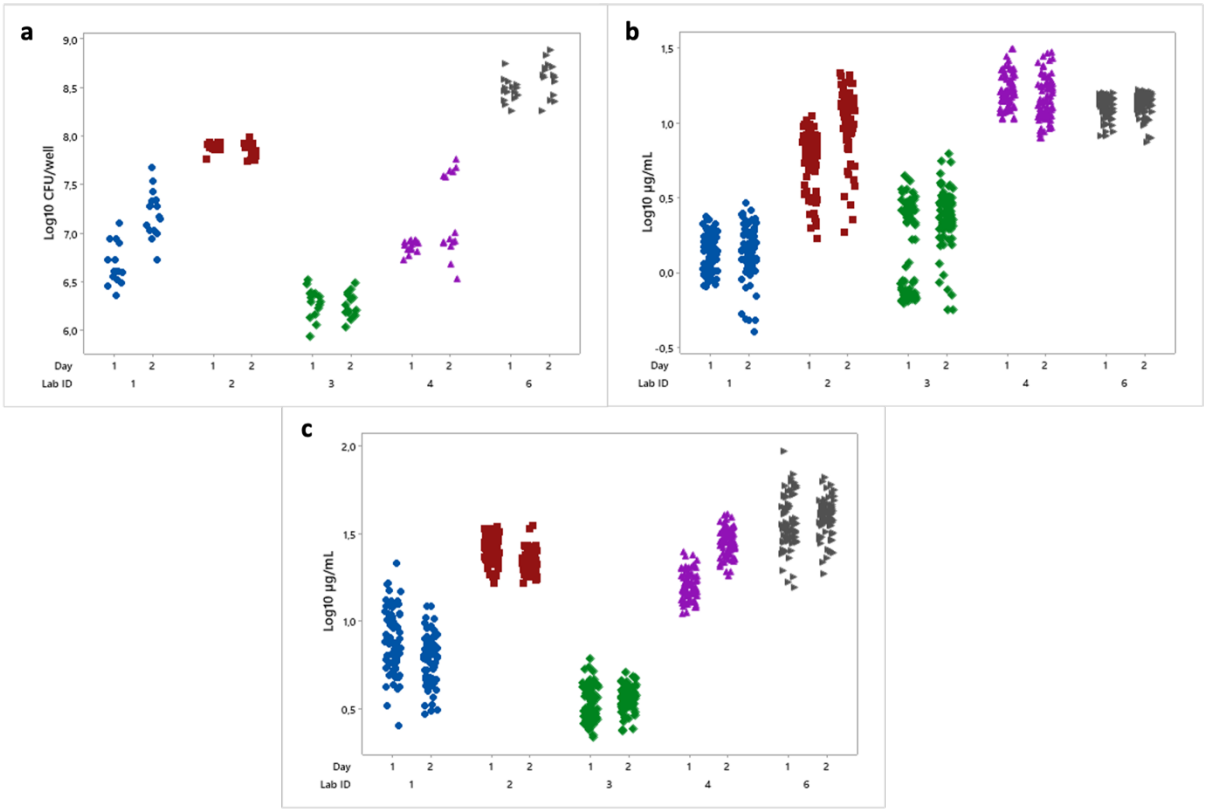


Figure 4.1. Control experiment data for the ILP protocol. Along the horizontal axis are listed the lab IDs and the two experimental days within each lab. Horizontal jitter has been applied to better visualize data points. **A. Plate count** - Each point in the graph is the log density (LD=log (CFU/well)) of biofilm bacteria grown on a single well. **B. Resazurin** - Each point in the graph is the log resorufin concentration (µg/mL) of biofilm bacteria grown on a single well. **C. Crystal violet** - Each point in the graph is the log crystal violet concentration (µg/mL) of biofilm bacteria grown on a single well.

When comparing the repeatability and reproducibility across all three methods based on the SD values in Table 4.1, the crystal violet method is most repeatable and the most reproducible of the three, followed by resazurin and finally the plate count method. As discussed in the Methods, it is possible to directly compare the SDs for the different methods in Table 4.1 because SDs of log responses are invariant to a change in units.

Table 4.1. Summary of statistical analysis for the ILP control data for each method. The table shows the main conclusions (mean Log values ± Standard Error (SE), variance and standard deviations for repeatability and reproducibility) for the control data using the ILP protocols only for all participating labs, excluding lab 5. Recall from the glossary and methods section that the Repeatability SD is the square root of the "Day + Error" variance, and the Reproducibility SD is the square root of the "total variance".

Method	Mean Log response ± SE	Units	Variance components		Standard deviation	
			Day + Error	Lab	Repeatability	Reproducibility
Plate count	7.32 ± 0.40	CFU/well	8.82%	91.18%	0.27	0.92
Resazurin	0.71 ± 0.22	µg/mL	15.89%	84.11%	0.21	0.53
Crystal Violet	1.13 ± 0.19	µg/mL	10.18%	89.82%	0.14	0.44

4.3.1.2. Interlaboratory protocol vs in-house protocol

One of the questions we wanted to answer in the present study was whether the protocol used for the assessment method made a difference in method outcomes. For this purpose, some of the labs, with established in-house protocols (IHP), performed them in parallel to the ILP protocol. For the plate count method only labs 2 and 6 performed IHPs (Figure S4.5.1). For the resazurin method, labs 4 and 6 performed IHPs (Figure S4.5.2) and for the crystal violet method labs 2, 4, and 6 performed IHPs (Figure S4.5.3).

A summary of the statistical comparison of ILP vs IHP for each three methods is given in Table 4.2. For each method, the ILP protocol has a better (smaller) reproducibility S_R. However, the mean LDs, log res and log CV between the ILP and IHP for each of the three methods were statistically significantly equivalent.[27]

Table 4.2. Comparison of the ILP and IHP control data for each method. The table shows the main conclusions (mean Log values ± Standard Error (SE), and standard deviations for repeatability and reproducibility, difference between means, and equivalency testing at 97.5% confidence with an equivalency margin of 0.5 logs) for the control experiments data using the ILP and IHP protocols.

Method	Protocol	Mean Log response ± SE	Units	Standard deviation		ILP - IHP	Result
				Repeatability	Reproducibility		
Plate count	ILP	8.20 ± 0.33	CFU/well	0.13	0.49	0.035	Equivalent
	IHP	8.17 ± 0.42	CFU/well	0.17	0.60		
Resazurin	ILP	1.15 ± 0.04	µg/mL	0.11	0.11	0.105	Equivalent
	IHP	1.05 ± 0.04	µg/mL	0.16	0.16		
Crystal Violet	ILP	1.42 ± 0.08	µg/mL	0.14	0.18	-0.095	Equivalent
	IHP	1.52 ± 0.23	µg/mL	0.19	0.42		

4.3.2. Treatment

The dose response curves with respect to the LRs are shown per lab in Figures S4.5.4, S4.5.5 and S4.5.6. The dose-response curves pooled over all labs are shown in Figure 4.2.

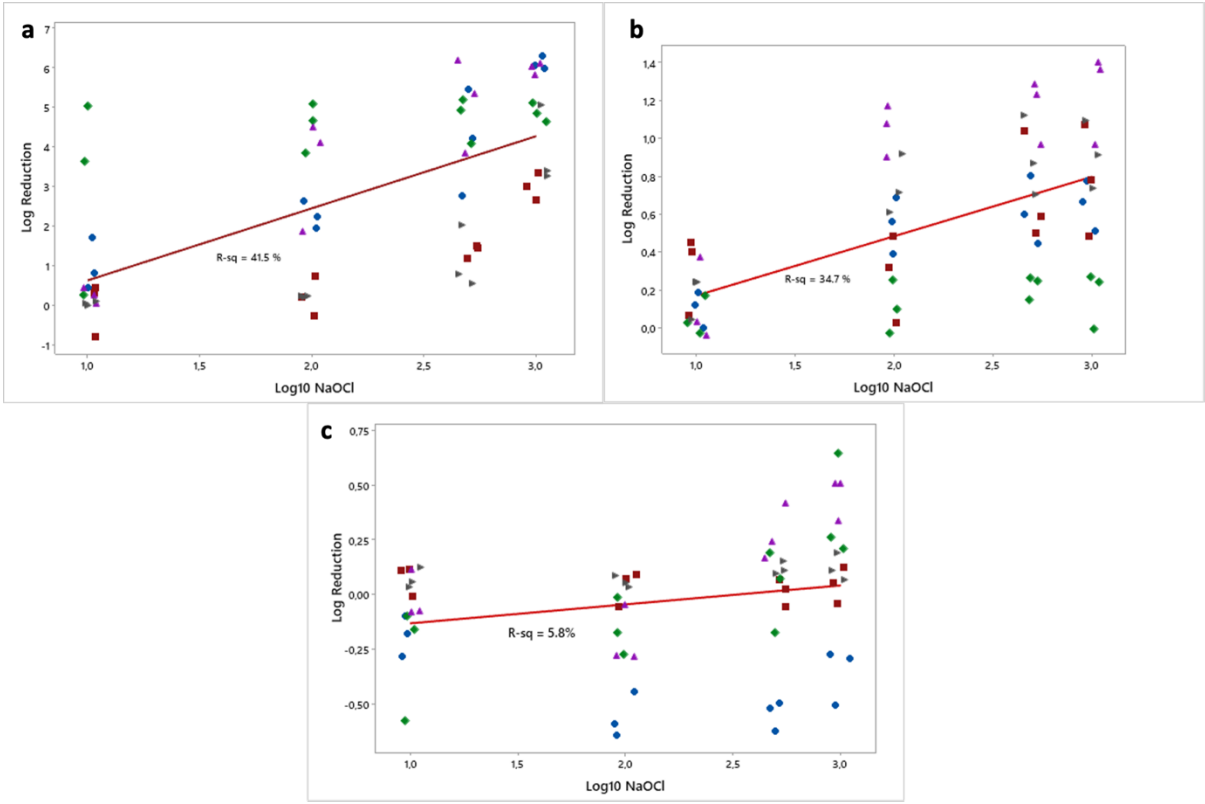


Figure 4.2. Dose response curves for treatment data. Each data point represents the mean LR per experimental day, per lab. The red curve indicates the regression line. **A.** Plate count **B.** Resazurin **C.** Crystal violet

A summary of the analysis is shown in Table 4.3. Based solely on the reproducibility S_R values from the table, crystal violet is the most reproducible method with an S_R = 0.28, followed by resazurin (S_R = 0.36) and plate counts (S_R = 1.79).

Table 4.3 Summary of the treatment data for each method. The table shows the S_R values for the LRs pooled over all concentrations of the NaOCl treatment when applying the ILP protocols only. In addition, the equation of the regression line (shown in Figure 4.2) that quantifies the dose response curve for each method. The slope in each equation quantifies responsiveness of each method, hence the column S_R/ Slope is a measure of the reproducibility of each method relative to the responsiveness.

Method	S_R	Equation	Slope/S_R
Plate count	1.79	LR = -1.196 + 1.823Log NaOCl	1.02
Resazurin	0.36	LR = -1.406 + 0.3132Log NaOCl	0.87
Crystal Violet	0.28	LR = -0.2186 + 0.08669Log NaOCl	0.31

However, the dose-response for the crystal violet method (Figure 4.2C) is quite different compared to the other two. Furthermore, in some instances crystal violet showed negative LR values for the treatment experiments (Figure 4.2C and Figure S4.5.6), which means that the treated biofilms had higher crystal violet concentrations than the side-by-side controls which suggests that there was an issue with the method despite the low S_R value. From the dose response relationship for each method in Table 4.3, the responsiveness of the method was evaluated based on the slope of regression line. The larger the slope, the more responsive the method is. Hence, the plate count method is the most responsive of the three, and crystal violet was the least responsive. As discussed in the Methods, it is possible to directly compare the mean LRs, SDs and slopes for the different methods because LRs, SDs and slopes of log responses are invariant to a change in units.

The reproducibility for all three methods is further illustrated in Figure 4.3. In these graphs the reproducibility SD was plotted as a frown shaped function of the mean LR for each NaOCl concentration for the cumulative data from all 5 labs. This is in contrast to the reproducibility SD presented in Table 4.3 that was calculated by pooling the data over all of the concentration of NaOCl. For the plate count method (Figure 4.3A), a similar relationship has been found for other antimicrobial tests methods and micoorganisms.[26] Figures 4.3B and 4.3C show, for the first time in the literature, that the reproducibility SDs for resazurin and crystal violet appear to have this same frown shaped pattern. This suggests that microbial LRs, whether from CFUs, resazurin or crystal violet, are most reproducible for ineffective and highly effective antimicrobials.

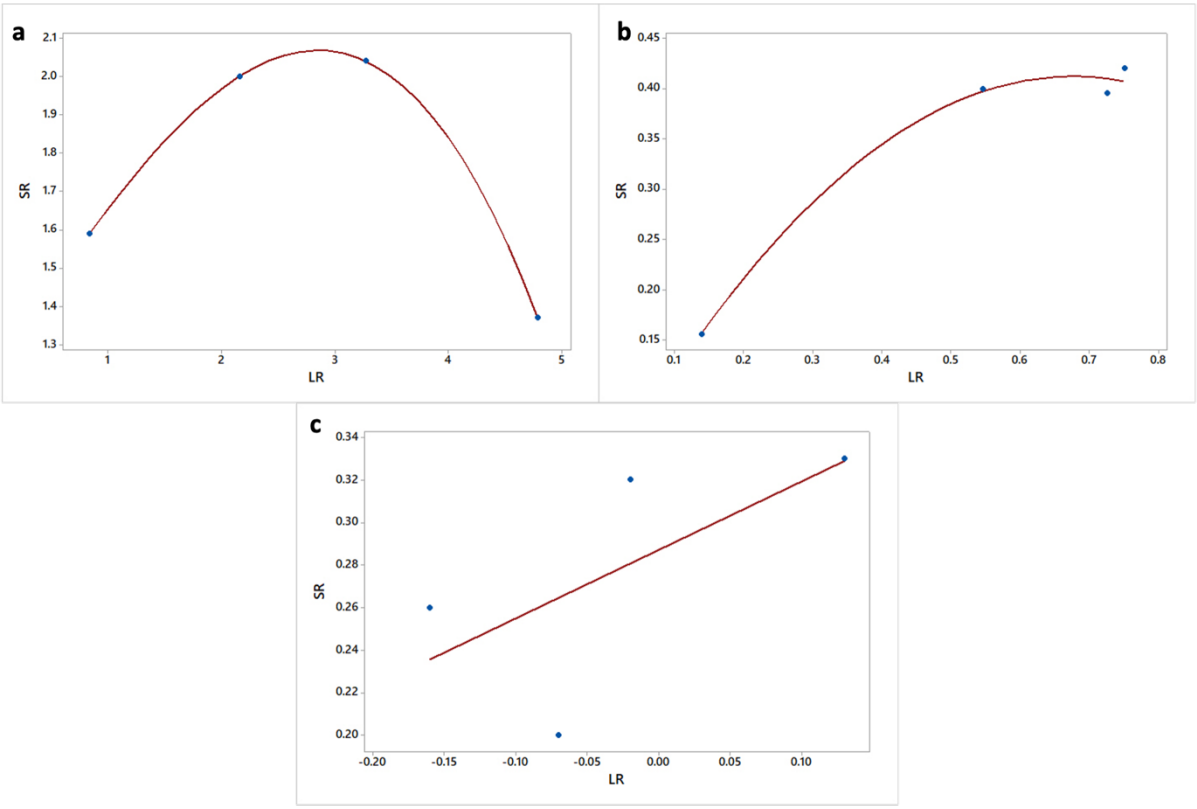


Figure 4.3. Reproducibility curves. Reproducibility SD (S_R) calculated as a function of the mean log reduction (LR) for all 4 concentrations of NaOCl across all 5 labs. **A.** Plate count **B.** Resazurin **C.** Crystal violet

To provide a comparison between the three methods that accounts for both responsiveness and reproducibility, we divided the slope of the dose-response curve by the reproducibility S_R (Slope/S_R), analogous to the signal to noise ratio (and inversely related to the relative standard error and the limit of quantitation) [28]. The higher this unitless value the better the method is at evaluating the effect of different concentrations of treatment. The plate count method performs best, even though it had the worst reproducibility SD, because of the large change in LRs as the concentration of NaOCl was changed (i.e., because of the steep slope). The crystal violet method performs the worst in spite of having the best reproducibility because of poor responsiveness; thus, in our setup it is not suitable for differentiating antimicrobials with different efficacy. A potential reason for this poor responsiveness may be a peculiar interaction noticed between the bleach, the plastic material of the microplate and the crystal violet dye (Figure S4.5.7). In follow up experiments, when bleach at different NaOCl concentrations (2% vol/vol and 0.1% vol/vol) was added to an empty microplate for 10 mins and the plate was subsequently stained with 0.1% vol/vol crystal violet, the stain bound to the wells, despite the lack of biofilm. One reason for this could

be that the bleach is corroding the surface of the wells and creates a rougher surface where the crystal violet can bind. Another possible explanation could be due to the redox reaction between crystal violet and bleach, which results in crystal violet losing colour. If any bleach is left behind following neutralisation, then it might be affecting the crystal violet dye. Hence, when choosing a suitable method, it is important to take into consideration any chemical interactions between the antimicrobial agent, the material used to grow the biofilm and any staining agents used in the assessment method.

Moreover, from the dose-response curves for the plate count method in Figure S4.5.4, it can be seen that for lab 3 the differences between the 4 NaOCl concentrations are not as pronounced. One reason for this difference in response was thought to be the relationship between the starting LD of the biofilm and the resulting LR, as biofilms with a lower starting LD are overall easier to kill by antimicrobial agents. [29] This can be seen in the graphs in Figure S4.5.8, where the Mean LR for each lab was plotted as a function of the mean LD. All 4 graphs show that for labs with lower mean LDs, the mean LR values are also lower. Hence, since lab 3 has overall lower LD values, the biofilm might be easier to kill with lower concentrations of NaOCl and saturation is reached, leading to no difference in LR between different treatments.

4.4. Discussion

Despite the extensive use of microplate methods in the biofilm research, little is known about their comparability, limitations, repeatability, and reproducibility. Due to this lack of information data collected from microplate methods often have little use outside of an individual study, or outside studies performed in the same laboratory. With this interlaboratory study we aimed to answer some of these questions: Is it possible to compare microplate data across different labs? How repeatable and reproducible are crystal violet, resazurin and plate counts in microplate experiments? How does the change in protocol for the assessment step affect reproducibility? How responsive and reproducible are these methods when performing an antimicrobial efficacy test?

First, our results clearly show that comparability between different labs is achievable. For the plate count method, this is very straightforward, however for resazurin and crystal violet extra steps and data transformations are necessary. Due to differences in plate reader outputs, to compare resazurin and crystal violet data across labs it was imperative that each lab calculate their own calibration curves. An attempt was made to compare “raw” data without the use of calibration curves, and the repeatability and reproducibility were

adversely affected [raw data available in Zenodo repository, https://doi.org/10.5281/zenodo.4450073]. The implementation of calibration curves enabled us to report the data as $\log_{10}$ concentration of crystal violet and resorufin, as opposed to the more commonly used optical density (OD) and fluorescence emission data. This is not common practice when using these methods and as such it is not possible to compare any published data from different research groups on these methods even if their protocols are similar or the same. Hence, the way in which scientific community treats and reports such data needs to change.

Once we were able to make the data comparable, we examined how repeatable and reproducible each of these three assessment methods were in microplates. If we focus solely on our study, the control experiment results showed that the crystal violet method is the most repeatable and reproducible method of the three, while plate counts were the least. However, previous studies on the repeatability of microplate methods seem to be in contradiction with our results. In the study by Jorge, *et al*., 2015, they evaluated the repeatability of the crystal violet, plate counts and XTT methods in assessing *Pseudomonas aeruginosa* biofilms. Their results showed issues with repeatability for all three methods, most noticeably for the crystal violet method.[7] Similarly, in the study by Kragh, *et al*., 2019 they report better repeatability for the plate count method when compared to crystal violet for 24 hr biofilms of *P. aeruginosa.* [8] The most probable explanation for these differences could be the way the data were treated. The studies mentioned above do not use calibration curves for the crystal violet data and instead report them as OD values and they also do not log transform their plate count data. These observed differences in repeatability could also be due to differences in the bacterial species used, as our study was performed on *S. aureus* biofilms, not *P. aeruginosa*. Moreover, they could also be attributable to differences in the biofilm growth protocol and assessment methods protocols.

As the plate count method is extensively used in previously validated standard methods, we can compare the reproducibility of our ILP protocol to that observed for other biofilm growing devices.[17,18,22] For example, the MBEC device which has been accepted as a standard method for antimicrobial testing on *P. aeruginosa* biofilms[20] is a modified microplate where the biofilm grows on pegs attached to the microplate lid. As such the environment and growth conditions within the MBEC device are similar to the microplate. For the MBEC device the ring trial results showed a control S_R = 0.67 and Mean Log CFU/mm^2 = 5.48.[17] If we convert our data to Log CFU/ mm^2, the Mean Log CFU/mm^2 = 5.14 and the control S_R remains the same at 0.92. Hence, our control data are not as

reproducible as the MBEC device. Nevertheless, acceptable repeatability and reproducibility SDs for plate counts from controls are < 0.5 and < 0.7, respectively.[30] In our case, the plate count method has very repeatable control data with an SD = 0.27, but less reproducible control data with an S_R = 0.92. No references are available for acceptable SD values for control data from the crystal violet and resazurin methods.

To compare the reproducibility of the LRs between microplates and the MBEC, we can compare the reproducibility curve in Figure 4.3A to the similar frown shaped curve showing reproducibility for the MBEC.[26,29] The reproducibility SD curve in Figure 4.3A is higher than for the MBEC over the same range of LRs, peaking at about S_R= 2 for the microtiter method compared to S_R= 1.5 for the MBEC. This indicates that LRs generated from CFUs from the 96-well microplate method are more variable than the MBEC when testing antimicrobials with a wide range of efficacy. One possible explanation for this could be the difference in the biofilm harvesting protocol. While the MBEC device relies on a mostly automated process by using sonication to remove the biofilm from the pegs, for the microplate the biofilm is removed by scrapping each well individually, which may increase the chance for human error. Moreover, as the ILP protocol did not go through the ruggedness testing that the MBEC device and other standard methods go through, further optimisation could help improve the reproducibility of the method.[17,31] Nonetheless, as these tests were performed for different species of bacteria, it is not certain if this difference in reproducibility is due to the different bacteria or the protocols used or the device itself.

Another question we wanted to explore was whether using a standardised protocol for the assessment method made a difference in results. For this purpose, groups with established in-house protocols performed them in parallel to our ILP. For all three methods, the results showed that the mean response was statistically significantly equivalent between the different protocols but that the reproducibility for the ILP was better (i.e., the S_R values for the ILP were smaller). One interesting detail to consider is that the biofilm growth protocol and the inoculum used was the same for both the ILP and the IHP protocols. This suggests that what contributes the most to variability is how the biofilm is grown and how the inoculum is prepared, rather than how the assessment of the biofilm is performed, an observation that is in agreement with previous research. [32]

Finally, we wanted to evaluate which assessment method would be the most appropriate to use for a treatment efficacy test. For this purpose, we chose an antimicrobial treatment and we evaluated NaOCl contact killing at four different NaOCl concentrations. An initial look at the results seemed to suggest that once again the crystal violet method was the most

reproducible and as such the better method to use. However, when performing treatment efficacy testing, the responsiveness of the method is crucial. Responsiveness indicates the ability of the method to differentiate between different concentrations of the treatment and evaluate it as a dose-response. The plate count method was the most responsive method of the three. Furthermore, when we looked at their reproducibility with respect to responsiveness, the plate count method performed the best out of the three. Unexpectedly, the resazurin method which measures the metabolic activity of the biofilm showed poor responsiveness compared to plate counts. This could be due to a high presence of viable but dormant cells in the treated wells or an unknown interaction of resazurin with the antimicrobial used. Another possibility is that the observed responsiveness of the plate counting method is artificially too high due to a large number of viable but non-culturable survivors on treated carriers.

In conclusion, the microplate is a versatile and easy-to-use biofilm reactor which shows good repeatability and reproducibility for different types of assessment methods. It can also allow for comparison of data between different labs. However, for comparisons across labs to be possible calibration curves are essential, hence there needs to be a change in the way we report microplate experiment data, especially for spectrophotometric and fluorometric type assays. This combined with more detailed reporting based on minimum information guidelines could add more value to results obtained from microplate experiments and open the possibility for comparison across studies and data mining applications

4.5. References

1. Goodman, S. N., Fanelli, D. & Ioannidis, J. P. What does research reproducibility mean? *Sci. Transl. Med.* **8**, 341ps12-341ps12 (2016).
2. Grimes, D. R., Bauch, C. T. & Ioannidis, J. P. Modelling science trustworthiness under publish or perish pressure. *R. Soc. Open Sci.* **5**, 171511 (2018).
3. Begley, C. G. & Ioannidis, J. P. Reproducibility in science: improving the standard for basic and preclinical research. *Circ. Res.* **116**, 116–126 (2015).
4. Baker, M. Reproducibility Crisis? *Nature* **533**, 26 (2016).
5. Peeters, E., Nelis, H. J. & Coenye, T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods* **72**, 157–165 (2008).
6. Sandberg, M., Määttänen, A., Peltonen, J., Vuorela, P. M. & Fallarero, A. Automating a 96-well microtitre plate model for Staphylococcus aureus biofilms: an approach to screening of natural antimicrobial compounds. *Int. J. Antimicrob. Agents* **32**, 233–240 (2008).
7. Jorge, P., Lourenço, A. & Pereira, M. O. Data Quality in Biofilm High-Throughput Routine Analysis: Intralaboratory Protocol Adaptation and Experiment Reproducibility. *J. AOAC Int.* **98**, 1721–1727 (2015).
8. Kragh, K. N., Alhede, M., Kvich, L. & Bjarnsholt, T. Into the well—A close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm* **1**, 100006 (2019).
9. Azeredo, J. *et al.* Critical review on biofilm methods. *Crit. Rev. Microbiol.* **43**, 313–351 (2017).
10. Skogman, M., Kanerva, S., Manner, S., Vuorela, P. & Fallarero, A. Flavones as Quorum Sensing Inhibitors Identified by a Newly Optimized Screening Platform Using Chromobacterium violaceum as Reporter Bacteria. *Molecules* **21**, 1211 (2016).
11. Melo, L. D. R. *et al.* Development of a Phage Cocktail to Control Proteus mirabilis Catheter-associated Urinary Tract Infections. *Front. Microbiol.* **7**, (2016).
12. Moreira, J. M. R. *et al.* The effect of glucose concentration and shaking conditions on Escherichia coli biofilm formation in microtiter plates. *Chem. Eng. Sci.* **94**, 192–199 (2013).
13. Gomes, L. C., Moreira, J. M. R., Simões, M., Melo, L. F. & Mergulhão, F. J. Biofilm localization in the vertical wall of shaking 96-well plates. *Scientifica* **2014**, 231083 (2014).
14. Coenye, T., Goeres, D., Van Bambeke, F. & Bjarnsholt, T. Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? *Clin. Microbiol. Infect.* **24**, 570–572 (2018).
15. Lourenço, A. *et al.* Minimum information about a biofilm experiment (MIABiE): standards for reporting experiments and data on sessile microbial communities living at interfaces. *Pathog. Dis.* **70**, 250–256 (2014).

16. Allkja, J. *et al.* Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates. *Biofilm* **2**, 100010 (2020).
17. Parker, A. E. *et al.* Ruggedness and reproducibility of the MBEC biofilm disinfectant efficacy test. *J. Microbiol. Methods* **102**, 55–64 (2014).
18. Goeres, D. M. *et al.* Development, standardization, and validation of a biofilm efficacy test: The single tube method. *J. Microbiol. Methods* **165**, 105694 (2019).
19. Goeres, D. M. *et al.* Drip flow reactor method exhibits excellent reproducibility based on a 10-laboratory collaborative study. *J. Microbiol. Methods* **174**, 105963 (2020).
20. ASTM E2799-17. Standard Test Method for Testing Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm using the MBEC Assay. *ASTM Int. West Conshohocken PA* (2017).
21. ASTM E2562. Standard Test Method for Evaluating Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method. (2019).
22. ASTM E2647-20. Standard Test Method for Quantification of a Pseudomonas aeruginosa Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. *ASTM Int. West Conshohocken PA* (2020).
23. Hamilton, M. A., Hamilton, G. C., Goeres, D. M. & Parker, A. E. Guidelines for the Statistical Analysis of a Collaborative Study of a Laboratory Method for Testing Disinfectant Product Performance. *J. AOAC Int.* **96**, 1138–1151 (2013).
24. Fritz, B. G., Walker, D. K., Goveia, D. E., Parker, A. E. & Goeres, D. M. Evaluation of Petrifilm™ aerobic count plates as an equivalent alternative to drop plating on R2A agar plates in a biofilm disinfectant efficacy test. *Curr. Microbiol.* **70**, 450–456 (2015).
25. Nelson, M. T., LaBudde, R. A., Tomasino, S. F. & Pines, R. M. Comparison of 3M Petrifilm Aerobic Count Plates to standard plating methodology for use with AOAC antimicrobial efficacy methods 955.14, 955.15, 964.02, and 966.04 as an alternative enumeration procedure: collaborative study. *J. AOAC Int.* **96**, 717–722 (2013).
26. Parker, A. E., Hamilton, M. A. & Goeres, D. M. Reproducibility of antimicrobial test methods. *Sci. Rep.* **8**, (2018).
27. Richter, S. J. & Richter, C. A Method for Determining Equivalence in Industrial Applications. *Qual. Eng.* **14**, 375–380 (2002).
28. Thompson, M. Uncertainty functions, a compact way of summarising or specifying the behaviour of analytical systems. *TrAC Trends Anal. Chem.* **30**, 1168–1175 (2011).
29. Stewart, P. S. Antimicrobial Tolerance in Biofilms. *Microbiol. Spectr.* **3**, (2015).
30. Parker, A. E. & Hamilton, M. A. KSA-SM-10: Assessing Resemblance, Repeatability, and Reproducibility for Quantitative Methods. Center for Biofilm Engineering at Montana State University, Bozeman, MT. (2011).

31. Hamilton, M. A. KSA-SM-03—Testing surface disinfectants: Desirable attributes of a standardized method. (2010).
32. Kragh, K. N. *et al.* The Inoculation Method Could Impact the Outcome of Microbiological Experiments. *Appl. Environ. Microbiol.* **84**, e02264-17 (2018).

Chapter 5.

Evaluation of multispecies biofilm methods

Abstract

Biofilms are often polymicrobial in nature, which can impact their behaviour and overall structure, often resulting in an increase in biomass and enhanced antimicrobial resistance. Microplate-based methods have often been used as a model for the study of CAUTI biofilms, hence we evaluated the repeatability of the plate count method in a microplate multispecies CAUTI model. Four species were selected: *Enterococcus faecalis*, *Escherichia coli, Candida albicans* and *Proteus mirabilis*. Eleven combinations of biofilms were grown on silicone coupons placed in 24-well plates for 24 hrs, 37 ºC, in AUM. The three bacterial species showed overall good repeatability (S_r values 0.1 - 0.54) in all combinations tested, whereas *C. albicans* had higher repeatability SD (S_r) values. To investigate the structure of these biofilms, fluorescence *in situ* hybridisation (FISH) was applied. The results showed that species undetected with the plate count method, could be seen within the biofilm and that despite the antagonistic relationship between the species, they were often co-aggregated. The images also showed that biofilm structures could differ depending on the position on the coupon. The study showed that the plate count method exhibits good repeatability overall, and that more sensitive, visual techniques can provide a qualitative analysis of biofilm structures which complement the plate count data and provide further information on spatial organisation.

Keywords

Biofilm, CAUTI, microplate, repeatability, FISH

5.1. Introduction

Biofilms are often polymicrobial in nature, i.e., more than one species of microorganism is present within the biofilm at the same time.[1] This can impact how the biofilm is formed, its properties, behaviour and overall structure, often resulting in an increase in biomass and enhanced capabilities.[2,3] An example of this, are multispecies biofilms in catheter associated urinary tract infections (CAUTIs), where microorganisms attach to the catheter surface both inside and outside the lumen causing infections in the urethra and bladder, or catheter blockage.[4–6] Through metagenomic and metaproteomic analysis we have been able to understand the composition of these multispecies biofilms. The most commonly found species in the consortia are *Escherichia coli* and *Enterbacter spp.* [7] Other prevalent microorganisms include *Pseudomonas aeruginosa, Klebsiella spp., Proteus spp., Candida spp.* and *Enterococcus spp*.[6,8–10]

Studies using dual or multi-species models of CAUTIs have shown the impact of these consortia in the growth and behaviour of the biofilm, as well as the challenge they can pose to treatment strategies. Polymicrobial interactions have been shown to facilitate biofilm growth, boost its matrix production and enhance virulence factors leading to a more severe form of infection. [11–13] For example, in the study by Tien *et al.* [14], the dual species *Enterococcus faecalis* - *E. coli* CAUTI model showed that the presence of *E. faecalis* increased the virulence of *E. coli* by facilitating tissue colonisation.[14] Additionally, polymicrobial consortia are inherently more resistant to treatment, thanks not only to the increase in biofilm formation which acts as a physical barrier but also the facilitated transfer of resistance genes between microorganisms. [15–18] Hence, the study of CAUTIs in multispecies models is important to ensure a better understanding of the biofilms and instruct more effective treatment strategies against them.

As discussed in previous chapters, the microplate is one of the more commonly used reactors for biofilm studies. Over the years it has also been employed for the study of CAUTI biofilms, where a coupon (generally made of a material commonly used in catheters e.g., silicone), or a piece of catheter tubing is placed inside the wells of the microplate reactor and biofilm formation on the coupon is assessed.[13,15,19–23] Similarly to single species microplate-based methods, the repeatability and reproducibility of multispecies models utilising these reactors are poorly described.

In this chapter we evaluate and report on the repeatability of the plate count method in a microplate system, for multispecies biofilms. As polymicrobial biofilms play a major role

in CAUTIs, we chose this as our model system. Four species were selected based on their recorded prevalence in CAUTIs and their diversity. Gram-positive (*E. faecalis*) and Gram-negative (*E. coli* and *Proteus mirabilis*) bacteria, as well as a yeast (*Candida albicans*) were selected. Per our definitions in chapter 2, methods like plate counts can only reach level 2 reproducibility. Investigating biofilm structure is crucial if we are to achieve the next level of reproducibility. As such, we applied fluorescence *in situ* hybridisation (FISH) in combination with confocal laser scanning microscopy (CLSM) to our biofilms to gain more insight into their spatial organisation and 3D structures.

5.2. Materials and Methods

The descriptions provided in this materials and methods section complies with the recently published guideline on reporting spectrophotometric and fluorometric methods to assess biofilms in microplates (Chapter 3). [24]

5.2.1. Biofilm formation

5.2.1.1. Culture maintenance and inoculum preparation

E. coli CECT 434 and *E. faecalis* CECT 184 were streak plated from -80 ºC glycerol stocks onto Tryptic soy agar (TSA) [Merck, 1.00550 (Broth) and VWR Chemicals USBIA0950 (Agar)] and grown overnight at 37 ºC ± 2 °C. *P. mirabilis* SGSC 3360 was streak plated from -80 ºC glycerol stocks onto CLED agar [VWR Chemicals, 84668] and grown overnight at 37 ºC ± 2 °C. *C. albicans* SC5314 was streak plated from -80 ºC glycerol stocks onto Seaboard's Dextrose Agar (SDA) [VWR Chemicals, 84685] and grown overnight at 37 ºC ± 2 °C.

One or two colonies from each plate were transferred into 15 mL Tryptic Soy Broth (TSB) [Merck, 1.00550] for the bacteria or 15 mL Yeast-extract peptone dextrose (YPD) [Edge BD Difco™, 242820] for the *Candida* and incubated at 37 ± 2 °C, 120 rpm, overnight. An aliquot was sub-inoculated in the respective fresh media at 37 ± 2 °C, 120 rpm until exponential growth phase was achieved [OD = 0.100 (620 nm) or 7.5 ± 0.5 Log CFU/mL for the bacteria], [OD = 1.400 (620 nm) or 7.5 ± 0.5 Log CFU/mL for Candida]. The inocula were centrifuged at 3000 *g* for 10 mins and resuspended in artificial urine medium (AUM) [According to the recipe Brooks and Keevil[25]] twice to ensure the previous media was removed. The resuspended inocula were diluted in AUM to a concentration of 5.5 ± 0.5 Log CFU/mL.

5.2.1.2. Biofilm growth

For the study, 11 different types of combinations were performed, including single species, dual species, three species and four species biofilms. ID designations for each combination can be found in Table 5.1.

Table 5.1. ID designation and description for each combination

Combination ID	Species
C1	*E. coli*
C2	*P. mirabilis*
C3	*E. faecalis*
C4	*C. albicans*
C5	*E. coli and P. mirabilis*
C6	*E. coli and E. faecalis*
C7	*E. coli and C. albicans*
C8	*E. coli and P. mirabilis and E. faecalis*
C9	*E. coli and P. mirabilis and C. albicans*
C10	*E. coli and E. faecalis and C. albicans*
C11	*All 4*

Silicone coupons, 1x1 cm [Neves & Neves Lda, Portugal] were cleaned and sterilised according to the procedure described by Allkja and Azevedo [26]. Briefly, coupons were cleaned for 30 mins with gentle stirring in warm water with detergent, subsequently washed 5x in ultra-pure water, then submerged in 90% ethanol with gentle stirring for 30 mins and finally washed with ultra-pure water and left to air dry. The coupons were then placed at the bottom of the wells in a flat bottom 24-well tissue culture plate [Orange Scientific, 4430300N] and sterilised in a flow chamber under ultraviolet (UV) light for 30 mins.

The inocula prepared at a concentration of 5.5 ± 0.5 Log CFU/mL were used for biofilm formation. From this, 1.5 mL/well were added to the plate, which was parafilmed and incubated at 37 ± 2 °C, no shaking for 24 hrs. In the case of multispecies biofilms, the inocula were mixed in equal parts according to the combinations in Table 5.1 prior to being added to the plate. Six coupons/combination were prepared, and three technical repeats were performed per combination. An additional coupon per experiment was prepared with AUM only to check for sterility. Extra coupons of C3 and C11 were prepared for the FISH method testing.

5.2.2. Plate count method

5.2.2.1. Selective media optimisation

For the mixed biofilms, selective media were created to be able to differentiate the plate counts for each species. In the case of *C. albicans,* 100 µg/mL of ampicillin [AppliChem GmbH, A0839] was incorporated into SDA which prevented bacterial growth for all three species (Figure S5.1.1). The presence of ampicillin did not impact plate count numbers for *C. albicans* (Table S5.1.1). For *E. faecalis* both bacterial and fungal growth needed to be supressed. Kanamycin [Eurobio Scientific, GABKAN00-6Z] at 50 µg/mL in TSA was selected to inhibit bacterial growth as it only affects Gram negative bacteria (Figure S5.1.2). To inhibit *C. albicans*, fluconazole was initially tested but was unsuccessful (Figure S5.1.2B). Amphotericin B [Sigma-Aldrich, A2942] at 10 µg/mL in TSA was tested as an alternative and it successfully inhibited *C. albicans* growth (Figure S5.1.3). The presence of both antibiotic and antifungal compounds did not impact *E. faecalis* growth (Table S5.1.2). *E. coli* and *P. mirabilis* media able to separate them into distinct colonies was necessary as selective inhibition using antibiotics would not be possible. While they grow different colonies in CLED agar when cultured separately, in a mixed culture the colonies were not distinguishable (Figure S5.1.4A). Subsequently, Chromocult® Coliform agar acc.to ISO 9308-1 [Merck, MERC1.10426.0500] was tested and could successfully separate the colonies. *E. coli* grew blue-purple colonies, while *P. mirabilis* grew white-orange colonies (Figure S5.1.4B). Plate counts in coliform agar were compared to the standard media, TSA for *E. coli* and CLED for *P. mirabilis,* and no changes were noticed (Table S5.1.3). The media was also successful in inhibiting E. faecalis and C. albicans growth without the need for antibiotic and antifungal addition (Figure S5.1.4 C and D).

5.2.2.2. Single tube method

The single tube method as described by Goeres *et al* [27] was adapted to harvest the biofilm from the coupons for each combination. Briefly, following 24-hr growth the supernatant was removed from the wells and coupons were washed once in 1.5 mL of 0.85% saline (vol/vol). They were then transferred to a falcon tube containing 10 mL of 0.85% saline (vol/vol). During initial testing it was observed that some *C. albicans* biofilm was left behind on the coupon, so 0.01 % Tween®80 (vol/vol) [Sigma-Aldrich*, P1754*] was added to the 10 mL of 0.85% saline (vol/vol) as a surfactant to ensure complete biofilm removal. The tubes underwent vortex/sonicate cycles of 30 seconds each, 5 steps in total beginning with vortex. The sonicator bath [VWR USC-1700T Ultrasonic cleaner, 142-0101] was degassed prior to use by turning it on for 5 minutes as advised in the STM protocol. The

suspensions were then serially diluted and 100 µL was spread plated (2x plates/sample) onto the respective selective media depending on the combination and species. The plates were incubated for 24 hrs at 37 ºC. Resulting colonies were counted, averaged across plates, multiplied by the dilution factor 10^d, divided by the spread plate volume (0.1 mL) and multiplied by the total volume (10 mL) divided by the coupon surface area (1 cm^2). This was then converted to a log density (LD) Log CFU/cm^2 following the formula (1) below:

(1) $LD = Log\ CFU/cm^2 = log\ [(10^d \times (average\ colonies) / 0.1\ mL) \times (10\ mL/1\ cm^2)]$

In the case of no colony growth, a value of 0.5 per plate was assigned at the lowest dilution counted in the previous formula. This resulted in our limit of detection (LOD) value, 1.699 Log CFU/cm^2.

5.2.3. Fluorescence *in situ* hybridisation

5.2.3.1. Probe testing

Four LNA/2'OMe probes [BioPortugal Lda, PT] targeting specific rRNA sequences on each species were used (Table 5.2). Each probe contained a different fluorochrome attached to it, selected to minimise spectral overlap (Figure S5.2.1). The *E. coli* and *E. faecalis* probes were provided by Andreia S. Azevedo. They were previously used and tested in mixed species cultures [Unpublished work]. The *P. mirabilis* and *C. albicans* probes were designed to match the hybridisation conditions of the existing probes as described in the work by Teixera *et al*. [28] Probe specificity and channel separation was tested in mixed suspensions containing all four species (Figure S5.2.1).

Table 5.2. List of LNA/2'OMe probes

Target	rRNA	Probe LNA/2´OMe (5´-3´)	Base pairs	Tag 5´
E. coli	16S	AcGTcAAtGAgCAaAGg	17	ATTO™ 655
E. faecalis	23S	gTTcTCtGCgTCtACcTC	18	Alexa Fluor™ 594
C. albicans	18S	cCCGcAtATcTacAa	15	Alexa Fluor™ 488
P. mirabilis	16-23S	cGGaTaTcAtCGGg	14	ATTO™ 550
LNA- lower case designation 2'OMe - upper case designation				

5.2.3.2. FISH in biofilms

The FISH in biofilms protocol as described by Allkja and Azevedo [26] was adapted for use in this study. Following 24-hr growth the supernatant was removed from the wells and coupons were washed once in 1.5 mL of 0.85% saline (vol/vol). The biofilm wax fixed and permeabilised by drying for 15 mins at 60 ºC, followed by 15-min immersion in 4 % (vol/vol) paraformaldehyde [Acros Organics, 30525-89-4], then 15 mins in 50 % (vol/vol) Ethanol and finally air drying at room temperature. The fixed biofilms were kept at +4 ºC until hybridisation. For the hybridisation, working solutions of the probes at 200 nM concentration were prepared in hybridisation buffer (0.5 M of urea [Merck, 1.08487], 50 mM Tris-HCl [Fisher BioReagents, BP153], 0.9 M NaCl [VWR Chemicals, 27808.297]; pH 7.5). In the case of multispecies biofilms, working solutions of probe mixtures at 200 nM according to the specific combination were prepared. 25 µL of the corresponding working solution was added to each coupon, covered with a coverslip, and incubated in a humidified container, at 60 ºC for 90 mins, in the dark. The coverslip was removed, and the coupons were immersed in pre-warmed wash buffer (5 mM Tris Base [Fisher BioReagents, BP152], 15 mM NaCl [VWR Chemicals, 27808.297] and 1% Triton X [Panreac, A9778]; pH 10) for 30 mins at hybridisation temperature. A negative control sample was performed containing only hybridisation buffer with no probe, to check autofluorescence (Figure S5.3.1). Moreover, DAPI staining was performed to verify that the probes were penetrating inside of the biofilm (Figure S5.3.2). For this, a drop of DAPI [Sigma-Aldrich, 165344] solution at 0.1 mg/mL was added to the coupon for 10 min in the dark, at room temperature following hybridisation, prior to washing. The coupons were left to air dry and transferred onto a 35 mm imaging dish [ibidi®, 81151] containing glycerol based mounting medium [ibidi®, 50001] ready for microscopy. Three biological and three technical replicated were performed per combination. Samples were stored for a maximum of 4 hrs at +4 ºC, prior to imaging.

5.2.3.3. Confocal Laser Scanning Microscopy

A Laser Scanning Confocal Leica SP5 microscope was used for image acquisition. Biofilms were observed under a 63x/1.30 glycerol immersion objective. Acquisition mode: XYZ; image format: 1024 x 1024 pixels; zoom factor of 1 or 2.5 or 4; pinhole size:1 Airy unit; line average: 6; Z-step of 1 μm. Five Z-stack images were acquired per coupon, per zoom factor (1 and 2.5). Sequential scanning was used for mixed species samples. Sequence one: ALEXA Fluor™488 (PMT detector, 488 nm laser,

emission range 498-553 nm) and ATTO™ 655 (PMT detector, 633 nm laser, emission range 665-741 nm) were detected. Sequence two: ATTO™ 550 (PMT detector, 561 nm laser, emission range 570-601 nm) and Alexa Fluor™ 594 (HyD detector, 561 nm laser, emission range 611-656 nm) were detected. The positions within the coupon were randomly selected to avoid bias. 3D projections and orthogonal views were generated using Imaris Viewer 9.7.2 [Bitplane AG] and LEICA Application Suite X (LASX).

5.2.4. Statistical analysis

To assess the repeatability of the plate count method for each species per combination, the LD data from all combination for each species was fit to a One-way ANOVA model comparing LD vs combination. This generated the Mean LDs and repeatability standard deviations (S_r) for each combination. Additionally, to investigate the variance components affecting the repeatability within each combination, the LD data was fit to a One-way ANOVA model comparing LD vs Day. The ANOVA was followed by Tukeys test (95% CI) to determine statistical equivalence at 97.5% confidence for the mean LDs between experimental days. The equivalency margin of 0.5 logs was chosen based on previous examples in the literature.[29,30] All statistical assessments were performed using Minitab v20® [Minitab 20.3 Statistical Software (2021). State College, PA: Minitab, Inc. (www.minitab.com)]. Graphs were generated using GraphPad Prism v9® [GraphPad Software, LLC Version 9.2.0 (2021)].

5.3. Results

5.3.1. Repeatability assessment of the plate count method

To investigate the repeatability of the plate count method for each species, per combination, the data collected across three experimental days, have been included in Figure 5.1. A summary of the statistical analysis results can be found in Table 5.3. Combinations IDs can be found in Table 5.1.

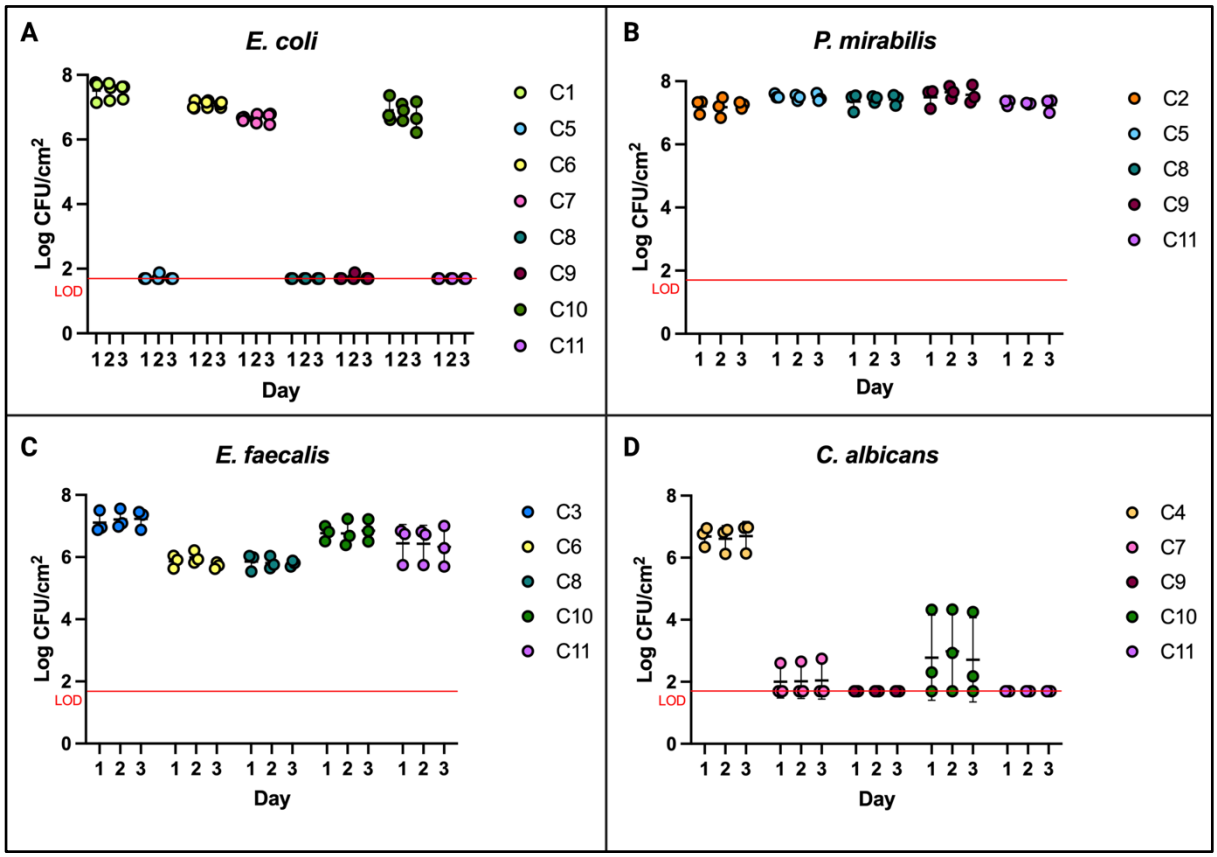


Figure 5.1. Plate count method data for each species per combination. Along the horizontal axis are listed the three experimental days within each combination. Each point in the graph is the log density (Log CFU/cm^2) on a single coupon. Error bars (Mean LD ± SD) have been plotted for each experimental day. LOD (1.699 Log CFU/cm^2) represented by the red line. **A.** *E. coli* **B.** *P. mirabilis* **C.** *E. faecalis* **D.** *C. albicans*

Table 5.3. Summary of statistical analysis for the plate count method for each species per combination. The table shows the main conclusions (Mean Log CFU/cm^2, S_r, variance components and equivalency of means testing at 97.5% confidence with an equivalency margin of 0.5 logs) for each species by combination.

Species	Combination	Mean Log CFU/cm^2	S_r	Equivalence of means*	Variance components	
					Day	Within plate
E. coli	C1	7.517	0.25	Yes	94.74%	5.26%
	C5	1.719	0.06	Yes	25.00%	75.00%
	C6	7.085	0.10	Yes	67.28%	32.72%
	C7	6.653	0.12	Yes	74.22%	25.78%
	C8	6.816	0.35	No	83.22%	16.78%
	C9	1.719	0.06	Yes	25.00%	75.00%
	C10	1.699	0	Yes	0%	0%
	C11	1.699	0	Yes	0%	0%
P. mirabilis	C2	7.209	0.20	Yes	60.57%	39.43%
	C5	7.497	0.09	Yes	73.48%	26.52%
	C8	7.405	0.18	Yes	52.37%	47.63%
	C9	7.570	0.24	Yes	78.78%	21.22%
	C11	7.287	0.12	Yes	24.73%	75.27%
E. faecalis	C3	7.184	0.28	No	83.87%	16.13%
	C6	5.859	0.19	Yes	48.62%	51.38%
	C8	5.859	0.18	Yes	43.38%	56.62%
	C10	6.797	0.31	No	92.32%	7.68%
	C11	6.400	0.54	No	93.42%	6.58%
C. albicans	C4	6.664	0.36	No	94.73%	5.27%
	C7	2.021	0.48	No	99.48%	0.52%
	C9	1.699	0	Yes	0%	0%
	C10	2.823	1.18	No	89.02%	10.98%
	C11	1.699	0	Yes	0%	0%

From Figure 5.1 and Table 5.3 we can see that the plate count method shows overall good repeatability for the three bacterial species in all the different combinations tested. Higher S_r values were observed for *C. albicans* overall compared to the bacteria, apart from combinations C9 and C11 where its growth was below the LOD, resulting in an S_r value of 0. The latter was also observed for *E. coli* in combinations C10 and C11. For *P. mirabilis* we can see that plate counts are not affected by the combination and the S_r values are low for all combinations. Whereas, if we look at the other three, we can see that the Log CFU/cm^2 values differ depending on the biofilm combination. In the case of *E. faecalis*, for the four species biofilms the repeatability is also affected. Moreover, we can see that in the majority of cases, the variability is mostly due to day-to-day sources rather than within

plate ones. We also evaluated the difference in means for each combination using a Tukeys test and overwhelmingly the means were statistically significantly equivalent.[7]

From the data, we can infer that *P. mirabilis* and *E. coli* have an antagonistic relationship where *P. mirabilis* inhibits *E. coli* growth within the biofilm. A similar relationship can be observed for *C. albicans* and *E. coli*, where the latter is more dominant. On the other hand, *E. faecalis* appears to have a mutualistic relationship as it grows well in all the different combinations, although overall Log CFU/cm^2 values are decreased in comparison its single species biofilm.

5.3.2. Application of FISH for the assessment of multispecies biofilms

5.3.2.1. Application of FISH in multispecies biofilms

To further investigate the biofilms for the 11 combinations tested, the FISH method was applied. Figure 5.2 illustrates the spatial organisation of one representative Z-stack collected from each combination.

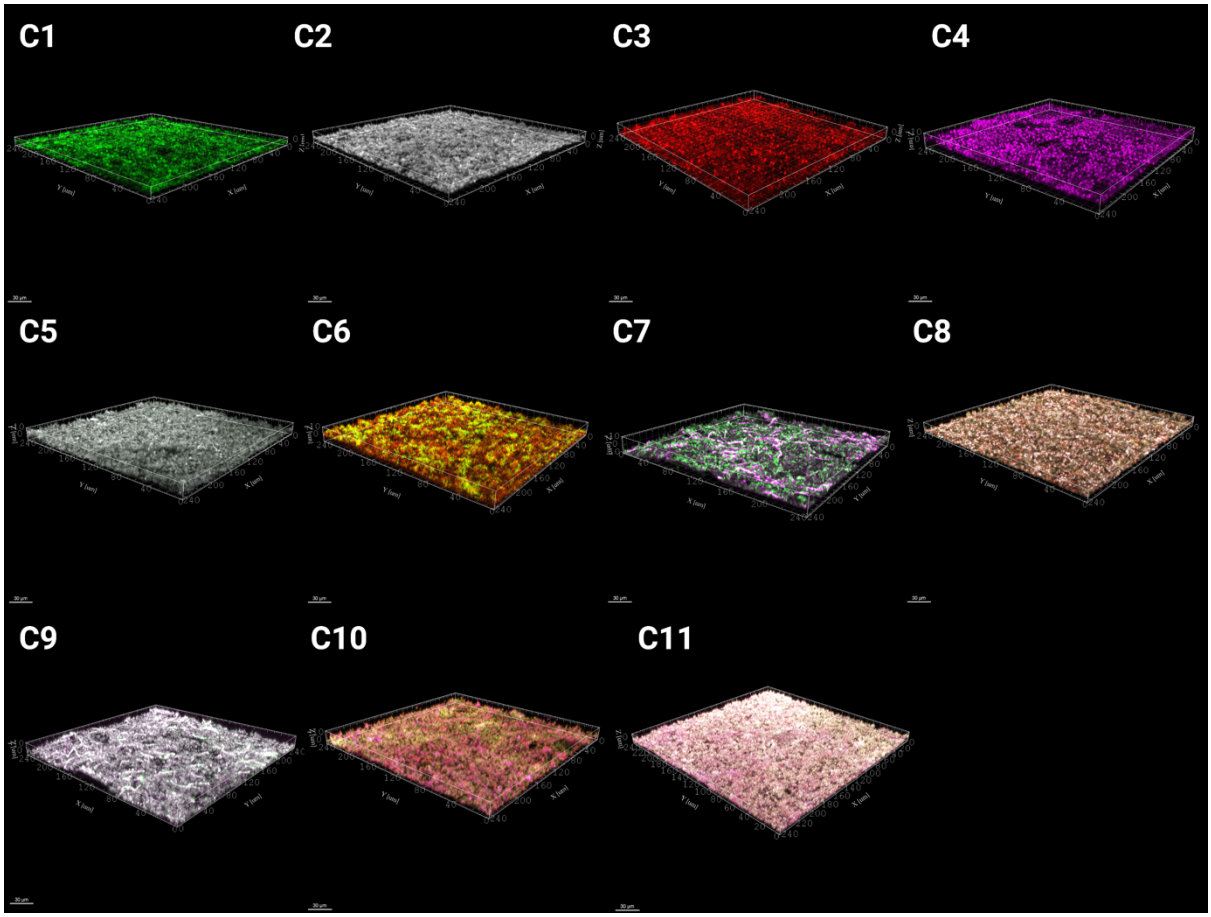


Figure 5.2. Spatial organization of biofilms per combination. Each species was tagged with a specific colour: *E. coli* (green), *P. mirabilis* (white), *E. faecalis* (red) and *C. albicans* (purple). Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. 3D projections generated with IMARIS viewer 9.7.2.

We can see that for single species biofilms (Figure 5.2 C1-4), the biofilms appear to have similar structures, in terms of heights and density. In the case of *C. albicans* (C4) biofilms, an interesting trend was observed during imaging. In the days with lower plate count values, more hyphae formation could be seen in the biofilm (Figure S5.3.3). For the dual-species biofilms (Figure 5.2 C5-7) we can see that the species are well mixed within the biofilm. For C5 this correlates to the plate count data as *P. mirabilis* is the dominant

species, however unlike the plate count method, with FISH we can detect a few *E. coli* cells dispersed within the biofilm (Figure S5.3.4). The C6 biofilm projection also correlates to the plate count data, as both species are abundant within the biofilm and appear well mixed in co-aggregated structures typical of a cooperative relationship. On the other hand, in the case of C7, we can see a lot more *C. albicans* presence within the biofilm than what the plate counts suggested. The species are also co-aggregated which is not typical for an antagonistic relationship.[32] Similar observations can be made for the three species and four species biofilms as well. These interactions are also reflected in the orthogonal view projections of the XY, XZ and YZ planes for each biofilm in Figure S5.3.5. The 3D structures also show an increase in biofilm density as the number of species increases.

5.3.2.2. Repeatability of biofilm architecture

As we saw in the case of *C. albicans*, the same biofilm can form very different structures depending on the experimental day. We wanted to investigate if we could see obvious structural differences between images acquired from the same coupon. Figure 5.3 below shows 3D projections and orthogonal views of C9 biofilm taken from 5 different locations within the coupon.

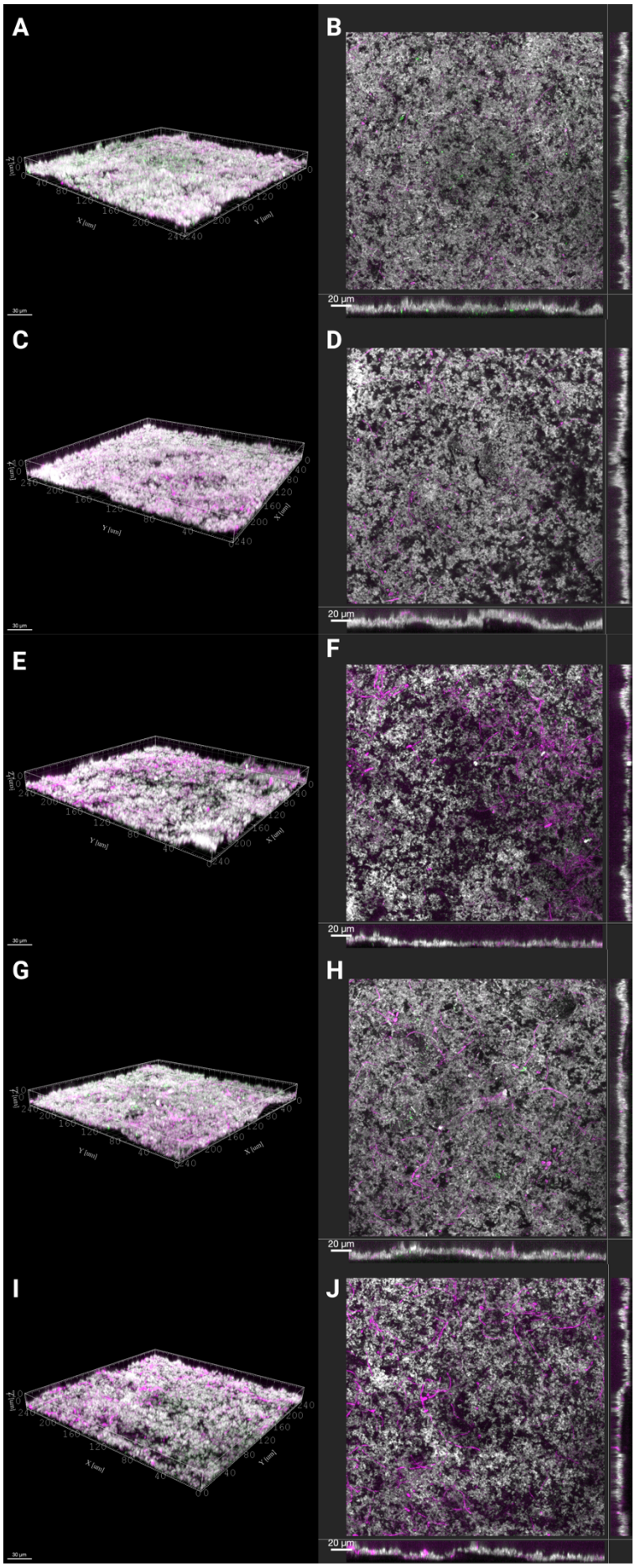


Figure 5.3. Repeatability of the structural organisation of the C9 biofilm within one coupon. A. 3D projection-position 1. **B.** Orthogonal views-position 1. **C.** 3D projection-position 2. **D.** Orthogonal views-position 2. **E.** 3D projection-position 3. **F.** Orthogonal views-position 3. **G.** 3D projection-position 4. **H.** Orthogonal views-position 4. **I.** 3D projection-position 5. **J.** Orthogonal views-position 5. *E. coli* (green), *P. mirabilis* (white), and *C. albicans* (purple). Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. 3D and orthogonal view projections generated with IMARIS viewer 9.7.2.

We can see that biofilm structure and species abundance differ within the coupon. For example, in positions 1, 2 and 4 (Figure 5.3A-D and G-H) there are few *C. albicans* cells present, while in positions 3 and 5 they are more abundant (Figure 5.3E-F and I-J). Another difference is the presence of noticeable peaks, e.g., position 1 (Figure 5.3A-B) appears flatter compared to position 2 (Figure 5.3C-D).

5.3.2.3. Proof of concept for dye separation multispecies biofilm samples

During the imaging we also observed some issues with autofluorescence (Figure S5.3.1), especially with regards to *C. albicans* which would appear on all four channels (Figure S5.3.6). Upon further investigation, we found that the *E. coli* probe could weakly bind to the *Candida* cells as some of the probe sequence could recognise them. This meant that *Candida* cells would often appear white on the merged images. Moreover, due to the closeness in spectrum between *E. faecalis* and *P. mirabilis,* they were not always easily distinguishable from one another. We attempted to correct these issues by using the manual dye separation tool in the LASX suite (Figure 5.4 and Figure S5.3.7).

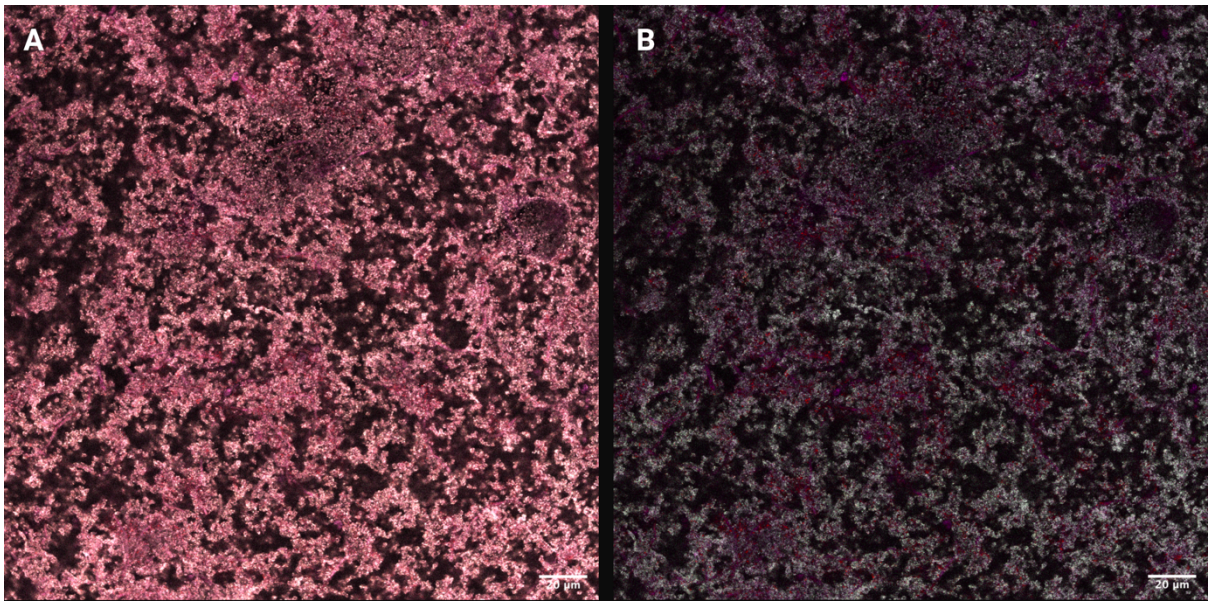


Figure 5.4. Manual dye separation of C11 biofilm staining. A. Raw image **B.** Dye separated image. Each species was tagged with a specific colour: *E. coli* (green), *P. mirabilis* (white), *E. faecalis* (red) and *C. albicans* (purple). Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. Images generated with LEICA Application Suite X (LASX).

The tool allows you to select your region of interest (ROI) for each species and it calculates the florescence intensity values per channel, for the selected regions. This generates a

reference matrix that can then separate the dyes based on the values in the matrix, using them as a threshold for exclusion. We performed this separation as proof of concept on the C11 biofilm. As it can be seen in Figure 5.4A, it is very difficult to distinguish between the four different species in the original image, however in the dye separated image (Figure 5.4B) we can clearly distinguish the *E. faecalis* cells (red), *P. mirabilis* (white) and *C. albicans* (purple) cells from one another. There were no *E. coli* cells present in this region, but they can be seen in Figure S5.3.7.

5.4. Discussion

In recent years, the importance of studying multispecies biofilms has become increasingly evident.[2] Microplate reactors are simple and easy to use, hence they can serve as a quick way to investigate interactions in simple multispecies models. They can also be adapted to create basic models to study biofilm formation on various materials e.g., silicone from catheters. With this study we wanted to evaluate the repeatability of the plate count method in a microplate based CAUTI model for four different species of microorganisms. Eleven different combinations of biofilms were tested, ranging from single to four species. This would allow us to investigate differences in repeatability for each species when increasing biofilm diversity. *E. coli* was used in all eleven combinations as it is the most commonly associated species with CAUTI. [7]

Our results showed good repeatability for all three bacterial species in all combinations. The recorded S_r values were comparable to the one for the plate count method control data in the interlaboratory study performed in chapter 4 (S_r=0.27). In contrast, the repeatability was not as good for *C. albicans*, with the highest S_r value being 1.18 for the C10 biofilm. From the plate count data, we can also see that the mean Log CFU/cm^2 values for *P. mirabilis* do not differ much between combinations, less than 0.5 log difference between the lowest and highest values. On the other hand, Log CFU/cm^2 values differed a lot depending on the combination, and for certain combinations the S_r values were higher e.g., *E. faecalis* in C11 and *E. coli* in C8.

These differences could be due to the interactions between the different species. *P. mirabilis* appears to be the more dominant species of the four. It inhibits the growth of *E. coli* and potentially *C. albicans* to below the LOD. The inhibitory effect of *P. mirabilis* on *C. albicans* has been previously observed in dual species biofilm models. [33] On the other hand, a co-culture study of *P. mirabilis* and *E. coli in vivo* showed an increase in biofilm formation by both species.[34] However, *in vitro* studies of *P. mirabilis* and urease negative

bacteria such as, *Klebsiella pneumoniae*, have shown that *P. mirabilis* inhibits the growth of these bacteria.[35] Considering that *E. coli* is also urease negative, a similar interaction might be occurring in this scenario as well. Another antagonistic interaction observed is the inhibitory effect of *E. coli* on *C. albicans* which has not been previously studied in a urinary catheter system. *E. faecalis* appears to have a more cooperative relationship with both *E. coli* and *P. mirabilis*, yet in C8 and C11 its presence does not affect the inhibition of *E. coli* by *P. mirabilis*.

While plate count data can provide information on the composition of the biofilm and overall abundance of each species within the biofilm, it cannot provide any information on the architecture of the biofilm or the spatial arrangement of the different species. For this purpose, the FISH method was used in combination with CLSM on all 11 biofilm combinations. As previously reported in the literature, the FISH method is more sensitive compared to traditional culture methods.[36,37] This was reflected in our results, as *E. coli* and *C. albicans* presence was observed in combinations with undetectable plate counts. The images also showed that despite the antagonistic relationship inferred by the plate count values, the species were co-aggregated which is not a typical presentation.[32] Additionally, the 3D projections indicate an increase in biofilm density as the number of species within the biofilm increases, which is consistent with polymicrobial biofilm descriptions in the literature.[38]

As the main objective of this study is to evaluate repeatability, we wanted to see what insights the biofilm images could offer on the plate count repeatability. Interestingly, we could see that for single species *C. albicans* biofilms the day-to-day variability might have been in part due to hyphae formation (Figure S5.3.3). In days with lower Log CFU/cm^2 values, hyphae formation was increased. In vitro studies of *C. albicans* CAUTI biofilms have shown that growth in urine media promotes hyphae formation.[39] In our study, we could see that this was not consistent from day-to-day. Since, microscopy allowed us to image different parts of the coupon we could also see that biofilm structures were different in different locations (Figure 5.3). This means that using microscopy we could potentially assess the intra-coupon repeatability for biofilm structures, which could help us understand how to get closer to level 3 reproducibility as defined in chapter 2. In figure 5.3 we could also see that the abundance of different species would vary depending on location. This means that if we were to use microscopy to quantify cell numbers, our results would be dependent on where the images were acquired, and the number of images acquired.

During the imaging we also observed some issues with regards to *C. albicans* which would appear on all four channels (Figure S5.3.6), and experienced difficulties distinguishing *E. faecalis* and *P. mirabilis* from one another due to the closeness in spectrum between their respective fluorochromes (Figure S5.2.1). These issues were not observed in the experiments performed in suspension (Figure S5.2.2) which suggests that the nature of the LNA/2'OMe probes might be a contributing factor. As the probes are negatively charged, their diffusion could be affected by the negatively charged biofilm matrix and lead to binding issues.[15,40] While this might not impact our ability to collect qualitative data from the biofilms, if we were to perform any quantification on the images it would impact the results. To correct these issues, we performed manual dye separation using the tool available in the LASX suite. We were able to successfully separate the dyes and could easily distinguish between the different species in a C11 biofilm. However, when the process was attempted using the automatic dye separation options in the LASX suite, it could not correctly separate the dyes [Data not shown]. This means that we would need to perform the dye separation process manually for each stack, which would be a very long and time-consuming process. Hence, to be able to quantify species specific parameters for the biofilm, a reliable automated dye separation tool, would need to be developed.

In conclusion, the microplate-based plate count method shows overall good repeatability for all the species tested, apart from *C. albicans*. It is a quick method for quantifying species abundance in multispecies biofilms, but it offers no information on the spatial distribution of these species and the overall structural organisation of the biofilm. The use of FISH and microscopy provided insights into the structural and organisational repeatability of our biofilm experiments. In the future, these techniques could help get us closer to level 3 reproducibility

5.5. References

1. Nadell, C. D., Drescher, K. & Foster, K. R. Spatial structure, cooperation and competition in biofilms. *Nat. Rev. Microbiol.* **14**, 589 (2016).
2. Burmølle, M., Ren, D., Bjarnsholt, T. & Sørensen, S. J. Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* **22**, 84–91 (2014).
3. Tan, C. H., Lee, K. W. K., Burmølle, M., Kjelleberg, S. & Rice, S. A. All together now: experimental multispecies biofilm model systems. *Environ. Microbiol.* **19**, 42–53 (2017).
4. Azevedo, A. S., Almeida, C., Melo, L. F. & Azevedo, N. F. Impact of polymicrobial biofilms in catheter-associated urinary tract infections. *Crit. Rev. Microbiol.* **43**, 423–439 (2017).
5. Chua, R. Y. R., Lim, K., Leong, S. S. J., Tambyah, P. A. & Ho, B. An in-vitro urinary catheterization model that approximates clinical conditions for evaluation of innovations to prevent catheter-associated urinary tract infections. *J. Hosp. Infect.* **97**, 66–73 (2017).
6. Flores-Mireles, A., Hreha, T. N. & Hunstad, D. A. Pathophysiology, Treatment, and Prevention of Catheter-Associated Urinary Tract Infection. *Top. Spinal Cord Inj. Rehabil.* **25**, 228–240 (2019).
7. Choe, H.-S. *et al.* Analysis of the distribution of bacteria within urinary catheter biofilms using four different molecular techniques. *Am. J. Infect. Control* **40**, e249–e254 (2012).
8. Norsworthy, A. N. & Pearson, M. M. From Catheter to Kidney Stone: The Uropathogenic Lifestyle of Proteus mirabilis. *Trends Microbiol.* **25**, 304–315 (2017).
9. Dworniczek, E. *et al.* Virulence factors and in Vitro adherence of Enterococcus strains to urinary catheters. *Folia Microbiol. (Praha)* **48**, 671–678 (2003).
10. Werneburg, G. T. *et al.* The Natural History and Composition of Urinary Catheter Biofilms: Early Uropathogen Colonization with Intraluminal and Distal Predominance. *J. Urol.* (2020) doi:10.1097/JU.0000000000000492.
11. Armbruster, C. E. *et al.* The Pathogenic Potential of Proteus mirabilis Is Enhanced by Other Uropathogens during Polymicrobial Urinary Tract Infection. *Infect. Immun.* **85**, e00808-16.
12. Flores-Mireles, A. L., Walker, J. N., Caparon, M. & Hultgren, S. J. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nat. Rev. Microbiol.* **13**, 269–284 (2015).
13. Cerqueira, L., Oliveira, J. A., Nicolau, A., Azevedo, N. F. & Vieira, M. J. Biofilm formation with mixed cultures of Pseudomonas aeruginosa/Escherichia coli on silicone using artificial urine to mimic urinary catheters. *Biofouling* **29**, 829–840 (2013).
14. Tien, B. Y. Q. *et al.* Enterococcus faecalis Promotes Innate Immune Suppression and Polymicrobial Catheter-Associated Urinary Tract Infection. *Infect. Immun.* **85**, e00378-17.

15. Azevedo, A. S., Almeida, C., Pereira, B., Melo, L. F. & Azevedo, N. F. Impact of Delftia tsuruhatensis and Achromobacter xylosoxidans on Escherichia coli dual-species biofilms treated with antibiotic agents. *Biofouling* **32**, 227–241 (2016).
16. Wang, J., Foxman, B., Mody, L. & Snitkin, E. S. Network of microbial and antibiotic interactions drive colonization and infection with multidrug-resistant organisms. *Proc. Natl. Acad. Sci.* **114**, 10467–10472 (2017).
17. Burmolle, M. *et al.* Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. *Appl. Environ. Microbiol.* **72**, 3916–3923 (2006).
18. Beaudoin, T. *et al.* Staphylococcus aureus interaction with Pseudomonas aeruginosa biofilm enhances tobramycin resistance. *Npj Biofilms Microbiomes* **3**, 25 (2017).
19. Azevedo, A. S. *et al.* Detection and discrimination of biofilm populations using locked nucleic acid/2'-O-methyl-RNA fluorescence in situ hybridization (LNA/2'OMe-FISH). *Biochem. Eng. J.* **104**, 64–73 (2015).
20. Pierce, C. G. *et al.* A novel small molecule inhibitor of Candida albicans biofilm formation, filamentation and virulence with low potential for the development of resistance. *Npj Biofilms Microbiomes* **1**, 1–8 (2015).
21. White, A. N., Learman, B. S., Brauer, A. L. & Armbruster, C. E. *Catalase Activity is Critical for Proteus mirabilis Biofilm Development, EPS Composition, and Dissemination During Catheter-Associated Urinary Tract Infection*. 2021.03.22.436542 https://www.biorxiv.org/content/10.1101/2021.03.22.436542v1 (2021) doi:10.1101/2021.03.22.436542.
22. Carvalho, F. M., Teixeira-Santos, R., Mergulhão, F. J. M. & Gomes, L. C. Effect of Lactobacillus plantarum Biofilms on the Adhesion of Escherichia coli to Urinary Tract Devices. *Antibiotics* **10**, 966 (2021).
23. Alves, D., Vaz, A. T., Grainha, T., Rodrigues, C. F. & Pereira, M. O. Design of an Antifungal Surface Embedding Liposomal Amphotericin B Through a Mussel Adhesive-Inspired Coating Strategy. *Front. Chem.* **7**, 431 (2019).
24. Allkja, J. *et al.* Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates. *Biofilm* **2**, 100010 (2020).
25. Brooks, T. & Keevil, C. W. A simple artificial urine for the growth of urinary pathogens. *Lett. Appl. Microbiol.* **24**, 203–206 (1997).
26. Allkja, J. & Azevedo, A. S. Characterization of Social Interactions and Spatial Arrangement of Individual Bacteria in MultiStrain or Multispecies Biofilm Systems Using Nucleic Acid Mimics-Fluorescence In Situ Hybridization. in *Fluorescence In-Situ Hybridization (FISH) for Microbial Cells: Methods and Concepts* (eds. Azevedo, N. F. & Almeida, C.) 97–109 (Springer US, 2021). doi:10.1007/978-1-0716-1115-9_7.

27. Goeres, D. M. *et al.* Development, standardization, and validation of a biofilm efficacy test: The single tube method. *J. Microbiol. Methods* **165**, 105694 (2019).
28. Teixeira, H., Sousa, A. L. & Azevedo, A. S. Bioinformatic Tools and Guidelines for the Design of Fluorescence In Situ Hybridization Probes. in *Fluorescence In-Situ Hybridization (FISH) for Microbial Cells: Methods and Concepts* (eds. Azevedo, N. F. & Almeida, C.) 35–50 (Springer US, 2021). doi:10.1007/978-1-0716-1115-9_3.
29. Fritz, B. G., Walker, D. K., Goveia, D. E., Parker, A. E. & Goeres, D. M. Evaluation of Petrifilm™ aerobic count plates as an equivalent alternative to drop plating on R2A agar plates in a biofilm disinfectant efficacy test. *Curr. Microbiol.* **70**, 450–456 (2015).
30. Nelson, M. T., LaBudde, R. A., Tomasino, S. F. & Pines, R. M. Comparison of 3M Petrifilm Aerobic Count Plates to standard plating methodology for use with AOAC antimicrobial efficacy methods 955.14, 955.15, 964.02, and 966.04 as an alternative enumeration procedure: collaborative study. *J. AOAC Int.* **96**, 717–722 (2013).
31. Richter, S. J. & Richter, C. A Method for Determining Equivalence in Industrial Applications. *Qual. Eng.* **14**, 375–380 (2002).
32. Elias, S. & Banin, E. Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol. Rev.* **36**, 990–1004 (2012).
33. Kart, D., Yabanoglu Ciftci, S. & Nemutlu, E. Altered metabolomic profile of dual-species biofilm: Interactions between Proteus mirabilis and Candida albicans. *Microbiol. Res.* **230**, 126346 (2020).
34. Alteri, C. J., Himpsl, S. D. & Mobley, H. L. T. Preferential Use of Central Metabolism In Vivo Reveals a Nutritional Basis for Polymicrobial Infection. *PLOS Pathog.* **11**, e1004601 (2015).
35. Juarez, G. E., Mateyca, C. & Galvan, E. M. Proteus mirabilis outcompetes Klebsiella pneumoniae in artificial urine medium through secretion of ammonia and other volatile compounds. *Heliyon* **6**, e03361 (2020).
36. Almeida, C. *et al.* Rapid detection of urinary tract infections caused by Proteus spp. using PNA-FISH. *Eur. J. Clin. Microbiol. Infect. Dis.* **32**, 781–786 (2013).
37. Azevedo, A. S., Sousa, I. M., Fernandes, R. M., Azevedo, N. F. & Almeida, C. Optimizing locked nucleic acid/2'-O-methyl-RNA fluorescence in situ hybridization (LNA/2'OMe-FISH) procedure for bacterial detection. *PLOS ONE* **14**, e0217689 (2019).
38. Røder, H. L., Sørensen, S. J. & Burmølle, M. Studying Bacterial Multispecies Biofilms: Where to Start? *Trends Microbiol.* **24**, 503–513 (2016).
39. Bella, A. A. L. *et al. Catheterized-bladder environment induces hyphal Candida albicans formation, promoting fungal colonization and persistence*. http://biorxiv.org/lookup/doi/10.1101/2021.06.01.446547 (2021) doi:10.1101/2021.06.01.446547.

40. Hobley, L., Harkins, C., MacPhee, C. E. & Stanley-Wall, N. R. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol. Rev.* **39**, 649–669 (2015).

Chapter 6.

Concluding remarks and future perspectives

This chapter provides a summary of the main findings and conclusions drawn from this thesis. Subsequently, proposals for future works that could be performed are also discussed. Further steps on how to increase the effectiveness of the minimum information guideline are considered, as well as how we could expand on it. The section also discusses new work that could be performed with respect to the reproducibility assessment of microplate methods. Finally, it deliberates the various approaches we can take to make the most use of our microscopy data, and how this could help us get closer to level 3 reproducibility.

6.1. Concluding remarks

The main aim of this thesis was to investigate different methods of addressing the reproducibility issue within the biofilm field and improve upon it. The lack of reproducibility among published studies is one of the major issues we are facing in science today. It is a multifactorial problem; hence different strategies are necessary to tackle it.

In **Chapter 2** we defined reproducibility within the context of biofilm research and proposed a new classification system. Level 1, represents no reproducibility, meaning that the biofilm varies significantly in the number of cells, structure, and EPS components between experiments and among different labs. Level 2 represents standard reproducibility, as currently defined by regulatory agencies and standard setting bodies and requires consistent numbers of viable cells. Level 3 represents a potential standard reproducibility and encompasses similarity in viable cell numbers, the concentration of EPS components and overall architecture of the biofilm (shape, structure). Level 4 refers to total reproducibility where, all biofilm elements (cells, architecture, EPS components, etc.) are present at the same number and location within the biofilm when the experiment is repeated within a single laboratory and in different laboratories. Both Level 3 and Level 4 have not yet been reached experimentally. It is also important to keep in mind that reproducible biofilms will have different meanings for different scientists and/or applications. Thus, it is important to consider which level of reproducibility is appropriate for your goals.

Microplate-based methods are by far the most widely used methods in the field, however little is reported on their repeatability and reproducibility. Additionally, there are many gaps in information when these types of experiments are reported in the literature, which makes it more difficult to reproduce the results. Thus, in **Chapter 3** we tackle this issue by introducing the "Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates". Guidelines instruct authors and reviewers on the necessary information that a manuscript should include for the experiments in a study to be clearly interpreted and independently reproduced. The guideline was built following several discussions between international groups working with these methods as well as an extensive review of the literature. This guideline was divided into 5 main sections, each containing a comprehensive set of recommendations: Experimental design, biofilm formation, biofilm assessment, statistical assessment, and bioinformatics. It also involves a guide on the appropriate data visualisation to be used for a full understanding of the results by the reader. The level of detail asked in the guideline

was selected based on the ability of most labs to be able to provide such data. This can help increase compliance with the requirements. Moreover, for ease of use the guideline and all its accompanying documents were made available online on the MIABiE website (http://miabie.org/introduction.php).

While improving reporting can help reproducibility, we also need to understand the methods, their repeatability and reproducibility, and their limitations. In **Chapter 4**, we performed the first interlaboratory study for microplate-based methods. The study included 5 different laboratories and evaluated the reproducibility of three different biofilm methods for single species *S. aureus* biofilms: plate counts, crystal violet and resazurin. The protocol was separated into three steps: biofilm growth, biofilm challenge, biofilm assessment. For control experiments participants performed the growth and assessment steps only. In control experiments, on the $\log_{10}$-scale, the reproducibility SD was 0.44 for crystal violet, 0.53 for resazurin, and 0.92 for the plate counts. The study also evaluated whether different protocols for the assessment part of the experiment would majorly affect the reproducibility of the methods, by having the participants perform their in-house protocols in parallel to the protocol developed for the trial. For all three methods, the results showed that the mean response was statistically significantly equivalent between the different protocols but that the reproducibility for the ILP was better.

For treatment experiments, all three steps were performed and the efficacy of sodium hypochlorite (NaOCl) in killing *S. aureus* biofilms was evaluated. Plate counts had the best responsiveness to different levels of efficacy and the best reproducibility with respect to responsiveness (Slope/S_R = 1.02), making it the more reliable method to use in an antimicrobial efficacy test. One crucial finding of the study was that data comparison for spectrophotometric and fluorometric type assays between different labs is possible, if calibration curves are performed. Hence, there needs to be a change in the way we report microplate experiment data for these types of assays.

The interlaboratory study provided a lot of information on single species biofilm methods, however we know that biofilms are often multispecies. These can play a major role in infections such as CAUTI, so being able to reliably study these types of biofilms in crucial. As such, in **Chapter 5** we investigated the repeatability of the plate count method for different multispecies biofilms. The study uses the urinary catheter as model system. Four species of microorganisms often associated with CAUTI were selected: *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis* and *Candida albicans*. Eleven combinations of biofilms were grown on silicone coupons embedded in 24-well plates for 24 hours, 37 ºC,

in AUM. The three bacterial species showed overall good repeatability in all combinations tested, whereas *C. albicans* had higher repeatability SD values. The data also showed that in most cases, the variability was mainly due to day-to-day sources rather than within plate, and that the day-to-day differences in means were mostly statistically significantly equivalent. To characterise the structure of these biofilms, fluorescence *in situ* hybridisation (FISH) was applied. The results showed that species undetected with the plate count method, could be seen within the biofilm and that despite the antagonistic relationship between the species they were often co-aggregated. The images also showed that, when evaluating biofilms, the position on the coupon where the images were acquired could result in different biofilm structures. During the imaging we also observed some issues with regards to dye separation as *C. albicans* would appear on all four channels, and *E. faecalis* and *P. mirabilis* were not easily distinguishable from one another due to the closeness in spectrum between their respective fluorochromes. We were able to successfully separate the dyes using the manual dye separation tool in the LASX suite and could easily distinguish between the different species in a C11 biofilm. However as this is a manual process it would not be feasible on a large scale. Hence, there is a need for a reliable automated dye separation tool.

In conclusion, biofilm reproducibility is a complex issue with no simple solution, but many different strategies can help us alleviate the problem. Minimum information guidelines can help us improve reporting of biofilm methods, ensuring a better understanding of how experiments were performed and their data. Microplate-based methods are among the most widely used methods in biofilm studies, and they show potential for standardisation as demonstrated by their good repeatability and reproducibility in the ring trial. The plate count method also showed good repeatability in assessing multispecies biofilms in a microplate-based system. Moreover, the application of the FISH method showed the potential of microscopy in assessing the spatial organisation and structure of the biofilm. This demonstrates the wealth of information we can obtain from microscopy and how it could help us understand and reach a new level of reproducibility.

6.2. Future perspectives

Minimum information guidelines are an effective tool for improving reporting, but their effectiveness is overly reliant on compliance by the scientific community.[1] A look into guideline compliance for metabolomic experiments, showed that reporting standards were followed when enforced by a repository or journal. [2] As such, dissemination and acceptance

by the biofilm community is very important in ensuring success. Moreover, while microplate-based methods are widely used, they are not the only biofilm methods being reported on. So, it necessary for new guidelines to be added to the MIABiE platform. These could expand on other MIABiE modules such as stirred flow reactors, *in vivo* models and even microscopy.[3]

Similar to guidelines, standard methods are validated for a specific use in a select reactor for a specific species. For example, ASTM protocols have been validated for *P. aeruginosa* disinfectant testing in the MBEC[4], CDC[5] and drip flow[6] reactors. To use these reactors for a different species, they were validated again using a ring trial e.g., *S. aureus*.[7] This means that while our interlaboratory study provides information on the repeatability and reproducibility of microplate methods for *S. aureus*, new studies need to be formed for other species. In broad terms these could be one study for a Gram-negative e.g., *P. aeruginosa,* and one study for a fungus e.g., *C. albicans*. Additionally, while acceptable reproducibility SD values have been defined for the plate count[8], there is no such standard for dye based methods like crystal violet and resazurin. So, further discussions are necessary in the field to reach a consensus on this topic.

Another area that currently has no defined standards for reproducibility is microscopy, which is essential in our quest for level 3 reproducibility. As we saw in our study, microscopy data can help us assess many different aspects of the biofilm structure, however quantifying these is not straightforward. First, we would need to decide on the parameter to be quantified. If we were to choose cell numbers, then the dye separation issue discussed in chapter 5 becomes essential. For this, attempts could be made to optimise the FISH probes to avoid channel overlap. An approach would be performing CLASI-FISH which could help in creating a unique tag for each species in a polymicrobial biofilm.[9] However, considering that some of the issues might be due to the biofilm matrix, bioinformatics tools are needed. In our experiments we showed the possibility of applying manual dye separation, but this would be impossible in a large-scale study. Existing image analysis software such as BiofilmQ could be adapted for this purpose. [10] This platform uses Matlab script and allows users to develop their own personalised script, hence it could be a viable option to explore. Another issue with cell number calculations is the question of *C. albicans*. As we have seen, they form hyphae in all the biofilms we tested. These structures are not easily quantifiable like bacterial cells. In most cases, a qualitative assessment of their morphology is performed. Quantifiable parameters include hyphal length and number of hyphae tips, which cannot be easily correlated to cell numbers.[11]

If we decide on quantifying spatial organisation, there are many possible parameters that could be selected. An example would be association matrices, where the directional information of each cell within the biofilm in relation to its neighbours is extracted.[12] Another parameter could be to adapt geo-statistics to map the abundance of microorganisms in various sections of the biofilm.[13] Finally, we could decide to quantify biofilm structure or architecture. This could be interpreted to mean many things, as discussed by *Beyenal et al.*, we could quantify biofilm texture which could quantify the structural heterogeneity of the biofilm.[14] Another approach would be to quantify structural parameters related to function, such as areal porosity and average diffusion distances.[14,15] Bioinformatics tools e.g., BiofilmQ also allow for other parameter calculations such as aspect ratios of height-to-width, height-to-length and width-to-length which could be used as measures for biofilm shape.[10] Once parameters have been selected, the repeatability needs to be tested for biofilm images. This would then need to be followed by an interlaboratory study to determine their reproducibility. Nonetheless, as previously discussed for resazurin and crystal violet, currently no agreed upon reproducibility standards exist for image quantification of biofilms so consensus within the field will be necessary.

6.3. References

1. Deutsch, E. W. *et al.* Minimum information specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE). *Nat. Biotechnol.* **26**, 305–312 (2008).
2. Spicer, R. A., Salek, R. & Steinbeck, C. Compliance with minimum information guidelines in public metabolomics repositories. *Sci. Data* **4**, 170137 (2017).
3. Lourenço, A. *et al.* Minimum information about a biofilm experiment (MIABiE): standards for reporting experiments and data on sessile microbial communities living at interfaces. *Pathog. Dis.* **70**, 250–256 (2014).
4. ASTM E2799-17. Standard Test Method for Testing Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm using the MBEC Assay. *ASTM Int. West Conshohocken PA* (2017).
5. ASTM E2562. Standard Test Method for Evaluating Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method. (2019).
6. ASTM E2647-20. Standard Test Method for Quantification of a Pseudomonas aeruginosa Biofilm Grown Using a Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. *ASTM Int. West Conshohocken PA* (2020).
7. ASTM. Standard Practice for Preparing a Pseudomonas aeruginosa or Staphylococcus aureus Biofilm Using the CDC Biofilm Reactor. (2018).
8. Hamilton, M. A. KSA-SM-11—How to decide whether the reproducibility standard deviation is small enough. (2011).
9. Valm, A. M., Welch, J. L. M. & Borisy, G. G. CLASI-FISH: Principles of Combinatorial Labeling and Spectral Imaging. *Syst. Appl. Microbiol.* **35**, 496–502 (2012).
10. Hartmann, R. *et al.* Quantitative image analysis of microbial communities with BiofilmQ. *Nat. Microbiol.* **6**, 151–156 (2021).
11. Barry, D. J. & Williams, G. A. Microscopic characterisation of filamentous microbes: towards fully automated morphological quantification through image analysis. *J. Microsc.* **244**, 1–20 (2011).
12. Shi, H. *et al.* Highly multiplexed spatial mapping of microbial communities. *Nature* (2020) doi:10.1038/s41586-020-2983-4.
13. Nunan, N., Wu, K., Young, I. M., Crawford, J. W. & Ritz, K. In Situ Spatial Patterns of Soil Bacterial Populations, Mapped at Multiple Scales, in an Arable Soil. *Microb. Ecol.* **44**, 296–305 (2002).
14. Beyenal, H., Lewandowski, Z. & Harkin, G. Quantifying Biofilm Structure: Facts and Fiction. *Biofouling* **20**, 1–23 (2004).

15. Beyenal, H., Donovan, C., Lewandowski, Z. & Harkin, G. Three-dimensional biofilm structure quantification. *J. Microbiol. Methods* **59**, 395–413 (2004).

APPENDIX

Chapter 3. Development of minimum information guidelines

S3.1. List of supplementary references

A list of the references used to help create the guideline is provided below. The list is divided into references of minimum information guidelines and references of articles related to the methods. References mentioned in the main article have not been included in the list below.

Minimum information guidelines

1. Brazma, A. et al. Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. Nature Genetics 29, ng1201-365–365 (2001).

2. Bustin, S. A. et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55, 611–622 (2009).

3. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLOS Biology 8, e1000412 (2010).

4. Janetzki, S. et al. "MIATA"—Minimal Information about T Cell Assays. Immunity 31, 527–528 (2009).

5. Martínez-Bartolomé, S., Binz, P.-A. & Albar, J. P. The Minimal Information About a Proteomics Experiment (MIAPE) from the Proteomics Standards Initiative. in Plant Proteomics (eds. Jorrin-Novo, J. V., Komatsu, S., Weckwerth, W. & Wienkoop, S.) 1072, 765–780 (Humana Press, 2014).

6. Taylor, C. F. Minimum Reporting Requirements for Proteomics: A MIAPE Primer. PROTEOMICS 6, 39–44 (2006).

Methods

1. Ahmed, A., Khan, A. K., Anwar, A., Ali, S. A. & Shah, M. R. Biofilm inhibitory effect of chlorhexidine conjugated gold nanoparticles against Klebsiella pneumoniae. Microb. Pathog. 98, 50–56 (2016).

2. Akbari, K. R. A. & Ali, A. A. Study of antimicrobial effects of several antibiotics and iron oxide nanoparticles on biofilm producing pseudomonas aeruginosa. Nanomed. J. 4, 37–43 (2017).

3. Akinbobola, A. B., Sherry, L., Mckay, W. G., Ramage, G. & Williams, C. Tolerance of Pseudomonas aeruginosa in in-vitro biofilms to high-level peracetic acid disinfection. Journal of Hospital Infection 97, 162–168 (2017).

4. Alves, D. et al. Co-immobilization of Palm and DNase I for the development of an effective anti-infective coating for catheter surfaces. Acta Biomaterialia 44, 313–322 (2016).

5. Bao, X. et al. Effect of polymyxin resistance (pmr) on biofilm formation of Cronobacter sakazakii. Microbial Pathogenesis 106, 16–19 (2017).

6. Basson, A., Flemming, L. A. & Chenia, H. Y. Evaluation of Adherence, Hydrophobicity, Aggregation, and Biofilm Development of Flavobacterium johnsoniae-Like Isolates. Microb Ecol 55, 1–14 (2008).

7. Batista, A. H. M. et al. Antimicrobial Effects of Violacein against Planktonic Cells and Biofilms of Staphylococcus aureus. Molecules 22, 1534 (2017).

8. Bordeleau, E., Atrin Mazinani, S., Nguyen, D., Betancourt, F. & Yan, H. Abrasive treatment of microtiter plates improves the reproducibility of bacterial biofilm assays. RSC Advances 8, 32434–32439 (2018).

9. Borra, R. C., Lotufo, M. A., Gagioti, S. M., Barros, F. de M. & Andrade, P. M. A simple method to measure cell viability in proliferation and cytotoxicity assays. Brazilian Oral Research 23, 255–262 (2009).

10. Borucki, M. K., Peppin, J. D., White, D., Loge, F. & Call, D. R. Variation in Biofilm Formation among Strains of Listeria monocytogenes. Appl. Environ. Microbiol. 69, 7336–7342 (2003).

11. Brackman, G. et al. The Quorum Sensing Inhibitor Hamamelitannin Increases Antibiotic Susceptibility of Staphylococcus aureus Biofilms by Affecting Peptidoglycan Biosynthesis and eDNA Release. Sci Rep 6, (2016).

12. Burmolle, M. et al. Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. Applied and Environmental Microbiology 72, 3916–3923 (2006).

13. Burton, E., Yakandawala, N., LoVetri, K. & Madhyastha, M. S. A microplate spectrofluorometric assay for bacterial biofilms. Journal of Industrial Microbiology & Biotechnology 34, 1–4 (2006).

14. Chapman, C. M. C., Gibson, G. R. & Rowland, I. Effects of single- and multi-strain probiotics on biofilm formation and in vitro adhesion to bladder cells by urinary tract pathogens. Anaerobe 27, 71–76 (2014).

15. Chavant, P., Gaillard-Martinie, B., Talon, R., Hebraud, M. & Bernardi, T. A new device for rapid evaluation of biofilm formation potential by bacteria. J. Microbiol. Methods 68, 605–612 (2007).

16. Coenye, T. & Nelis, H. J. In vitro and in vivo model systems to study microbial biofilm formation. Journal of Microbiological Methods 83, 89–105 (2010).

17. Coffey, B. M. & Anderson, G. G. Biofilm formation in the 96-well microtiter plate. Methods Mol. Biol. 1149, 631–641 (2014).

18. Costa Quishida, C. C. et al. Susceptibility of multispecies biofilm to photodynamic therapy using Photodithazine (R). Lasers Med. Sci. 30, 685–694 (2015).

19. Crémet, L. et al. Comparison of three methods to study biofilm formation by clinical strains of Escherichia coli. Diagnostic Microbiology and Infectious Disease 75, 252–255 (2013).

20. Czerwonka, G. et al. The role of Proteus mirabilis cell wall features in biofilm formation. Arch Microbiol 198, 877–884 (2016).

21. Dalecki, A. G., Crawford, C. L. & Wolschendorf, F. Targeting Biofilm Associated Staphylococcus aureus Using Resazurin Based Drug-susceptibility Assay. J. Vis. Exp. e53925 (2016). doi:10.3791/53925

22. de Breij, A. et al. Prevention of Staphylococcus aureus biomaterial-associated infections using a polymer-lipid coating containing the antimicrobial peptide OP-145. Journal of Controlled Release 222, 1–8 (2016).

23. Deighton, M. A., Capstick, J., Domalewski, E. & Van Nguyen, T. [17] Methods for studying biofilms produced by staphylococcus epidermidis. in Methods in enzymology 336, 177–195 (Elsevier, 2001).

24. Devi, K. R. et al. In vitro and in vivo efficacy of rosmarinic acid on quorum sensing mediated biofilm formation and virulence factor production in Aeromonas hydrophila. Biofouling 32, 1171–1183 (2016).

25. Dieser, S. A., Fessia, A. S., Ferrari, M. P., Raspanti, C. G. & Odierno, L. M. Streptococcus uberis: In vitro biofilm production in response to carbohydrates and skim milk. Rev. Argent. Microbiol. 49, 305–310 (2017).

26. Djordjevic, D., Wiedmann, M. & McLandsborough, L. A. Microtiter Plate Assay for Assessment of Listeria monocytogenes Biofilm Formation. Appl. Environ. Microbiol. 68, 2950–2958 (2002).

27. Doll, K., Jongsthaphongpun, K. L., Stumpp, N. S., Winkel, A. & Stiesch, M. Quantifying implant-associated biofilms: Comparison of microscopic, microbiologic and biochemical methods. Journal of Microbiological Methods 130, 61–68 (2016).

28. Dvořáčková, M., Růžička, F., Benešík, M., Pantůček, R. & Dvořáková-Heroldová, M. Antimicrobial effect of commercial phage preparation Stafal® on biofilm and planktonic forms of methicillin-resistant Staphylococcus aureus. Folia Microbiol (2018). doi:10.1007/s12223-018-0622-3

29. Dworniczek, E. et al. Virulence factors and in Vitro adherence of Enterococcus strains to urinary catheters. Folia Microbiol 48, 671–678 (2003).

30. Elkhatib, W. F., Khairalla, A. S. & Ashour, H. M. Evaluation of different microtiter plate-based methods for the quantitative assessment of Staphylococcus aureus biofilms. Future Microbiol. 9, 725–735 (2014).

31. Extremina, C. I., Costa, L., Aguiar, A. I., Peixe, L. & Fonseca, A. P. Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. Journal of Microbiological Methods 84, 167–173 (2011).

32. Fatani, E. J. et al. In vitro assessment of stainless steel orthodontic brackets coated with titanium oxide mixed Ag for anti-adherent and antibacterial properties against Streptococcus mutans and Porphyromonas gingivalis. Microbial Pathogenesis 112, 190–194 (2017).

33. Fernández-Barat, L. et al. Assessment of in vivo versus in vitro biofilm formation of clinical methicillin-resistant Staphylococcus aureus isolates from endotracheal tubes. Scientific Reports 8, 11906 (2018).

34. Flores, V. da C. et al. Antibiofilm effect of antimicrobials used in the therapy of mycobacteriosis. Microbial Pathogenesis 99, 229–235 (2016).

35. Foerster, S., Desilvestro, V., Hathaway, L. J., Althaus, C. L. & Unemo, M. A new rapid resazurin-based microdilution assay for antimicrobial susceptibility testing of Neisseria gonorrhoeae. J Antimicrob Chemother 72, 1961–1968 (2017).

36. Fredheim, E. G. A. et al. Biofilm Formation by Staphylococcus haemolyticus. J. Clin. Microbiol. 47, 1172–1180 (2009).

37. Fujishige, N. A., Kapadia, N. N., De Hoff, P. L. & Hirsch, A. M. Investigations of Rhizobium biofilm formation. FEMS Microbiol Ecol 56, 195–206 (2006).

38. Gjermansen, M., Nilsson, M., Yang, L. & Tolker-Nielsen, T. Characterization of starvation-induced dispersion in Pseudomonas putida biofilms: genetic elements and molecular mechanisms. Molecular Microbiology 75, 815–826 (2010).

39. Gomes, L. C. et al. Macroscale versus microscale methods for physiological analysis of biofilms formed in 96-well microtiter plates. Journal of Microbiological Methods 95, 342–349 (2013).

40. Gomes, L. C., Moreira, J. M. R., Simões, M., Melo, L. F. & Mergulhão, F. J. Biofilm localization in the vertical wall of shaking 96-well plates. Scientifica (Cairo) 2014, 231083 (2014).

41. Gomez, G. F., Huang, R., Eckert, G. & Gregory, R. L. Effect of phototherapy on the metabolism of Streptococcus mutans biofilm based on a colorimetric tetrazolium assay. Journal of Oral Science 60, 242–246 (2018).

42. Hanafi, A. et al. Molecular and Proteomic Analysis of Levofloxacin and Metronidazole Resistant Helicobacter pylori. Front Microbiol 7, 2015 (2016).

43. Haney, E. F., Trimble, M. J., Cheng, J. T., Vallé, Q. & Hancock, R. E. W. Critical Assessment of Methods to Quantify Biofilm Growth and Evaluate Antibiofilm Activity of Host Defence Peptides. Biomolecules 8, 29 (2018).

44. Haque, F., Sajid, M., Cameotra, S. S. & Battacharyya, M. S. Anti-biofilm activity of a sophorolipid-amphotericin B niosomal formulation against Candida albicans. Biofouling 0, 1–12 (2017).

45. Hassan, A. et al. Evaluation of different detection methods of biofilm formation in the clinical isolates. The Brazilian Journal of Infectious Diseases 15, 305–311 (2011).

46. Hayat, S. et al. In vitro antibiofilm and anti-adhesion effects of magnesium oxide nanoparticles against antibiotic resistant bacteria. Microbiology and Immunology 62, 211–220

47. He, Z. et al. Anti-biofilm Activities from Resveratrol against Fusobacterium nucleatum. Front. Microbiol. 7, (2016).

48. Hiltunen, A., Skogman, M., Vuorela, P. M. & Fallarero, A. Exploration of Microbial Communities Using the Thermo Scientific Varioskan LUX Multimode Reader and the Invitrogen EVOS FL Cell Imaging System. BioTechniques 63, 236–237 (2017).

49. Hussain, M. S. & Oh, D. H. Substratum attachment location and biofilm formation by Bacillus cereus strains isolated from different sources: Effect on total biomass production and sporulation in different growth conditions. Food Control 77, 270–280 (2017).

50. Jackson, D. W. et al. Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of Escherichia coli. Journal of Bacteriology 184, 290–301 (2002).

51. Jakobsen, T. H. et al. Complete genome sequence of the cystic fibrosis pathogen Achromobacter xylosoxidans NH44784-1996 complies with important pathogenic phenotypes. PloS one 8, e68484 (2013).

52. Jin, Y., Samaranayake, L. P., Samaranayake, Y. & Yip, H. K. Biofilm formation of Candida albicans is variably affected by saliva and dietary sugars. Arch. Oral Biol. 49, 789–798 (2004).

53. Jin, Y., Yip, H. K., Samaranayake, Y. H., Yau, J. Y. & Samaranayake, L. P. Biofilm-forming ability of Candida albicans is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. J. Clin. Microbiol. 41, 2961–2967 (2003).

54. Jorge, P., Grzywacz, D., Kamysz, W., Lourenço, A. & Pereira, M. O. Searching for new strategies against biofilm infections: Colistin-AMP combinations against Pseudomonas aeruginosa and Staphylococcus aureus single- and double-species biofilms. PLoS One 12, (2017).

55. Jorge, P., Lourenço, A. & Pereira, M. O. Data Quality in Biofilm High-Throughput Routine Analysis: Intralaboratory Protocol Adaptation and Experiment Reproducibility. Journal of AOAC International 98, 1721–1727 (2015).

56. Joshi, S. G. et al. Control of methicillin-resistant Staphylococcus aureus in planktonic form and biofilms: A biocidal efficacy study of nonthermal dielectric-barrier discharge plasma. American Journal of Infection Control 38, 293–301 (2010).

57. Kaur, G., Balamurugan, P., Vasudevan, S., Jadav, S. & Princy, S. A. Antimicrobial and Antibiofilm Potential of Acyclic Amines and Diamines against Multi-Drug Resistant Staphylococcus aureus. Front Microbiol 8, (2017).

58. Kipanga, P. N. & Luyten, W. Influence of serum and polystyrene plate type on stability of Candida albicans biofilms. Journal of Microbiological Methods 139, 8–11 (2017).

59. Knezevic, P. & Petrovic, O. A colorimetric microtiter plate method for assessment of phage effect on Pseudomonas aeruginosa biofilm. Journal of Microbiological Methods 74, 114–118 (2008).

60. Knight, D. B., Rudin, S. D., Bonomo, R. A. & Rather, P. N. Acinetobacter nosocomialis: Defining the Role of Efflux Pumps in Resistance to Antimicrobial Therapy, Surface Motility, and Biofilm Formation. Front Microbiol 9, (2018).

61. Kolouchová, I. et al. Resveratrol, pterostilbene, and baicalein: plant-derived anti-biofilm agents. Folia Microbiol. (Praha) (2017). doi:10.1007/s12223-017-0549-0

62. Krom, B. P. & Willems, H. M. E. In Vitro Models for Candida Biofilm Development. in Candida Species 95–105 (Humana Press, New York, NY, 2016). doi:10.1007/978-1-4939-3052-4_8

63. LaPlante, K. L. & Mermel, L. A. In Vitro Activities of Telavancin and Vancomycin against Biofilm-Producing Staphylococcus aureus, S. epidermidis, and Enterococcus faecalis Strains. Antimicrobial Agents and Chemotherapy 53, 3166–3169 (2009).

64. Lee, J.-H., Cho, M. H. & Lee, J. 3-indolylacetonitrile decreases Escherichia coli O157:H7 biofilm formation and Pseudomonas aeruginosa virulence. Environ. Microbiol. 13, 62–73 (2011).

65. Lee, J.-H., Kim, Y.-G. & Lee, J. Carvacrol-rich oregano oil and thymol-rich thyme red oil inhibit biofilm formation and the virulence of uropathogenic Escherichia coli. J. Appl. Microbiol. (2017). doi:10.1111/jam.13602

66. Lee, J.-S., Bae, Y.-M., Lee, S.-Y. & Lee, S.-Y. Biofilm Formation of Staphylococcus aureus on Various Surfaces and Their Resistance to Chlorine Sanitizer. Journal of Food Science 80, M2279–M2286

67. Lehman, S. M. & Donlan, R. M. Bacteriophage-Mediated Control of a Two-Species Biofilm Formed by Microorganisms Causing Catheter-Associated Urinary Tract Infections in an In Vitro Urinary Catheter Model. Antimicrob Agents Chemother 59, 1127–1137 (2015).

68. Li, Y.-H. et al. A Quorum-Sensing Signaling System Essential for Genetic Competence in Streptococcus mutans Is Involved in Biofilm Formation. Journal of Bacteriology 184, 2699–2708 (2002).

69. Lijuan, C., Xing, Y., Minxi, W., Wenkai, L. & Le, D. Development of an aptamer-ampicillin conjugate for treating biofilms. Biochem. Biophys. Res. Commun. 483, 847–854 (2017).

70. Lima-e-Silva, A. A. et al. Sub-Inhibitory Concentrations of Rifampicin Strongly Stimulated Biofilm Production in S. aureus. Open Microbiol J 11, 142–151 (2017).

71. Liu, Y. et al. Anti-biofilm Activities from Bergenia crassifolia Leaves against Streptococcus mutans. Front Microbiol 8, 1738 (2017).

72. Lopes, S. P., Machado, I. & Pereira, M. O. Role of planktonic and sessile extracellular metabolic by products on Pseudomonas aeruginosa and Escherichia coli intra and interspecies relationships. J Ind Microbiol Biotechnol 38, 133–140 (2011).

73. Magalhães, A. P., Lopes, S. P. & Pereira, M. O. Insights into Cystic Fibrosis Polymicrobial Consortia: The Role of Species Interactions in Biofilm Development, Phenotype, and Response to In-Use Antibiotics. Front Microbiol 7, (2017).

74. Mampel, J. et al. Planktonic replication is essential for biofilm formation by Legionella pneumophila in a complex medium under static and dynamic flow conditions. Appl. Environ. Microbiol. 72, 2885–2895 (2006).

75. Manner, S., Goeres, D. M., Skogman, M., Vuorela, P. & Fallarero, A. Prevention of Staphylococcus aureus biofilm formation by antibiotics in 96-Microtiter Well Plates and Drip Flow Reactors: critical factors influencing outcomes. Scientific Reports 7, srep43854 (2017).

76. Martinez, L. R. & Casadevall, A. Susceptibility of Cryptococcus neoformans Biofilms to Antifungal Agents In Vitro. Antimicrobial Agents and Chemotherapy 50, 1021–1033 (2006).

77. Martinez, L. R. & Casadevall, A. Cryptococcus neoformans biofilm formation depends on surface support and carbon source and reduces fungal cell susceptibility to heat, cold, and. Appl. Environ. Microbiol. 73, 4592–4601 (2007).

78. McBain, A. J. Chapter 4 In Vitro Biofilm Models: An Overview. in Advances in Applied Microbiology 69, 99–132 (Academic Press, 2009).

79. McLay, R. B. et al. Level of fimbriation alters the adhesion of Escherichia coli bacteria to interfaces. Langmuir (2017). doi:10.1021/acs.langmuir.7b02447

80. Mehdi, H. & Giti, E. Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. International Biodeterioration & Biodegradation 62, 170–178 (2008).

81. Merritt, J. H., Kadouri, D. E. & O'Toole, G. A. Growing and Analyzing Static Biofilms. Curr Protoc Microbiol 0 1, Unit-1B.1 (2005).

82. Minardi, D. et al. Efficacy of Tigecycline and Rifampin Alone and in Combination against Enterococcus faecalis Biofilm Infection in a Rat Model of Ureteral Stent. Journal of Surgical Research 176, 1–6 (2012).

83. Moreira, J. M. R. et al. The effect of glucose concentration and shaking conditions on Escherichia coli biofilm formation in microtiter plates. Chemical Engineering Science 94, 192–199 (2013).

84. Müsken, M., Di Fiore, S., Römling, U. & Häussler, S. A 96-well-plate–based optical method for the quantitative and qualitative evaluation of Pseudomonas aeruginosa biofilm formation and its application to susceptibility testing. Nat. Protocols 5, 1460–1469 (2010).

85. Nett, J. E., Cain, M. T., Crawford, K. & Andes, D. R. Optimizing a Candida Biofilm Microtiter Plate Model for Measurement of Antifungal Susceptibility by Tetrazolium Salt Assay. J. Clin. Microbiol. 49, 1426–1433 (2011).

86. Oja, T. et al. Effective Antibiofilm Polyketides against Staphylococcus aureus from the Pyranonaphthoquinone Biosynthetic Pathways of Streptomyces Species. Antimicrobial Agents and Chemotherapy 59, 6046–6052 (2015).

87. Oliveira, F., França, Â. & Cerca, N. Staphylococcus epidermidis is largely dependent on iron availability to form biofilms. International Journal of Medical Microbiology 307, 552–563 (2017).

88. Ommen, P., Zobek, N. & Meyer, R. L. Quantification of biofilm biomass by staining: Non-toxic safranin can replace the popular crystal violet. Journal of Microbiological Methods 141, 87–89 (2017).

89. Overhage, J. et al. Human Host Defense Peptide LL-37 Prevents Bacterial Biofilm Formation. Infection and Immunity 76, 4176–4182 (2008).

90. Palanisamy, S. K. A., Ramirez, M. A., Lorenz, M. & Lee, S. A. Candida albicans PEP12 Is Required for Biofilm Integrity and In Vivo Virulence. Eukaryot Cell 9, 266–277 (2010).

91. Parker, A., Cureoglu, S., Lay, N. D., Majdalani, N. & Gottesman, S. Alternative pathways for Escherichia coli biofilm formation revealed by sRNA overproduction. Molecular Microbiology 105, 309–325 (2017).

92. Pearson, M. M., Laurence, C. A., Guinn, S. E. & Hansen, E. J. Biofilm Formation by Moraxella catarrhalis In Vitro: Roles of the UspA1 Adhesin and the Hag Hemagglutinin. Infection and Immunity 74, 1588–1596 (2006).

93. Peeters, E., Nelis, H. J. & Coenye, T. Evaluation of the efficacy of disinfection procedures against Burkholderia cenocepacia biofilms. Journal of Hospital Infection 70, 361–368 (2008).

94. Pérez, D. L. et al. Experimental and statistical methods to evaluate antibacterial activity of a quaternary pyridinium salt on planktonic, biofilm-forming, and biofilm states. Biofouling 33, 222–234 (2017).

95. Perumal, P., Mekala, S. & Chaffin, W. L. Role for cell density in antifungal drug resistance in Candida albicans biofilms. Antimicrob. Agents Chemother. 51, 2454–2463 (2007).

96. Peterson, S. B. et al. Different Methods for Culturing Biofilms In Vitro. in Biofilm Infections 251–266 (Springer, New York, NY, 2011). doi:10.1007/978-1-4419-6084-9_15

97. Pierce, C. G. et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nature Protocols 3, 1494–1500 (2008).

98. Pitts, B., Hamilton, M. A., Zelver, N. & Stewart, P. S. A microtiter-plate screening method for biofilm disinfection and removal. Journal of Microbiological Methods 54, 269–276 (2003).

99. Ramachandran, G., Aheto, K., Shirtliff, M. E. & Tennant, S. M. Poor biofilm-forming ability and long-term survival of invasive Salmonella Typhimurium ST313. Pathog Dis 74, (2016).

100. Ramage, G. Comparing apples and oranges: considerations for quantifying candidal biofilms with XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] and the need for standardized testing. Journal of Medical Microbiology 65, 259–260 (2016).

101. Ramage, G., Saville, S. P., Wickes, B. L. & López-Ribot, J. L. Inhibition of Candida albicans Biofilm Formation by Farnesol, a Quorum-Sensing Molecule. Appl. Environ. Microbiol. 68, 5459–5463 (2002).

102. Ramage, G., Walle, K. V., Wickes, B. L. & López-Ribot, J. L. Standardized Method for In Vitro Antifungal Susceptibility Testing of Candida albicans Biofilms. Antimicrob. Agents Chemother. 45, 2475–2479 (2001).

103. Rampersad, S. N. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. Sensors 12, 12347–12360 (2012).

104. Redding, S. et al. Inhibition of Candida albicans biofilm formation on denture material. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 107, 669–672 (2009).

105. Rodrigues, M. E. et al. Polymicrobial Ventilator-Associated Pneumonia: Fighting In Vitro Candida albicans-Pseudomonas aeruginosa Biofilms with Antifungal-Antibacterial Combination Therapy. PLOS ONE 12, e0170433 (2017).

106. Saito, Y. et al. Stimulation of Fusobacterium nucleatum biofilm formation by Porphyromonas gingivalis. Oral Microbiology and Immunology 23, 1–6 (2008).107.

108. Salta, M. et al. Anti-Biofilm Performance of Three Natural Products against Initial Bacterial Attachment. International Journal of Molecular Sciences 14, 21757–21780 (2013).

109. Savijoki, K. et al. Penicillin G increases the synthesis of a suicidal marker (CidC) and virulence (HlgBC) proteins in Staphylococcus aureus biofilm cells. International Journal of Medical Microbiology 306, 69–74 (2016).

110. Shakeri, S., Kermanshahi, R. K., Moghaddam, M. M. & Emtiazi, G. Assessment of biofilm cell removal and killing and biocide efficacy using the microtiter plate test. Biofouling 23, 79–86 (2007).

111. Simoes, L. C., Simoes, M. & Lima, N. Kinetics of biofilm formation by drinking water isolated Penicillium expansum. Biofouling 31, 349–362 (2015).

112. Singh, A. K. et al. Curcumin Quantum Dots Mediated Degradation of Bacterial Biofilms. Front Microbiol 8, 1517 (2017).

113. Skogman, M., Kanerva, S., Manner, S., Vuorela, P. & Fallarero, A. Flavones as Quorum Sensing Inhibitors Identified by a Newly Optimized Screening Platform Using Chromobacterium violaceum as Reporter Bacteria. Molecules 21, 1211 (2016).

114. Stepanović, S., Vuković, D., Dakić, I., Savić, B. & Švabić-Vlahović, M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of microbiological methods 40, 175–179 (2000).

115. Stiefel, P. et al. Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. Appl. Microbiol. Biotechnol. 100, 4135–4145 (2016).

116. Todorov, S. D., de Paula, O. A. L., Camargo, A. C., Lopes, D. A. & Nero, L. A. Combined effect of bacteriocin produced by Lactobacillus plantarum ST8SH and vancomycin, propolis or EDTA for controlling biofilm development by Listeria monocytogenes. Revista Argentina de Microbiología (2017). doi:10.1016/j.ram.2017.04.011

117. Toledo-Arana, A. et al. The Enterococcal Surface Protein, Esp, Is Involved in Enterococcus faecalis Biofilm Formation. Appl. Environ. Microbiol. 67, 4538–4545 (2001).

118. Torelli, R. et al. Different effects of matrix degrading enzymes towards biofilms formed by E. faecalis and E. faecium clinical isolates. Colloids and Surfaces B: Biointerfaces 158, 349–355 (2017).

119. Trafny, E. A., Lewandowski, R., Zawistowska-Marciniak, I. & Stepinska, M. Use of MTT assay for determination of the biofilm formation capacity of microorganisms in metalworking fluids. World J. Microbiol. Biotechnol. 29, 1635–1643 (2013).

120. Udine, C. et al. Phenotypic and Genotypic Characterisation of Burkholderia cenocepacia J2315 Mutants Affected in Homoserine Lactone and Diffusible Signal Factor-Based Quorum Sensing Systems Suggests Interplay between Both Types of Systems. PLoS One 8, (2013).

121. Van den Driessche, F., Rigole, P., Brackman, G. & Coenye, T. Optimization of resazurin-based viability staining for quantification of microbial biofilms. Journal of Microbiological Methods 98, 31–34 (2014).

122. Van kerckhoven, M. et al. Characterizing the in vitro biofilm phenotype of Staphylococcus epidermidis isolates from central venous catheters. Journal of Microbiological Methods 127, 95–101 (2016).

123. Vandecandelaere, I., Acker, H. V. & Coenye, T. A Microplate-Based System as In Vitro Model of Biofilm Growth and Quantification. in Bacterial Persistence 53–66 (Humana Press, New York, NY, 2016). doi:10.1007/978-1-4939-2854-5_5

124. Vandecandelaere, I., Van Nieuwerburgh, F., Deforce, D. & Coenye, T. Metabolic activity, urease production, antibiotic resistance and virulence in dual species biofilms of Staphylococcus epidermidis and Staphylococcus aureus. PLoS One 12, (2017).

125. Vandenbosch, D. et al. Genome wide screening for genes involved in biofilm formation and miconazole susceptibility in Saccharomyces cerevisiae. FEMS Yeast Res 13, 720–730 (2013).

126. Varga, J. J. et al. Genotypic and phenotypic analyses of a Pseudomonas aeruginosa chronic bronchiectasis isolate reveal differences from cystic fibrosis and laboratory strains. BMC Genomics 16, (2015).

127. Vestby, L. K., Møretrø, T., Langsrud, S., Heir, E. & Nesse, L. L. Biofilm forming abilities of Salmonella are correlated with persistence in fish meal- and feed factories. BMC Veterinary Research 5, 20 (2009).

128. Vila, T. & Lopez-Ribot, J. L. Screening the Pathogen Box for Identification of Candida albicans Biofilm Inhibitors. Antimicrobial Agents and Chemotherapy 61, e02006-16 (2017).

129. Vittal, J. B. A. and R. R. Antimicrobial, Biofilm Inhibitory and Anti-infective Activity of Metallic Nanoparticles Against Pathogens MRSA and Pseudomonas aeruginosa PA01. Pharmaceutical Nanotechnology (2017).

130. Vuong, C., Saenz, H. L., Götz, F. & Otto, M. Impact of the agr Quorum-Sensing System on Adherence to Polystyrene in Staphylococcus aureus. J Infect Dis 182, 1688–1693 (2000).

131. Wakimoto, N. et al. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative Escherichia coli. Am. J. Trop. Med. Hyg. 71, 687–690 (2004).

132. Yadav, M. K., Chae, S.-W. & Song, J.-J. Effect of 5-azacytidine on in vitro biofilm formation of Streptococcus pneumoniae. Microb. Pathog. 53, 219–226 (2012).

133. Zaborowska, M. et al. Biofilm formation and antimicrobial susceptibility of staphylococci and enterococci from osteomyelitis associated with percutaneous orthopaedic implants. Journal of Biomedical Materials Research Part B: Applied Biomaterials 105, 2630–2640 (2017).

134. Zhang, Y., Brackman, G. & Coenye, T. Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on Pseudomonas aeruginosa and Acinetobacter baumannii biofilms. PeerJ 5, (2017).

135. Zhao, T. & Liu, Y. N-acetylcysteine inhibit biofilms produced by Pseudomonas aeruginosa. BMC Microbiology 10, 140 (2010).

136. Zheng, J. et al. Characterization of biofilm formation by Enterococcus faecalis isolates derived from urinary tract infections in China. Journal of Medical Microbiology 67, 60–67 (2018).

S3.2. Terminology definitions text box

Extra guideline content available on MIABiE website.

DISTINCTION BETWEEN TECHNICAL, INDEPENDENT & BIOLOGICAL REPLICATES

TECHNICAL replication means samples representing the same test condition for an experiment were collected from the same microtiter plate. Concurrent controls should be included in every plate.

INDEPENDENT replication means that the experiment was performed on different days.

BIOLOGICAL replicates use a different inoculum, although the experiment may be performed on the same day.

Chapter 4. Evaluation of single species biofilm methods

S4.1. Protocol optimisation

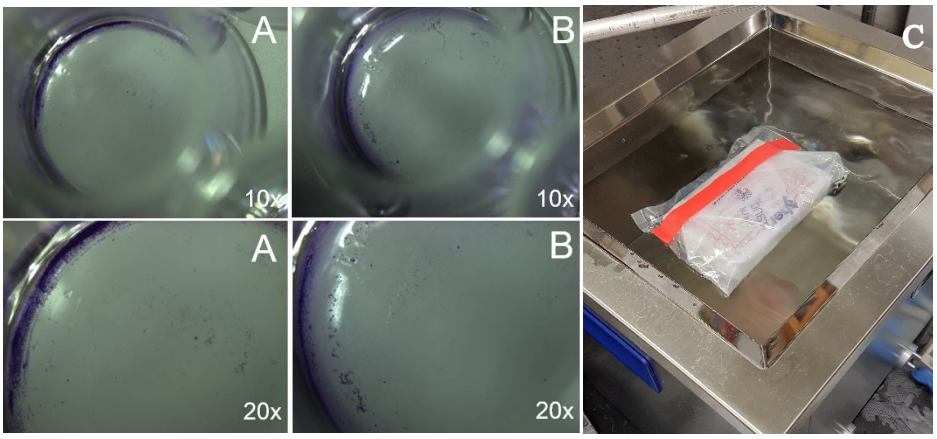


Figure S4.1.1. Sonication test. A. Wells stained with 0.05% (v/v) crystal violet pre-sonication. **B**. Wells stained with 0.05% (v/v) crystal violet post sonication. **C**. Microtiter plate in sonication bath. Microscopy images courtesy of L. Lorenz.

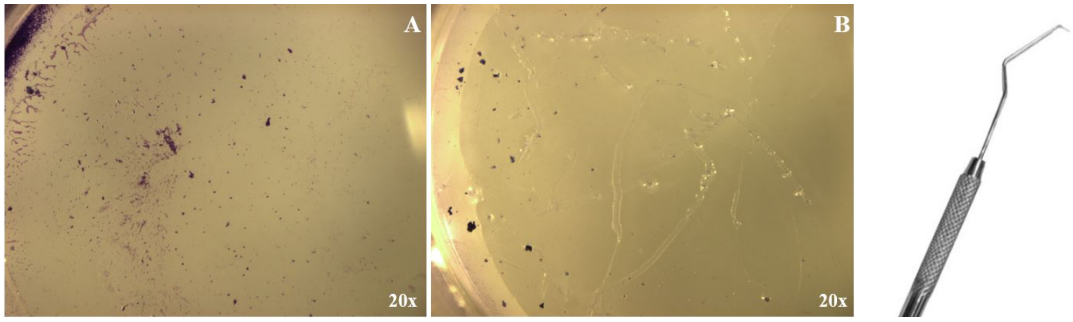


Figure S4.1.2. Scraping test (Dental tool). A. Wells stained with 0.05% (v/v) crystal violet pre-scraping. **B**. Wells stained with 0.05% (v/v) crystal violet post scraping. Microscopy images courtesy of L. Lorenz.

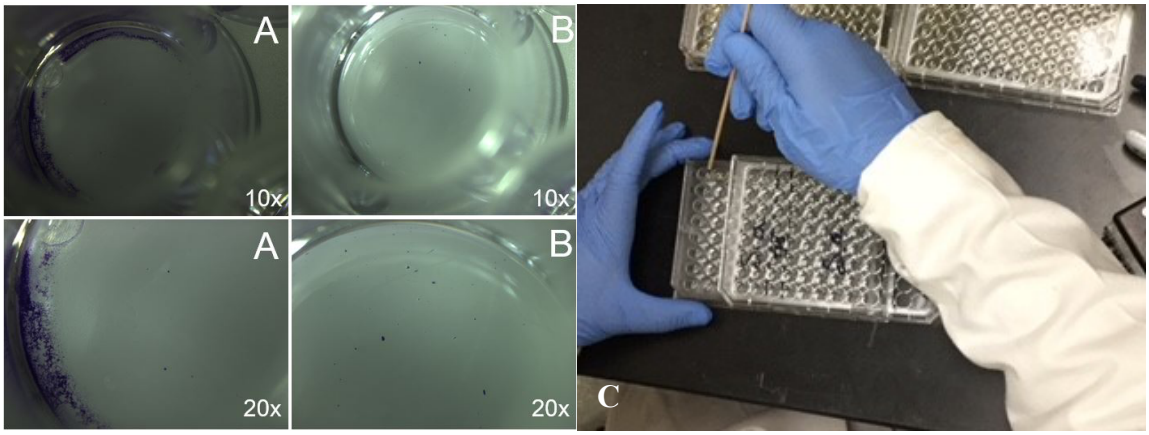


Figure S4.1.3. Scraping test (Wooden Applicator stick). A. Wells stained with 0.05% (v/v) crystal violet pre-scraping. **B**. Wells stained with 0.05% (v/v) crystal violet post scraping. **C**. Scraping method. Microscopy images courtesy of L. Lorenz.

Table S4.1.1 Plate count method day to day variability test. The experiments were performed in 2 separate days. For each day 15 individual wells were scraped and counted. The values have been converted to a log scale. The mean and standard deviation of all 15 counts

Day	Mean Log CFU/well	STDEV
1	7.48	0.22
2	7.34	0.31

Table S4.1.2 Plate count wooden applicator stick efficacy. For this experiment the contents of 6 individual wells were scraped and counted. Additionally, the amount of biofilm left behind on their respective wooden applicator sticks were quantified and added to their values before log conversion. The mean and standard deviation of the log values are represented in the table.

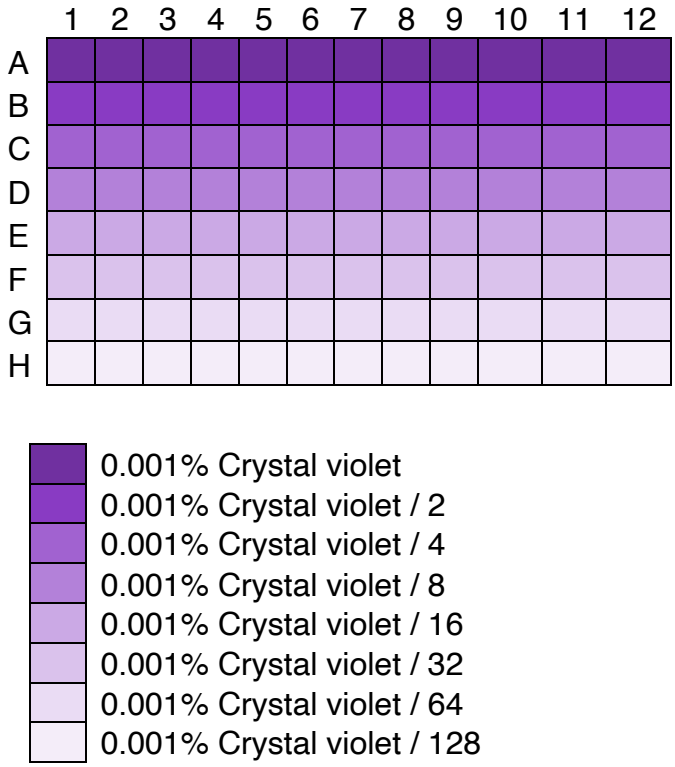
	Wells only	Wells + stick	Difference
Mean Log CFU/well	7.08	7.11	0.03
STDEV	0.30	0.28	0.03

S4.2. Collection of protocols used in interlaboratory study
Detailed description of the ILP protocols developed and used in the ring trial study, as well as in-house protocols from participating laboratories.

S4.2.1. Inter-lab Protocols

Prior to performing the experiments for the interlaboratory study, conduct the following tests:

1- Crystal violet: Make 0.001% crystal violet in laboratory grade (deionized water) and serially dilute by a factor of 2. Add 200 µL/well of each dilution to a microtiter plate using the following layout:



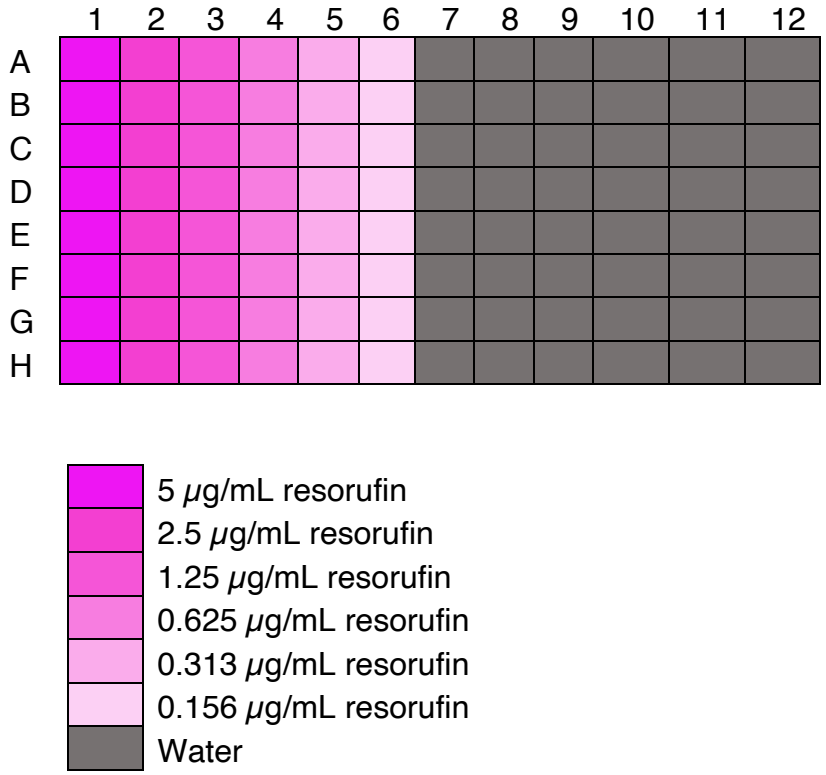
Read the plate at 595 nm and record the data in the spreadsheet provided.

2- Resorufin: Make 5 µg/mL resorufin solution. Resorufin can be made by reducing resazurin using sodium dithionite (also known as sodium hydrosulfite) [Sigma Aldrich; #7775-14-6] or by using commercially available resorufin powder [Sigma Aldrich; #34994-50-8].

To reduce resazurin we suggest making a stock of 1 g/L resazurin in MilliQ water and adding sodium dithionite one grain at a time until the solution turn dark pink or red (Using stocks of lower concentrations will increase chances of over-reducing the resazurin until it

becomes colorless). To keep this compound stable, it should be prepared in anaerobic conditions and autoclaved.

Serially dilute by a factor of 2 in MilliQ water and add 200 µL/well of each dilution to a microtiter plate using the following layout:



Measure fluorescence at $\lambda_{excitation}$ = 560 nm; $\lambda_{emission}$ = 590 nm and record the data in the spreadsheet provided.

Experimental design

Each laboratory needs to perform 3 different biofilm assessment protocols: crystal violet, resazurin and CFU count. All experiments must be performed by only one person per lab. For each protocol 2 different plate layouts will be tested. A control plate to assess the variability within the plate and a treatment plate to be used for a disinfectant activity test, to assess the sensitivity of the methods. Each protocol lasts 4 days, expect for the CFU count which lasts for 5 days. The first three days are the biofilm formation part of the protocol and as such they will be the same for all methods. When performing the control plate experiments, we ask that each laboratory uses their own protocol (IHP) on the 4th day (data collection day) in parallel with the inter-lab protocol (ILP), if available. Repeat the experiments twice for the control plates

and 3 times for the treatment plates. Below you can find a suggested timeline for the ring trial. In total all experiments should take 6 weeks to be completed.

Table S4.2.1. Model week layout

Monday	Tuesday	Wednesday	Thursday	Friday
Day 1	Day 2	Day 3	Data collection day	
	Day 1	Day 2	Day 3	Data collection day

Table S4.2.2. Suggested ring trial timeline

Data collection day 1	Data collection day 2	Data collection day 3	Data collection day 4	Data collection day 5	Data collection day 6	Data collection day 7	Data collection day 8	Data collection day 9	Data collection day 10	Data collection day 11	Data collection day 12
Control plate Crystal violet ILP + IHP	Control plate Resazurin ILP + IHP	Control plate CFU count ILP + IHP	Control plate Crystal violet ILP + IHP	Control plate CFU count ILP + IHP	Control plate Resazurin ILP + IHP	Treated plate CFU count + Crystal violet	Treated plate Resazurin	Treated plate CFU count + Crystal violet	Treated plate Resazurin	Treated plate CFU count + Crystal violet	Treated plate Resazurin

Disinfectant preparation protocol

Sodium hypochlorite (NaOCl) or bleach will be used to challenge the biofilm. The disinfectant will be diluted to 1000 mg/L fresh every day, no more than 2 hours prior to use. Additionally, a titration test needs to be performed to make sure the total concentration of chlorine within your solution is 1000 ±10 mg/L. To perform the titration test you can use a digital titrator [Hach; Mfr. No. 1690001] or any commercially available total chlorine test that falls within this range.

Dilution

Bleach [Pure Bright] stock concentration 6 % i.e. 60000 mg/L. Calculate amount of stock needed to make 30 mL of 1000 mg/L solution using $C_1V_1=C_2V_2$ formula. Dilute 0.5 mL of stock bleach solution into 29.5 mL of filter sterilized MilliQ water. **Make sure the container**

used is sterile and try to avoid plastic containers. Perform a titration test to confirm the solution is within the 10 % range as described above.

Perform the following dilutions:

- 5 mL of 1000 mg/L + 5 mL of sterile MilliQ water = 500 mg/L
- 1 mL of 1000 mg/L + 9 mL of sterile MilliQ water = 100 mg/L
- 0.1 mL of 1000 mg/L + 9.9 mL of sterile MilliQ water = 10 mg/L
- Keep an extra 15 mL of sterile MilliQ water to use for the experiment

Make sure to adjust the calculations depending on the concentration stated on the label of your bleach bottle and perform the titration test every day.

Biofilm growth Protocol

Day 1

Materials: Tryptic Soy Agar (TSA) [Difco, Cat #236290] plate.

Staphylococcus aureus subsp. aureus (ATCC® 25923™) glycerol stock, stored at -80 °C.

Protocol: Using a sterile inoculation loop pick up some of the *Staphylococcus aureus subsp. aureus* (ATCC® 25923™) glycerol stock and streak it on a TSA plate. Incubate the plate at 37 ± 2 °C for 24 hours. The colonies should be round, smooth, slightly raised and light yellow/creamy white in colour. Typical colony appearance and size is shown in Figure S4.2.1. If colonies formed are too small, too large, or drastically different in appearance and morphology, use fresh stock.

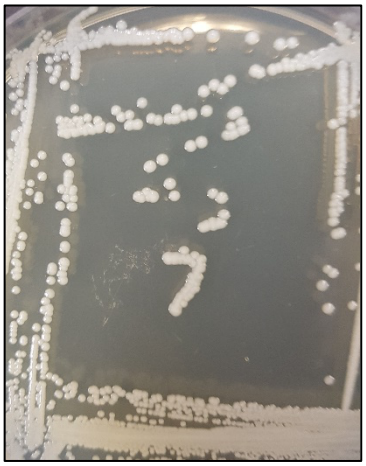


Figure S4.2.1. *Staphylococcus aureus subsp. aureus* (ATCC® 25923™) colony morphology

If possible use stocks only once per experiment. Alternatively, if stocks are re-used make sure that no contamination is present and colony morphology is consistent.

Day 2

Materials and equipment: Tryptic Soy Broth (TSB) [Difco, Cat # 211822] conc. 30 g/L.
Sterile 50 mL Falcon tube
Orbital shaker

Protocol: Prefill falcon tube with 15 mL of TSB.

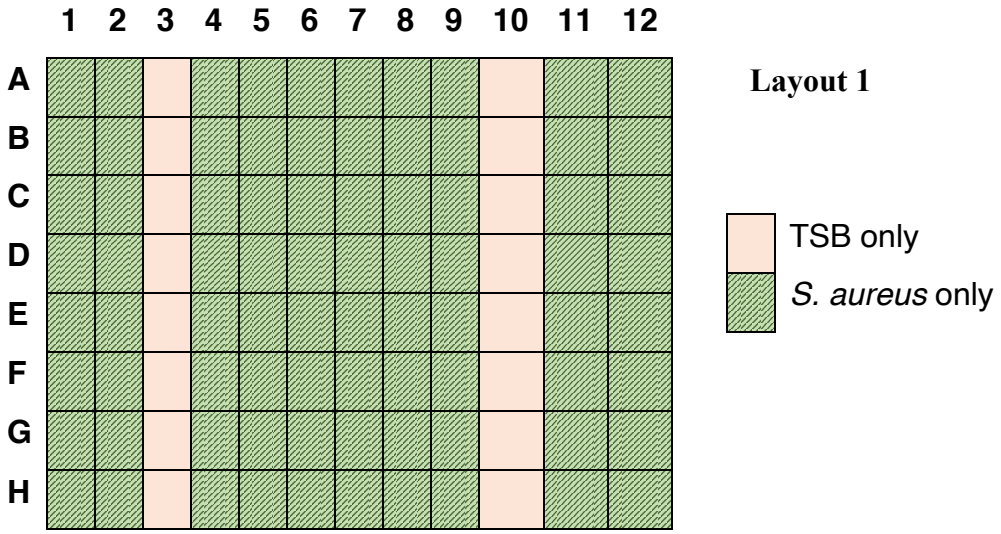
Using a sterile inoculation loop pick one colony from the day 1 streak plate and transfer it into the falcon tube. Incubate the inoculum at 37 ± 2 °C, 125 rpm, 1.9 cm orbital diameter for 18 hours. (Suggested time to incubate 15:00)

Day 3

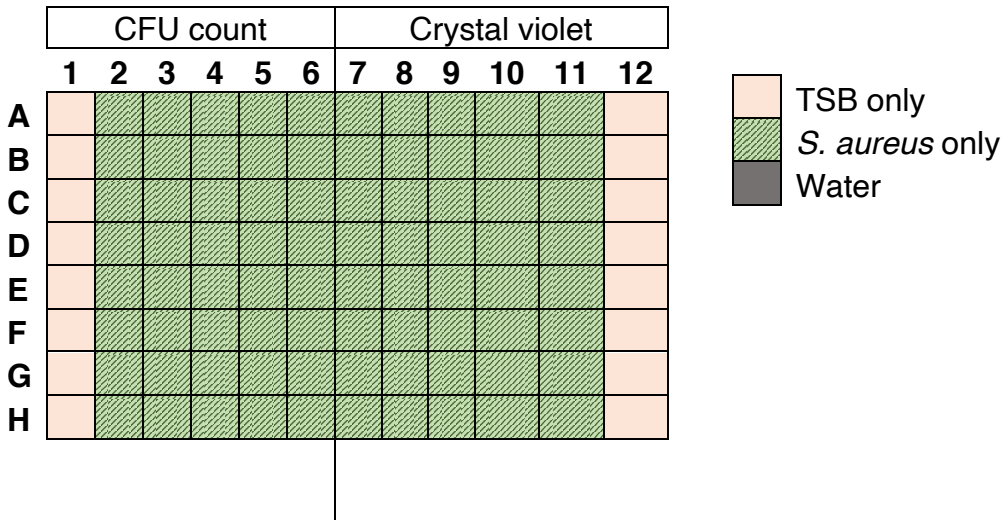
Materials and Equipment: Tryptic Soy Broth (TSB) [Difco, Cat # 211822] conc. 30 g/L.
Sterile 50 mL Falcon tube
Thermo Scientific Nunc Flat bottom sterile 96 well plate (Cat # 12-566-202)
Dilution tubes
Spectrophotometer
Vortexer
Orbital shaker

Protocol: Prefill 14 dilution tubes with 9 mL of TSB and 1 falcon tube with 9.9 mL TSB.

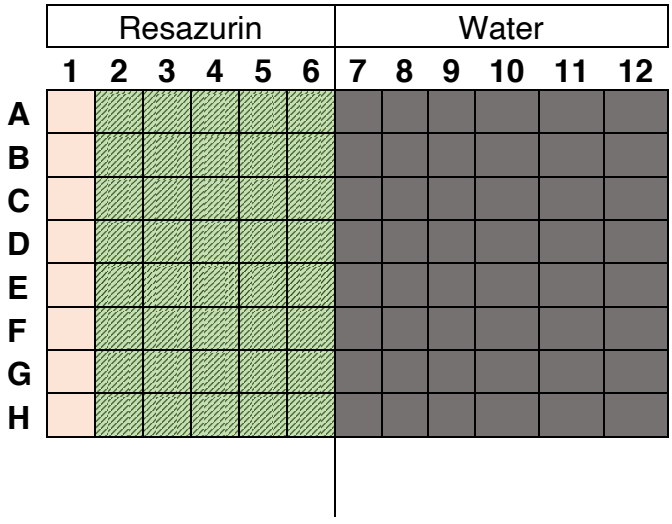
Remove the day 2 inoculum from incubation and vortex to mix. Pipette 100 µL of the inoculum into 9.9 mL of TSB. Incubate at 37 ± 2 °C, 125 rpm, 1.9 cm orbital diameter for approx. 3 hours or until optical density at 595 nm reaches 0.300 ± 0.020 (Use TSB as blank). Dilute the new inoculum 1:100 (total volume/plate = 20 mL) in fresh TSB and vortex to mix. Pipette 200 µL/well of the 1:100 dilution into two microtiter plates, following layout 1 for the control plate. Alternatively, for the treatment plate follow layouts 2 or 3 (depending on the assessment method to be performed) and only prepare 1 microtiter plate.



Layout 1

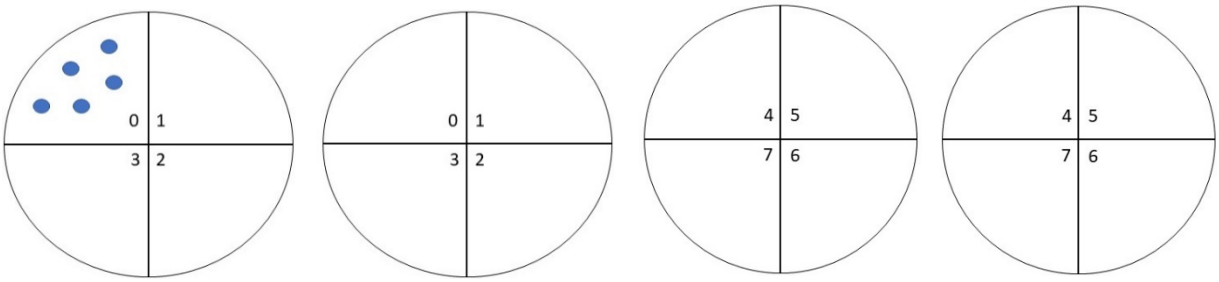


Layout 2



Layout 3

Serially dilute both inoculums by a factor of 10 up to 10^{-7}, and drop plate (10 µL/drop), 5 drops/ concentration, 2 plates per concentration as shown on TSA plates below:



Incubate TSA plates at 37 ± 2 °C for 24 hours and count colonies the next day. Input the colony count numbers in the spreadsheet provided. Resulting CFUs for the OD592 = 0.300 inoculums should be in the range of 7.5 ± 0.5 Log CFU/mL and the 1:100 dilutions should be in the range of 5.5 ± 0.5 Log CFU/mL.

For the control plates: Incubate one microtiter plate following your own laboratory's protocol. For the second microtiter plate, parafilm the sides and incubate at 37 ± 2 °C, no shaking for 24 hours.

For the treatment plates: Parafilm the sides of the plate and incubate at 37 ± 2 °C, no shaking for 24 hours.

If you notice condensation on your plates or if the climate in your location is very dry, put a tray of water at the bottom of the incubator.

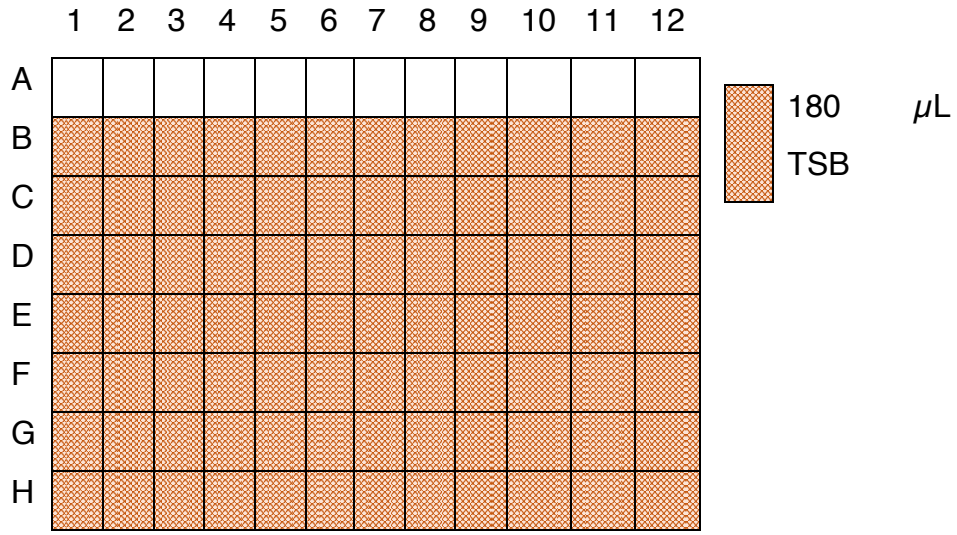
Day 4 (Data collection day) Control plate

For microtiter plate 1 if applicable use your own laboratory's protocol and run parallel to the corresponding ILP protocol. Run the protocols according to the plan in table 2.

CFU count ILP

Materials: Thermo Scientific Nunc Flat bottom sterile 96 well plates (Cat # 12-566-202)
Tryptic Soy Broth (TSB) [Difco, Cat # 211822] conc. 30 g/L.
Sterile wooden applicator sticks [Fisher brand, Cat # 01-340]
Phosphate Buffered Saline (PBS) [pH 7.4, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4]

Protocol: Prefill 96 well plates with fresh TSB (yellow wells), 180 µL/well according to the following layout:



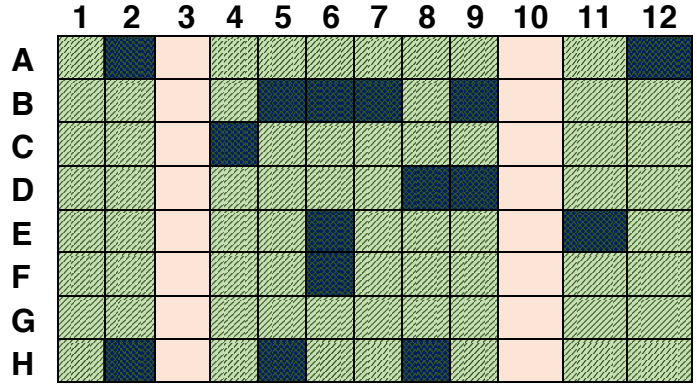
Remove planktonic suspension from the microtiter plate by pipetting. Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well. **(If any contaminated control wells are present discard the plate!)**

Wash the plate twice with 250 µL/well of PBS. Pipette the PBS in and out making sure not to disturb the biofilm. Leave the plates to dry for 10-15 mins (lid off, in laminar flow).

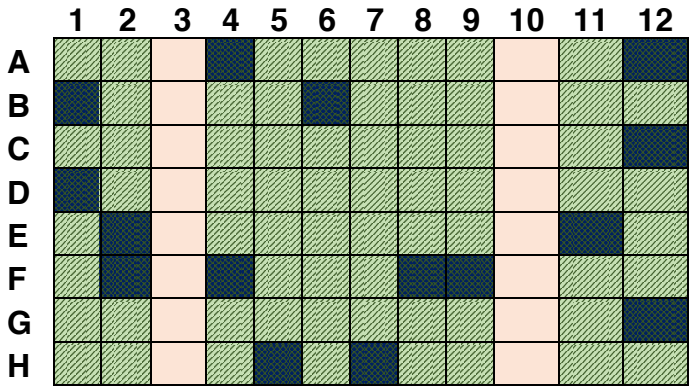
Add 200 µL/well of fresh TSB to each well. Use a wooden applicator stick to scrape the biofilm. Scrape the sides of the well and the bottom (3x each), shake the stick in the well after scraping to remove some of the biofilm left behind on the stick. **(Video guide available at: Training video)**

Scrape 15 wells/plate using the layouts below:

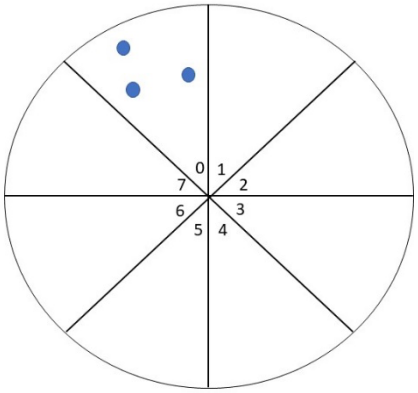
Pattern 1



Pattern 2



Mix the samples by pipetting and transfer to the top row of the prefilled plate. Serially dilute each sample by a factor of 10 going from row A (10^0) to row H (10^{-7}). Drop plate (10 µL/drop) on TSA plates using the following layout:



Incubate the plates at 37 ± 2 °C, for 24 hours. Count colonies the next day and input values in the spreadsheet provided.

Crystal violet ILP

Materials and equipment: PBS

3.5 g/L (vol/vol) Aqueous Crystal violet [Millipore Sigma; Cat #65092A-95]

100 % Ethanol

Sterile water

Plate reader

Protocol: Remove planktonic suspension from the microtiter plate by pipetting. Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well. **(If any contaminated control wells are present discard the plate!)**

Wash the plate twice with 250 µL/well of PBS. Pipette the PBS in and out making sure not to disturb the biofilm. Leave the plates to dry for 10-15 mins (lid off, in laminar flow).

Add 200 µL/well of 99-100 % Ethanol to the plate and incubate for 15 mins at room temperature (20 ± 5 °C), no shaking. Then remove the ethanol by pipetting. (Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well.)

Leave the plates to **dry fully** (lid off, in laminar flow) until no more ethanol is present in the wells.

Add 200 µL/well of 0.1 % (vol/vol) Crystal violet solution (diluted from stock in deionized water) and incubate at room temperature (20 ± 5 °C), no shaking for 15 min. Then remove the stain by pipetting. (Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well.)

Alternative Crystal violet stocks may be used. Make sure they are diluted to 0.1 % in deionized water!

Wash the plate twice with 250 µL/well of sterile water. Pipette the water in and out making sure not to disturb the biofilm. **(Make sure no drops of crystal violet are left behind on the sides or rims of the wells!)** Leave the plates to dry for 10-15 mins (lid off, in laminar flow).

Add 200 µL/well of 99-100 % Ethanol to the plate and incubate for 30 mins at room temperature (20 ± 5 °C), 125 rpm. Read the absorbance at 595 nm and input raw data in the spreadsheet provided. If values are above 1.300 dilute in 99-100 % ethanol and provide dilution factor with the data.

Resazurin ILP

Materials and equipment: PBS

MilliQ water

Resazurin (stock 0.1 mg/mL) [BTC; Cat #214215-1G]

Protocol: Remove planktonic suspension from the microtiter plate by pipetting. Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well. **(If any contaminated control wells are present discard the plate!)**

Wash the plate twice with 250 µL/well of PBS. Pipette the PBS in and out making sure not to disturb the biofilm. Leave the plates to dry for 10-15 mins (lid off, in laminar flow).

Make a 1:20 dilution (working solution = 5 µg/mL) of Resazurin stock in MilliQ water or sterile PBS, under sterile conditions and keep away from light.

Add 200 µL/well of working solution to the microtiter plate. Cover the plate in foil and incubate at room temperature (20 ±5 °C) for 60-90 minutes **(Depending on lab conditions this might take less or more time. Monitor color change and adjust time accordingly!)**, 125 rpm.

Measure fluorescence $\lambda_{excitation}$ = 560 nm; $\lambda_{emission}$ = 590 nm. Input the raw data into the spreadsheet provided.

Other resazurin solutions can be used if the concentration of resazurin or the recommended fluorescence are the same as above.

Day 4 (Data collection day) Treatment plate

CFU count / Crystal violet

Materials and equipment: PBS

3.5 g/L (vol/vol) Aqueous Crystal violet [Millipore Sigma; Cat #65092A-95]

100 % Ethanol

TSB

Wooden applicator sticks

Bleach

MilliQ water

Sterile water

Plate reader

Protocol: Prefill 96 well plates with 180 µL/well as on page 11.

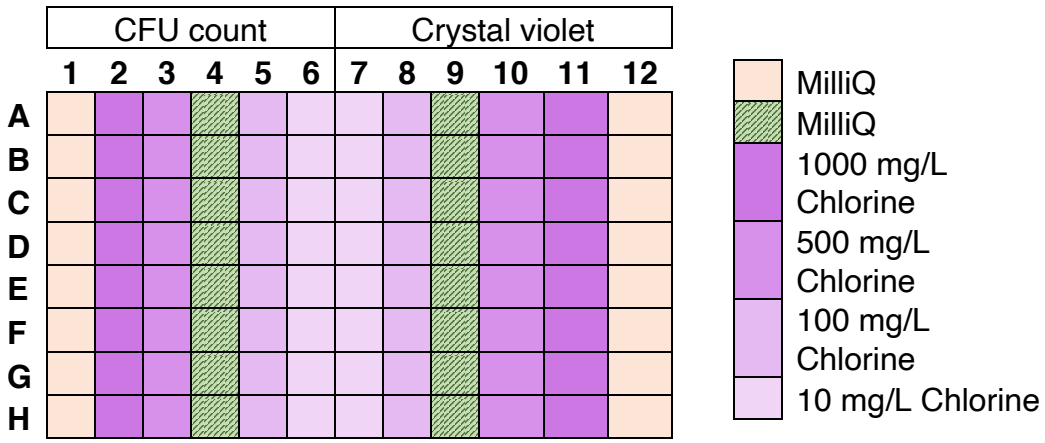
Start on the crystal violet side of the plate (Columns 7 to 12).

Remove planktonic suspension from the microtiter plate by pipetting. Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well. **(If any contaminated control wells are present discard the plate!)**

Wash the plate once with 250 µL/well of PBS. Pipette the PBS in and out making sure not to disturb the biofilm.

Repeat the process on the CFU count side (Columns 1-6).

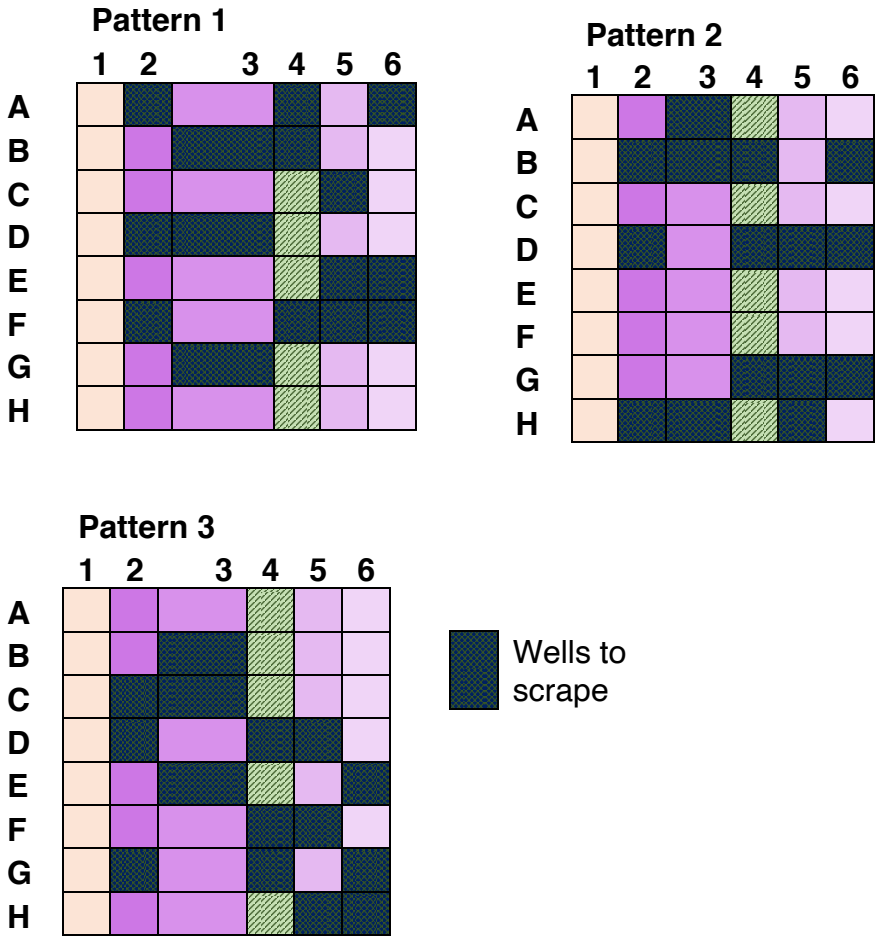
Starting on the Crystal violet side add 250 µL/well of disinfectant following the layout below:



Use a multichannel pipette and add each solution 2 minutes apart. When exactly 10 minutes have passed remove the disinfectant and wash twice with 250 µL/well of PBS. **(If you find that 2-minute intervals are too short you can also increase it to 3 minutes apart.)**

Leave the crystal violet side of the plate to dry with the lid off while you repeat the process on the CFU count side. Once done add 200 µL/well of 99-100 % ethanol to columns 7-12 and incubate on the bench for 15 minutes. Then remove the ethanol by pipetting.

While the ethanol dries add 200 µL/well of TSB to the wells in column (1-6) following the patterns below:



Scrape the wells, serially dilute and drop plate using the same technique described on pages 11-12. Counts the plates the next day and record the data in the spreadsheet provided.

When done with the CFU counts, add 200 µL/well of 0.1 % (vol/vol) Crystal violet solution (diluted from stock in deionized water) and incubate at room temperature (20 ± 5 °C), no shaking for 15 min. Then remove the stain by pipetting. (Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well.)

Wash the plate twice with 250 µL/well of sterile water. Pipette the water in and out making sure not to disturb the biofilm. **(Make sure no drops of crystal violet are left behind on the sides or rims of the wells!)** Leave the plates to dry for 10-15 mins (lid off, in laminar flow).

Add 200 µL/well of 99-100 % Ethanol to the entire plate and incubate for 30 mins at room temperature (20 ± 5 °C), 125 rpm. Read the absorbance at 595 nm and input raw data in the spreadsheet provided. If absorbance values are above 1.300 dilute in 99-100 % ethanol and provide dilution factor with the data.

Resazurin

Materials and equipment: PBS

MilliQ water

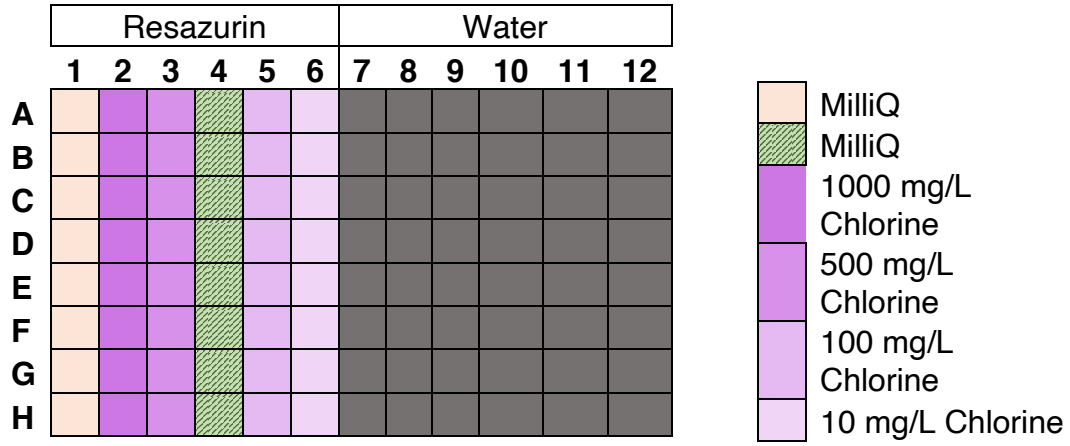
Bleach

Resazurin (stock 0.1 mg/mL) [BTC; Cat #214215-1G]

Plate reader

Protocol: Remove planktonic suspension from columns 1-6 of the microtiter plate by pipetting. Do not tilt plate and insert the pipette tip at a 45° angle making sure not to touch the sides or the bottom of the well. **(If any contaminated control wells are present discard the plate!)** Wash the plate once with 250 µL/well of PBS. Pipette the PBS in and out making sure not to disturb the biofilm.

Add 250 µL/well of disinfectant following the layout below:



Use a multichannel pipette and add each solution 2 minutes apart. When exactly 10 minutes have passed remove the disinfectant and wash twice with 250 µL/well of PBS. Leave to dry for 10-15 with the lid off under laminar flow. **(If you find that 2-minute intervals are too short you can also increase it to 3 minutes apart.)**

Make a 1:20 dilution (working solution = 5 µg/mL) of Resazurin stock in MilliQ water or sterile PBS, under sterile conditions and keep away from light.

Add 200 µL/well of working solution to the microtiter plate. Cover the plate in foil and incubate at room temperature (20 ±5 °C) for 1-2 hours **(Depending on lab conditions this might take less or more time. Monitor color change and adjust time accordingly!)**, 125 rpm.
Measure fluorescence $\lambda_{excitation}$ = 560 nm; $\lambda_{emission}$ = 590 nm. Input the raw data into the spreadsheet provided.

Supply list

Item	Supplier / Brand	Concentration	Storage
Bleach	Pure Bright	6 % NaOCl v/v	Shelf, Room temperature
Crystal violet	Millipore Sigma; Cat #65092A-95	3.5 g/L (0.35%) v/v	Shelf, Room temperature
Deionized water	Laboratory water system	N/A	N/A
Ethanol	Decon™ Labs, Cat # 2705HC	200 Proof (100%)	Shelf, Room temperature
Flat bottom, sterile, polystyrene, 96 well plates	Thermo Scientific, Cat # 12-566-202	N/A	Shelf, Room temperature
MilliQ water	Laboratory water system	N/A	N/A
Phosphate buffered Saline	Made in-house Sterilize by autoclaving or filtration	137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4. pH 7.4	Shelf, Room temperature
Resazurin	BTC; Cat #214215-1G	Stocks made at 0.1 mg/mL in PBS	Powder (Shelf) Stock (+ 4 °C; dark)
Sodium dithionite	Sigma Aldrich; Cat #7775-14-6	N/A	Shelf

Staphylococcus aureus subsp. aureus	ATCC® 25923™	Glycerol stocks	-80 °C
Syringe filters, Sterile	Corning, Cat # 431224	0.22 *μ*m Nylon pore	Shelf
Tryptic Soy Agar	Difco, Cat #236290	40 g/L	Shelf
Tryptic Soy Broth	Difco, Cat # 211822	30 g/L	Shelf
Wooden applicator sticks	Fisher brand, Cat # 01-340	N/A	Drawer

S4.2.2. Protocols UGhent

Crystal violet staining

After growing a biofilm in a 96-well microtiter plate:

- Remove the supernatant
- Wash with 100 µL physiological saline
- Add 100 µL 99% methanol to each well (denaturation and dehydration of the cells => fixation)
- After 15 min, remove the methanol by turning the plate upside down above the special container in the sink
- Dry plates in the incubator (37°C) until all methanol is evaporated (place microtiter plate without lid in an incubator)
- Add 100 µL of a 0.1% crystal violet solution to each well (stock solution is 0.5%)
- Let stand on the bench for 20 min
- After 20 min, put on gloves and remove the crystal violet solution by turning the plate above the container in the sink
- Wash under running tap water and dry plates by placing them on absorbent paper
- Add 150 µL of a 33% acetic acid solution
- Incubate the microtiter plate on a rotator (450 rpm) for at least 20 minutes (all crystal violet should be in solution)
- Measure the absorbance of all wells at 590 nm (EnVision protocol for round bottomed plates: KV abs @ 590 nm)

Comments:

It is not necessary to work sterile!

Average absorbances at 590 nm are calculated and the net absorbances are determined by taking into account the absorbance values of the negative growth controls (=blanc values).

Resazurin staining

After growing a biofilm in a 96-well microtiterplate:

- Remove the supernatant
- Wash with 100 µL physiological saline
- Prepare a resazurin solution by adding 10.5 mL physiological saline to 2.1 mL aliqots of resazurin stored in the freezer (-20°C) (1/6 dilution of commercial available CTB solution Promega)
- Add 120 µL resazurin solution to each well (first the blanks)
- Incubate 1 hour (or more depending on the strain using) at 37°C (also strain dependent) **protected from light**

  (time: 20min for *P. acnes,* 30min for *S. aureus,* 1h for *B. cenocepacia*)
- Measure fluorescence (l_{ex}: 560 nm and l_{em}: 590 nm (Nvision: CTB Heleen)
  Calculate the mean of different wells and correct for blank fluorescence values

Work sterile!

CFU count for biofilms from 96 well plate

After growing/treating biofilm

- Remove supernatant
- Wash biofilms with 100 µL physiological saline
- Add 100 µL physiological saline to each well
- Replace the lid with a sealing film
- Vortex the 96 well plate for 5 min at 900 rpm
- Place the 96 well plate in the sonificator (42 Hz ± 6%) for 5 min
- Pipette the 100 µL out of the wells and add to 1.8 mL physiological saline
- Add 100 µL physiological saline to each well
- Vortex the 96 well plate for 5 min at 900 rpm
- Place the 96 well plate in the sonificator (42 Hz ± 6%) for 5 min

- Pipette the 100 µL out of the wells and add to the same 1.8 mL physiological saline
- Pipette 1 mL from the now 2 mL physiological saline (10^-1) and add to 9mL physiological saline (10^-2)
- Repeat until desired dilution(s)
- Pipette 1 mL of desired dilution(s) into petri dish
- Add 15-20 mL of agar to petri dish and mix to distribute cells (MHA for *S. aureus*)

S4.2.3. Protocols UMC, Amsterdam

Culturing; 96 wells plate Biofilm dispersion by sonication

- Sonicate the micro titer plate for 5 minutes in a ultrasonic water bath (Elma; Transsonic 460; 35 Hz):
    - Use a sealing film (Greiner; easy seal) to cover the micro titer plate (press carefully on each well), put a lid on the plate and seal it with parafilm. Put the micro titer plate in a plastic bag, which must also be sealed to prevent contamination during sonication. (This construction can float in the ultrasonic water bath.)

- After sonication pipet several times check visually (microscopically if necessary) if the biofilm has been dispersed properly.
- Take ~100 µl from the sonicated biofilm plate and add it to the first column of a fresh plate to make dilutions.
- Plate dilutions on blood agar plates (for example 2 x 10 µ).

96 wells plate Biofilm detection using Crystal violet staining

- Wash the biofilm very carefully 2x (or more depending on you assay) by pipetting off the supernatant (only in the corner with your tip) and adding PBS. Repeat once, second wash only remove the PBS.

- Stain adherent bacteria with 125 µl of 1% crystal violet for **10 min** at RT.

- Wash wells 4x with water and dry plate on paper towels.

- Add 150 µl 96% ethanol to each well to dissolve crystal violet. Incubate **10 min** at RT.

- Transfer 100 µl from each well to a new 96-wells flat bottom plate. Measure optical density at 595 nm.

S4.2.4. Protocols UHelsinki

B1. Resazurin staining

- Make a 1:20 dilution of the resazurin stock in PBS
- Wash the biofilms once with 200 µL PBS (or MQ-water), carefully to avoid harming the biofilms
- Add 200 µL of the resazurin diluted solution per well
- Incubate in RT, darkness, 200 rpm for about 45 min (for *S. aureus 25923* but *P. aeruginosa* needs about 1h30)
- Measure fluorescence at $\lambda_{excitation}$ = 560nm and $\lambda_{emission}$ = 590nm

B2. Crystal violet staining

- Remove the resazurin stain from the plate
- Fix the biofilms with EtOH by adding 200 µL per well
- Incubate 15 min, RT, no shaking
- Remove the ethanol carefully and let the wells dry COMPLETELY (remove lid, takes about 30 min)
- Add 190 µL crystal violet (100X diluted) stain carefully without touching the walls of the wells
- Stain for 5 min, RT, no shaking
- Remove the stain carefully
- Wash the wells 2 times with MQ-water
- Let air dry (for 5-10 min)
- Solubilize the stain in 100% ethanol and incubate in RT for at least 1h (but no more than 3h) (I do 1h30)
- Measure absorbance at 595 nm

S4.2.5. Protocols UAntwerp

Resazurin assay *Staphylococcus* Biofilms in 96-well plates
Keywords

S. aureus,S. epidermidis, biofilm, resazurin

Materials

- 96-well plates, polystyrene, flat bottom (greiner bio-one BVBA, 655182) with *Staphylococcus* biofilm
- Automatic multichannel pipette
- Vacusafe 8-channel Vacuum pump system adapted for 96-well plates (afzuigtoestel bacterio)
- E.p. tips 2-200 µL, order number 0030.000.870 (= tips Vacusafe)
- E.p. tips 50-1250 µL, order number 0030.000.935 (= tips automatic multichannel pipette)
- Tecan
- Multistep pipette (eppendorf)

Reference number	Firm	product	LMPH number
14190-094	Gibco, lifetechnologies	PBS (sterile)	
		Resazurin 	
Lab M, Lab 004	International medical products	Trypton Soy Broth (TSB)	

Methods

- Discard the medium using the Vacusafe system (be careful you don't touch the bottom of the wells in order to avoid disruption of the biofilm)
- Add 100 µL PBS to each well using an automatic 8-channel pipette
- Discard the PBS using the Vacusafe system (be careful you don't touch the bottom of the wells in order to avoid disruption of the biofilm)
- Add 100 µL PBS to each well using an automatic 8-channel pipette
- Discard the PBS using the Vacusafe system (be careful you don't touch the bottom of the wells in order to avoid disruption of the biofilm)
- Add 200 µL TSB to each well using an automatic 8-channel pipette
- Add 10 µL Resazurin to each well using a multistep pipette
- Incubate for 30 minutes at 37°C in the dark
- Measure fluorescence (λ_{ex} 550nm – λ_{em} 590nm)

Remarks

For 24-well plates use 1mL PBS/well, 2mL TSB/well and 100 µL Resazurin/well

Crystal Violet assay *Staphylococcus* Biofilms in 96-well plates

Keywords

Staphylococcus, biofilm, crystal violet

Materials

- 96-well plates, polystyrene, flat bottom (greiner bio-one BVBA, 655182) with *Staphylococcus* biofilm
- Automatic multichannel pipette
- Vacusafe 8-channel Vacuum pump system adapted for 96-well plates (afzuigtoestel bacterio)
- E.p. tips 2-200 µL, order number 0030.000.870 (= tips Vacusafe)
- E.p. tips 50-1250 µL, order number 0030.000.935 (= tips automatic multichannel pipette)
- Telix (Multiskan MCC 340 Microplate reader, Labsystems)
- Chemical Fume hood

Reference number	Firm	product	LMPH number
		PBS (non-sterile)	
20903.368	VWR international	Technical Methanol 	202
94448-2.5L-F	Merck	Gram's Crystal Violet solution	185
MERC1.000 63.2500	Merck, VWR	Glacial Acetic Acid	443
		DemiQ (non-sterile)	

Methods

- Discard the medium using the Vacusafe system (be careful you don't touch the bottom of the wells in order to avoid disruption of the biofilm)
- Add 100 µL PBS to each well using an automatic 8-channel pipette
- Discard the PBS using the Vacusafe system (be careful you don't touch the bottom of the wells in order to avoid disruption of the biofilm)
- Add 100 µL PBS to each well using an automatic 8-channel pipette
- Discard the PBS using the Vacusafe system (be careful you don't touch the bottom of the wells in order to avoid disruption of the biofilm)
- Add 150 µL methanol to each well using an automatic 8-channel pipette (in chemical fume hood polyvalent lab)
- Incubate for 15 minutes at room temperature
- Remove the methanol by turning upside down the plate above a suitable box in order to collect the methanol
- Remove the residual methanol by gently dabbing the plate on a green tissue
- Air dry plates in chemical fume hood
- In the meantime, you can prepare the 33% (v/v) Glacial Acetic Acid and 0,005% crystal violet solution
- Add 200 µL 0,005% crystal violet to each well using an automatic 8-channel pipette in chemical fume hood
- Incubate for 5 minutes at room temperature
- Remove the crystal violet by turning upside down the plate above a suitable

box in order to collect the crystal violet
- rinse the plate under running tap water and collect the crystal violet through a funnel in an appropriate waste recipient
- Remove the residual crystal violet by gently dabbing the plate on a green tissue
- Air dry plates (the wells have to be dry completely)
- Add 250 µL 33% Glacial Acetic Acid to each well using an automatic 8-channel pipette
- Incubate for 15 minutes at room temperature
- Measure OD_{570nm} with telix (mix 45 seconds before measuring)

Preparation of 0,005% (v/v) Crystal violet (fume hood):

Dilute Gram's Crystal Violet solution 1:200
in non-sterile DemiQ Protect it from light
by wrapping the bottle in tinfoil

Preparation of 33% (v/v) Glacial Acetic Acid (fume hood):

Add 330 mL Glacial Acetic Acid to 670 mL non-sterile DemiQ

Remarks
You can perform this assay in a non-sterile way

S4.3. Control experiments data with Lab 5

Data analysis was not performed for the treatment experiments as there was missing data necessary for the analysis. Including lab 5 data in the statistical analysis increases the reproducibility SD for all three methods. The biggest effect is observed for the plate count method, where the lab 5 dataset stands out as an outlier (~ 2.25 log difference from the mean). It was not possible to verify if the data was properly input in the datasheet due to lack of lab book records.
Summary of the control experiments results when data collected by Lab 5 were included.

Table S4.3.1 Summary of analysis of the control data for the interlab protocol (ILP) with Lab 5 data included.

Method	Mean Log ± SE	Units	Variance components		Standard deviation	
			Day + Error	Lab	Repeatability	Reproducibility
Plate count	7.77 ± 0.56	CFU/well	3.3%	96.7%	0.25	1.37
Resazurin	0.56 ± 0.24	µg/mL	10.5%	89.5%	0.19	0.61
Crystal Violet	1.24 ± 0.19	µg/mL	7.7%	92.3%	0.13	0.48

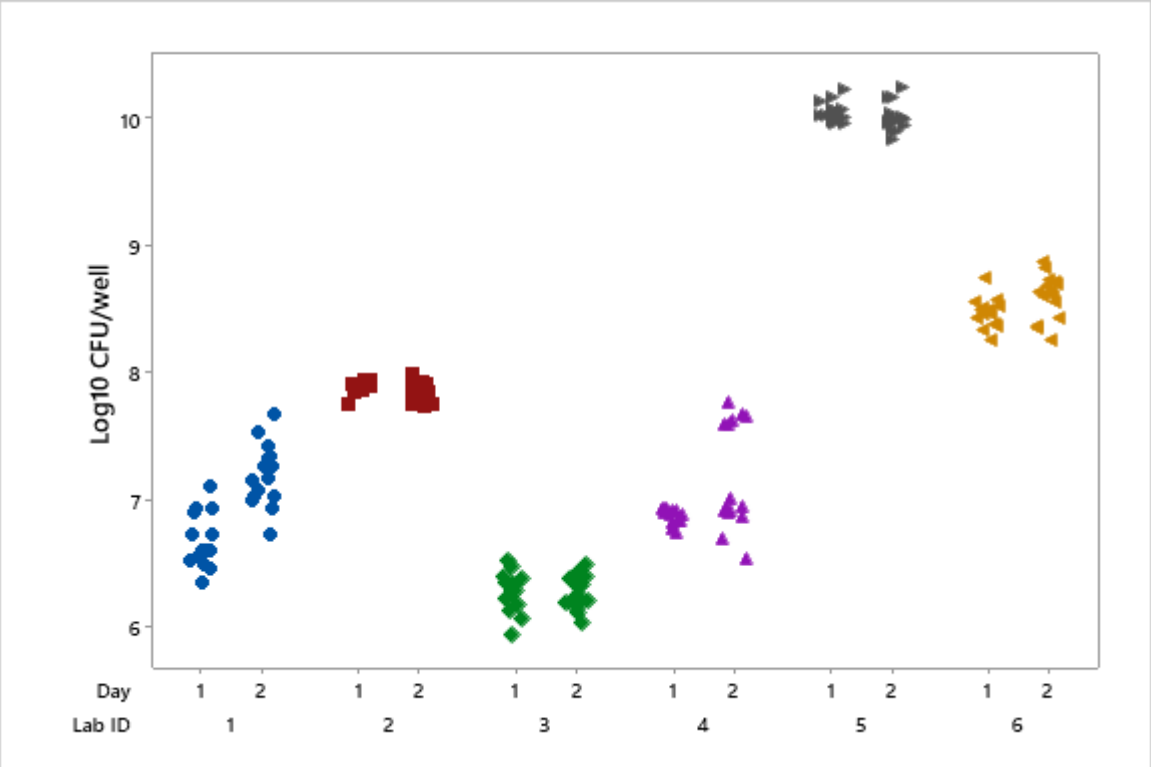


Figure S4.3.1. Individual value plot of control experiment results for the plate count method with Lab 5 data included Along the horizontal axis are listed the lab IDs and the two experimental days within each lab. Horizontal jitter has been applied to better visualize data points.

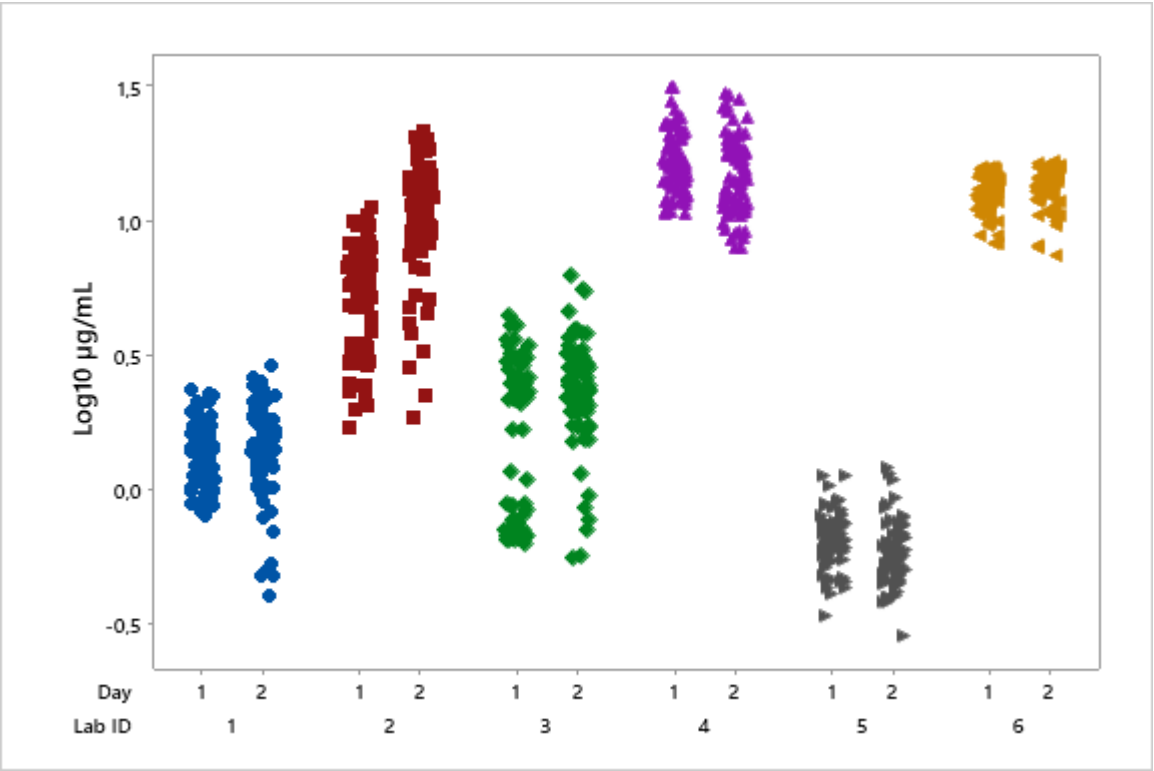


Figure S4.3.2 Individual value plot of control experiment results for the resazurin method with Lab 5 data included Along the horizontal axis are listed the lab IDs and the two experimental days within each lab. Horizontal jitter has been applied to better visualize data points.

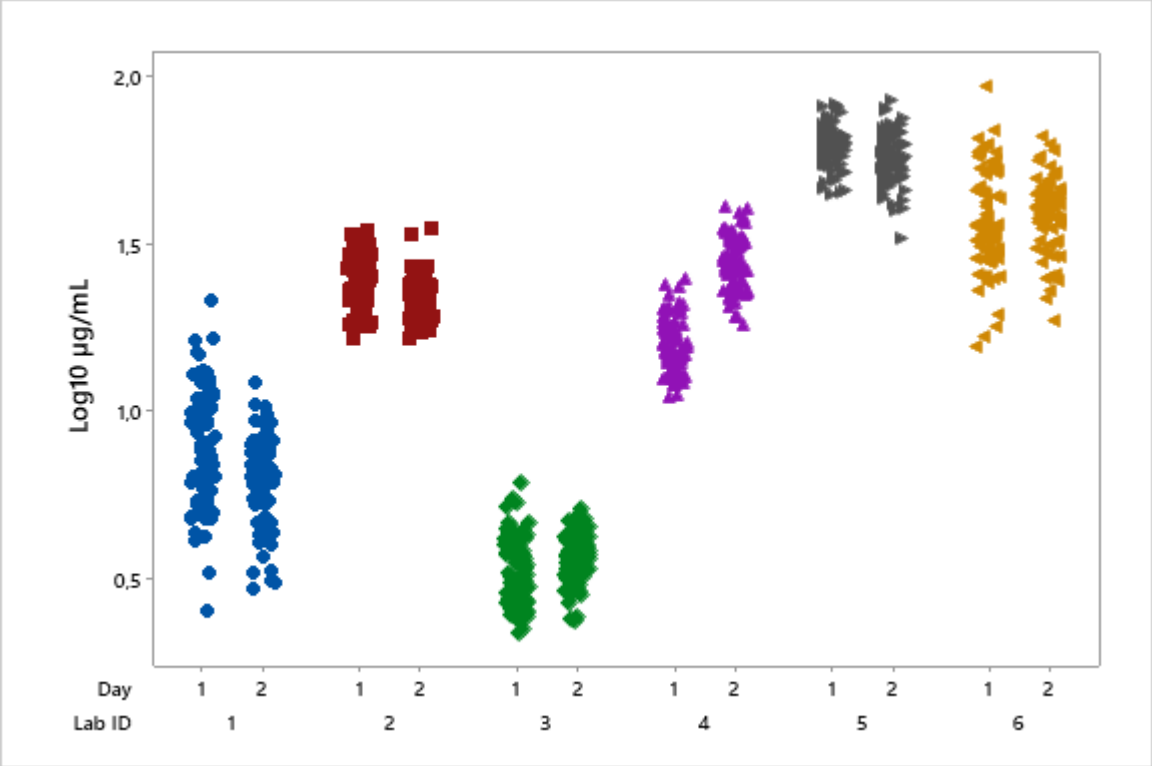


Figure S4.3.3. Individual value plot of control experiment results for the crystal violet method with Lab 5 data included Along the horizontal axis are listed the lab IDs and the two experimental days within each lab. Horizontal jitter has been applied to better visualize data points.

S4.4. Plate reader test results

Summary of the results for plate reader tests performed for crystal violet and resazurin. Standard curves and equations are shown for each individual laboratory.

S4.4.1. Resazurin

The fluorescence data collected from the plate reader test with the chemically reduced resazurin was used to plot the standard curves for each laboratory. Based on this data, a regression analysis was performed for the following:

- Fluorescence vs Resorufin concentration
- Log Fluorescence vs Log Resorufin concentration
- Log Fluorescence vs Resorufin concentration
- Fluorescence vs Log Resorufin concentration

Based on the regression analysis, Log Fluorescence vs Log Resorufin was chosen as the standard curve for the resazurin data. The residual plots (not shown) from the regression analysis indicated better fit of the Log vs Log model to the data, and better adherence to the modelling assumptions (normality and constant variance). This allows for better prediction of

data points outside the range of the standard curve, which was necessary in the next steps of the statistical analysis.

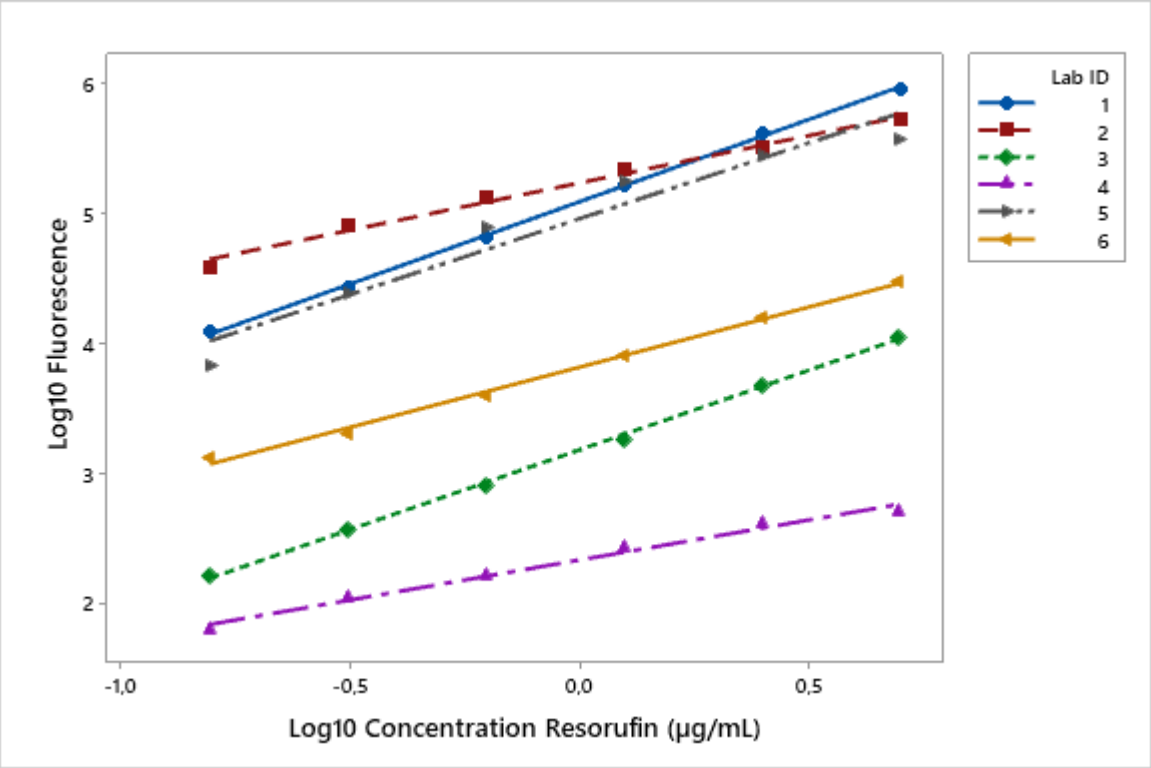


Figure S4.4.1. Scatter plot of Log Fluorescence vs Log Resorufin concentration for all 6 participating laboratories.

The equations for each lab were generated by linear regression analysis and they were used to transform the fluorescence outputs into log resorufin concentration.

Equations per Lab

Lab ID			
1	Log Fluorescence	=	5.0866 + 1.2650 Log Concentration
2	Log Fluorescence	=	5.2335 + 0.7284 Log Concentration
3	Log Fluorescence	=	3.1730 + 1.2260 Log Concentration
4	Log Fluorescence	=	2.3248 + 0.6157 Log Concentration
5	Log Fluorescence	=	4.9561 + 1.1667 Log Concentration
6	Log Fluorescence	=	3.8140 + 0.9248 Log Concentration

S4.4.2. Crystal Violet

The optical density (OD) data collected from the plate reader test was used to plot the standard curves as a function of crystal violet concentration for each lab. Regression analysis for the Absorbance vs Crystal violet concentration was performed.

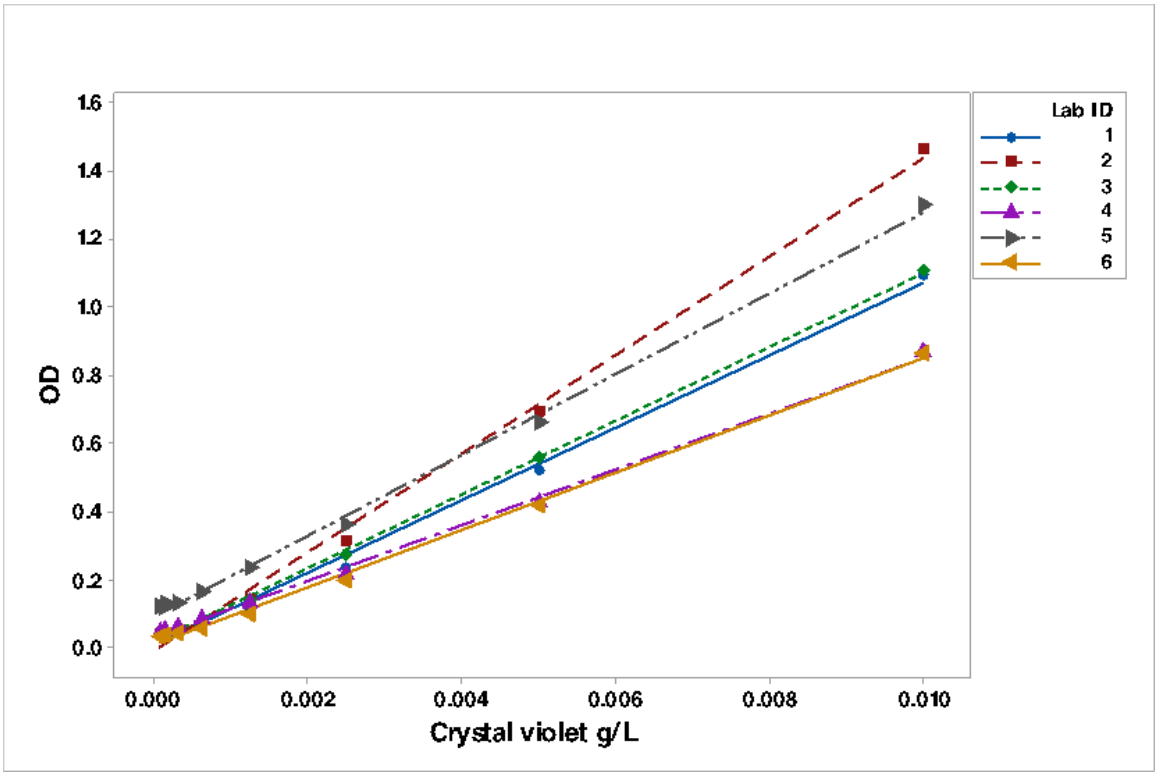


Figure S4.4.2. Scatter plot of OD vs Crystal violet concentration for all 6 participating laboratories.

The equations for each lab were generated by linear regression analysis and were then used to transform the absorbance data to crystal violet concentrations. The crystal violet concentrations were further transformed from g/L to µg/mL.

Equations per Lab

Lab ID			
1	OD	=	0.00485 + 106.70 Concentration
2	OD	=	-0.01042 + 145.04 Concentration
3	OD	=	0.01581 + 108.60 Concentration
4	OD	=	0.02924 + 82.41 Concentration
5	OD	=	0.09051 + 119.00 Concentration
6	OD	=	0.00662 + 84.23 Concentration

S4.5. Supplementary figures.

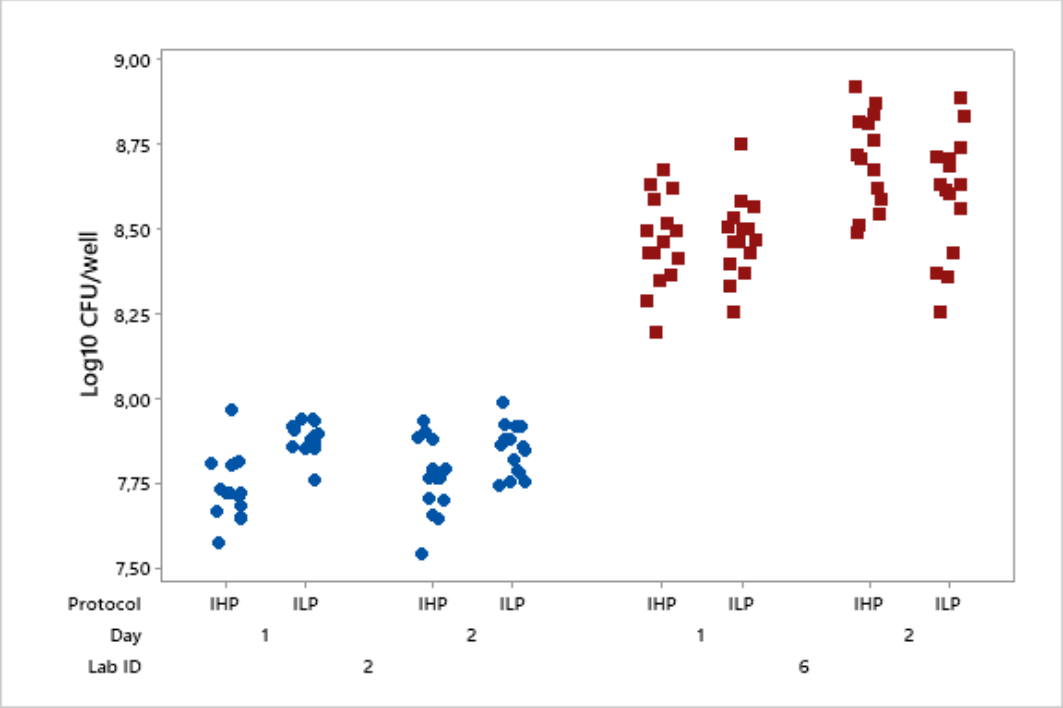


Figure S4.5.1. Control experiment data comparing ILP and IHP protocols. Each point in the graph is the log density (LD=log(CFU/well)) of biofilm bacteria grown on a single well. Along the horizontal axis are listed the lab IDs, the two experimental days within each lab and the two protocols within each experimental day.

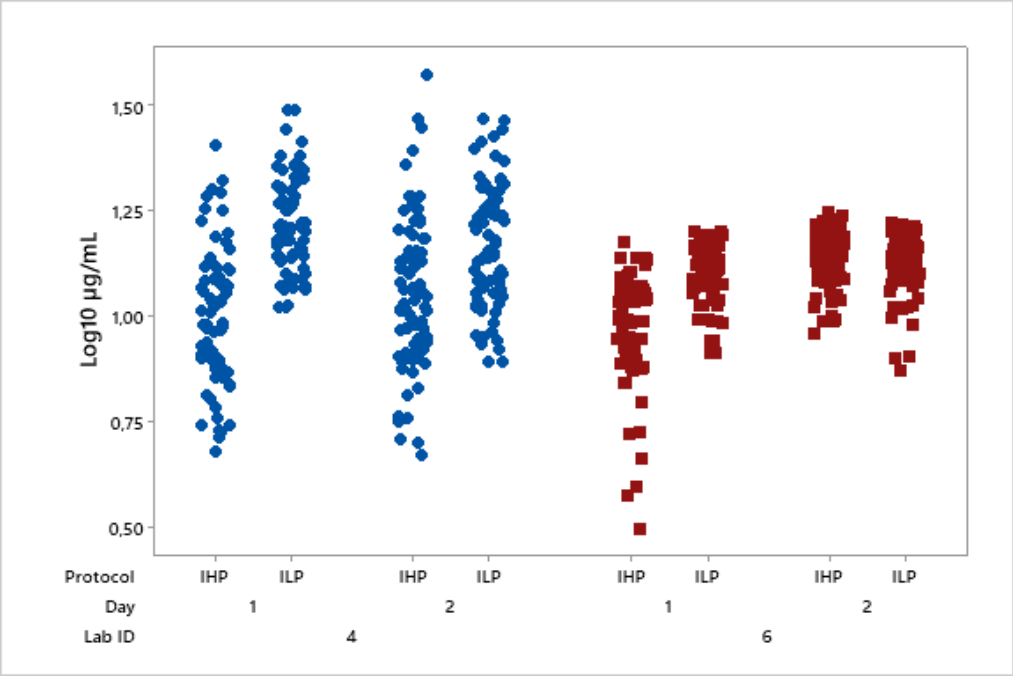


Figure S4.5.2. Control experiment data comparing IHP and ILP protocols. Each point in the graph is the log resorufin concentration (µg/mL) of biofilm bacteria grown on a single well. Along the horizontal axis are listed the lab IDs, the two experimental days within each lab and the two protocols within each experimental day.

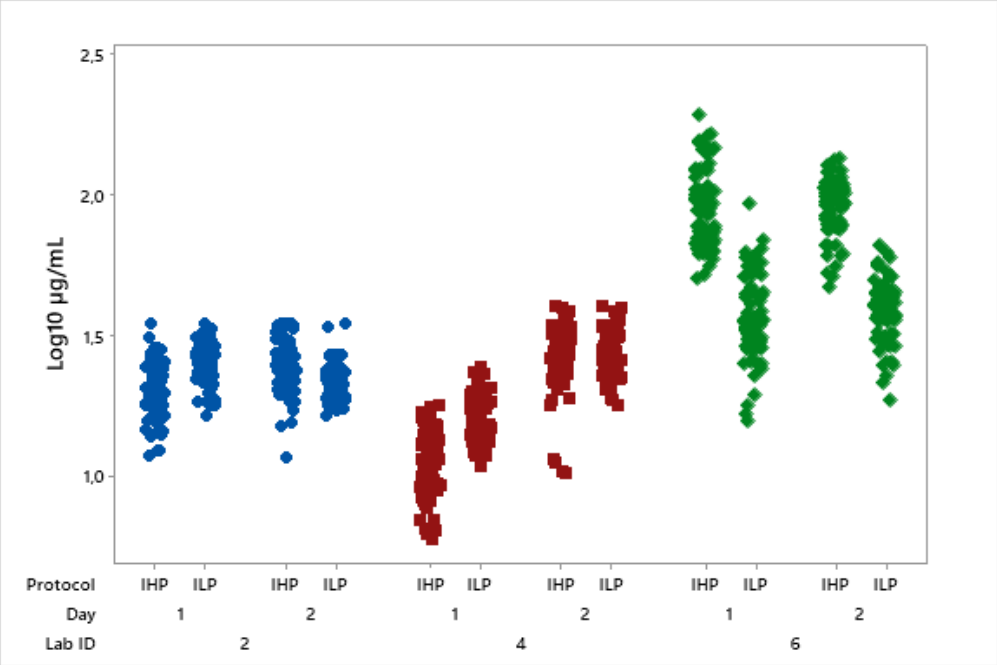


Figure S4.5.3. Control experiment data comparing IHP and ILP protocols. Each point in the graph is the log crystal violet concentration (µg/mL) of biofilm bacteria grown on a single well. Along the horizontal axis are listed the lab IDs, the two experimental days within each lab and the two protocols within each experimental day.

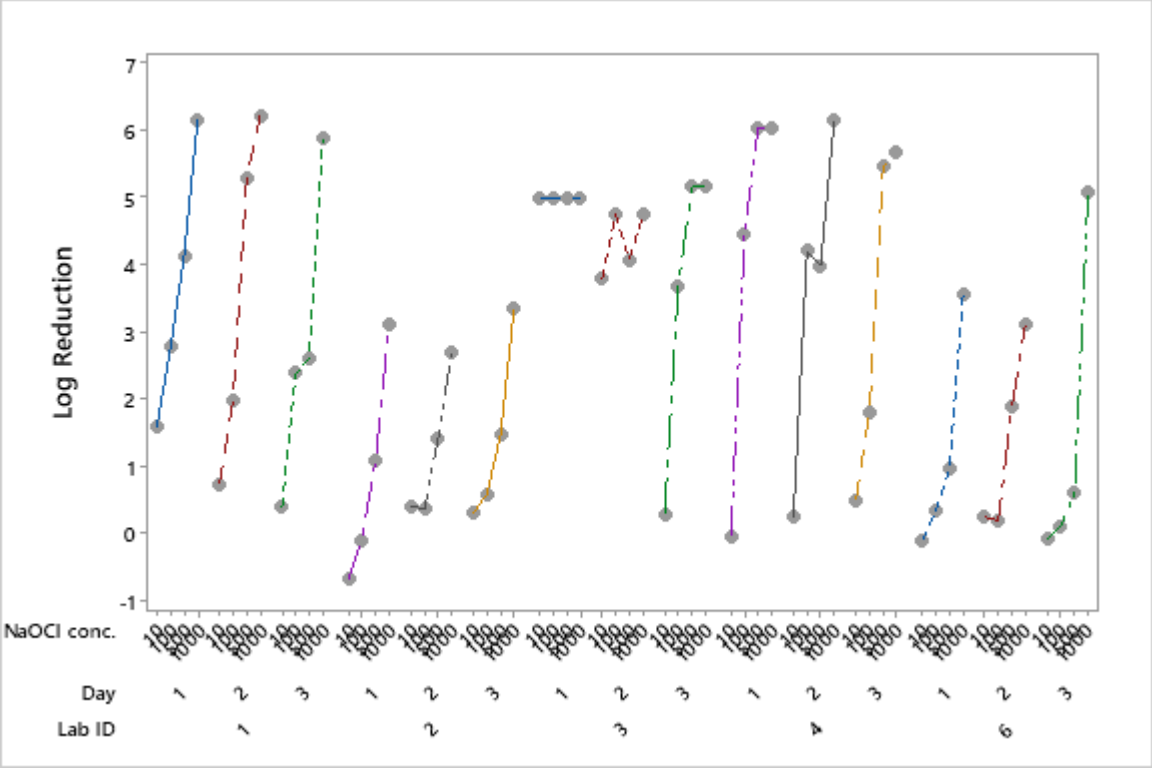


Figure S4.5.4. Treatment experiment data for the plate count method. Log reductions (LR) for the NaOCl treatment in the multi-lab study. The horizontal axis lists all lab IDs, the three experimental days and the 4 NaOCl concentrations tested. Each point in the figure is the mean LR for a single concentration of disinfectant in a single experiment.

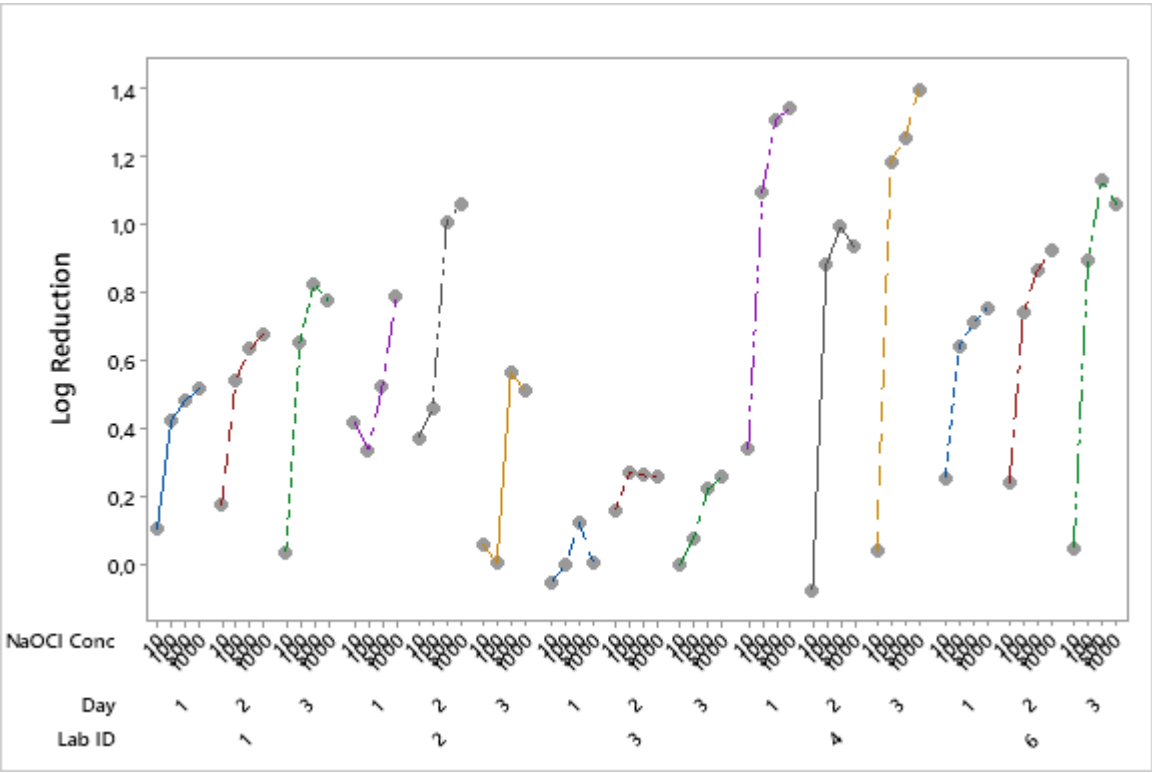


Figure S4.5.5. Treatment experiment data for the resazurin method. Log reductions (LR) for the NaOCl treatment in the multi-lab study. The horizontal axis lists all lab IDs, the three experimental days and the 4 NaOCl concentrations tested. Each point in the figure is the mean LR for a single concentration of disinfectant in a single experiment.

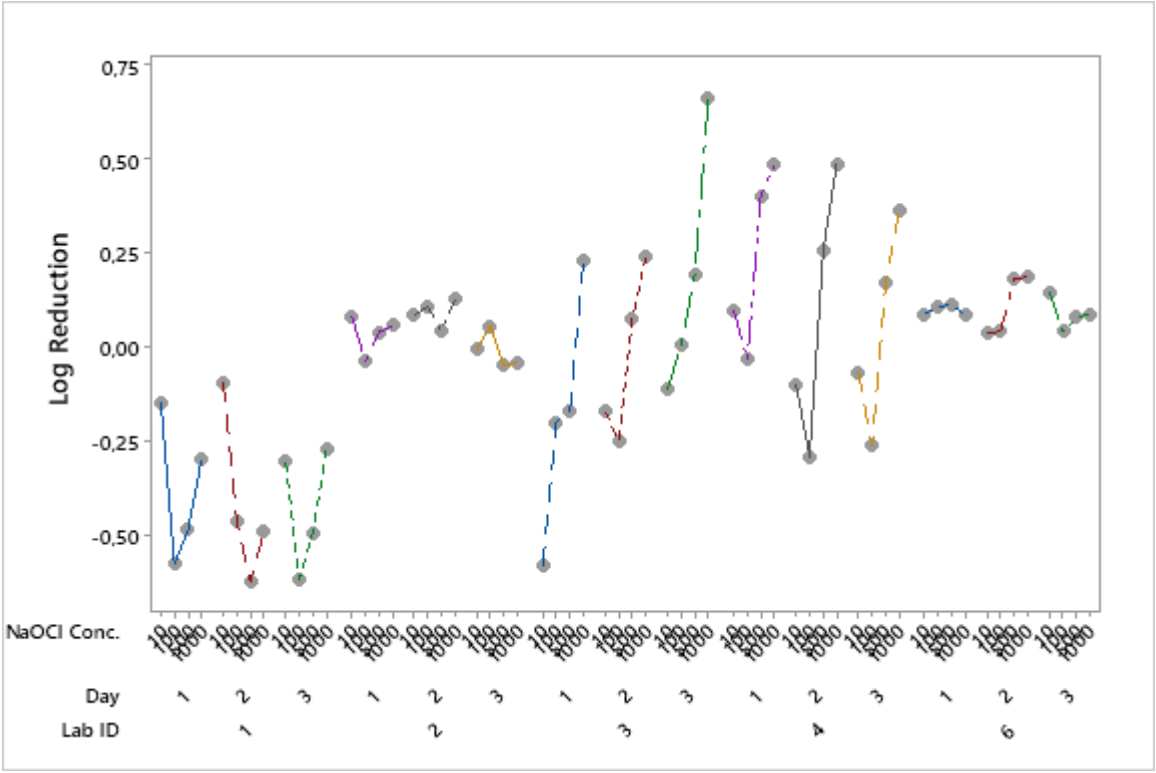


Figure S4.5.6. Treatment experiment data for the crystal violet method. Log reductions (LR) for the NaOCl treatment in the multi-lab study. The horizontal axis lists all lab IDs, the three experimental days and the 4 NaOCl concentrations tested. Each point in the figure is the mean LR for a single concentration of disinfectant in a single experiment.

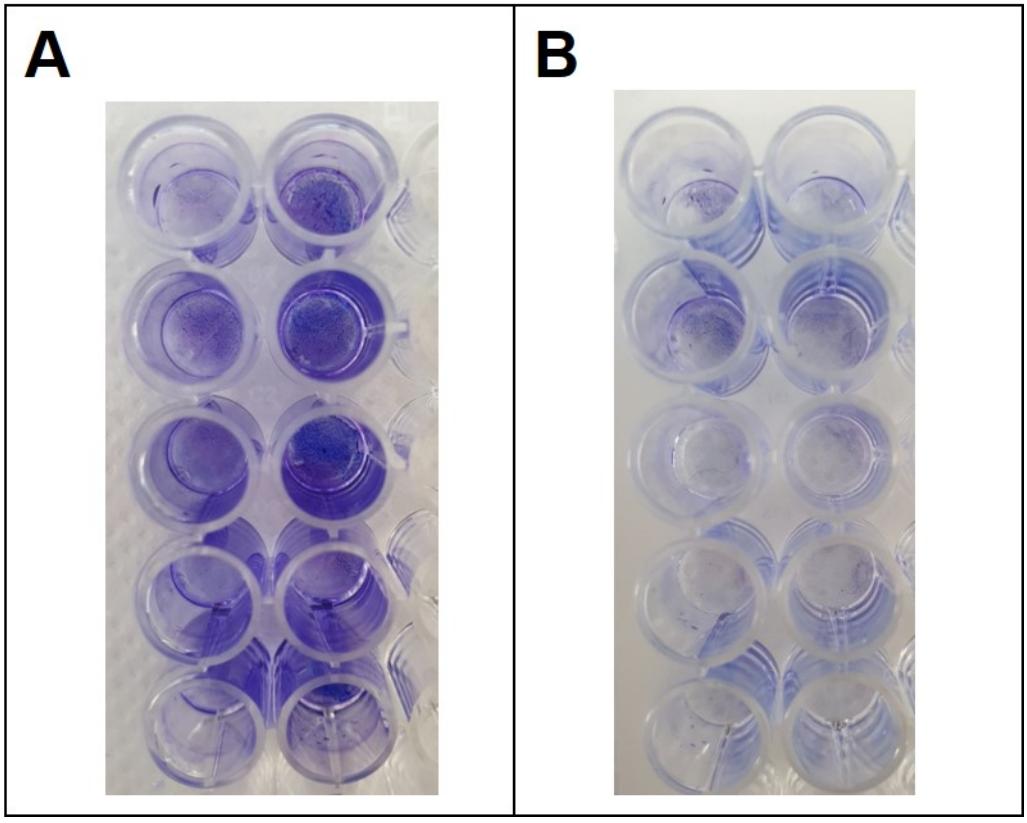


Figure S4.5.7. Interaction between NaOCl and crystal violet. Crystal violet staining of 96-well plates post incubation with different concentrations of NaOCl measured as Cl **A.** 2% vol/vol; **B.** 0.1 % vol/vol.

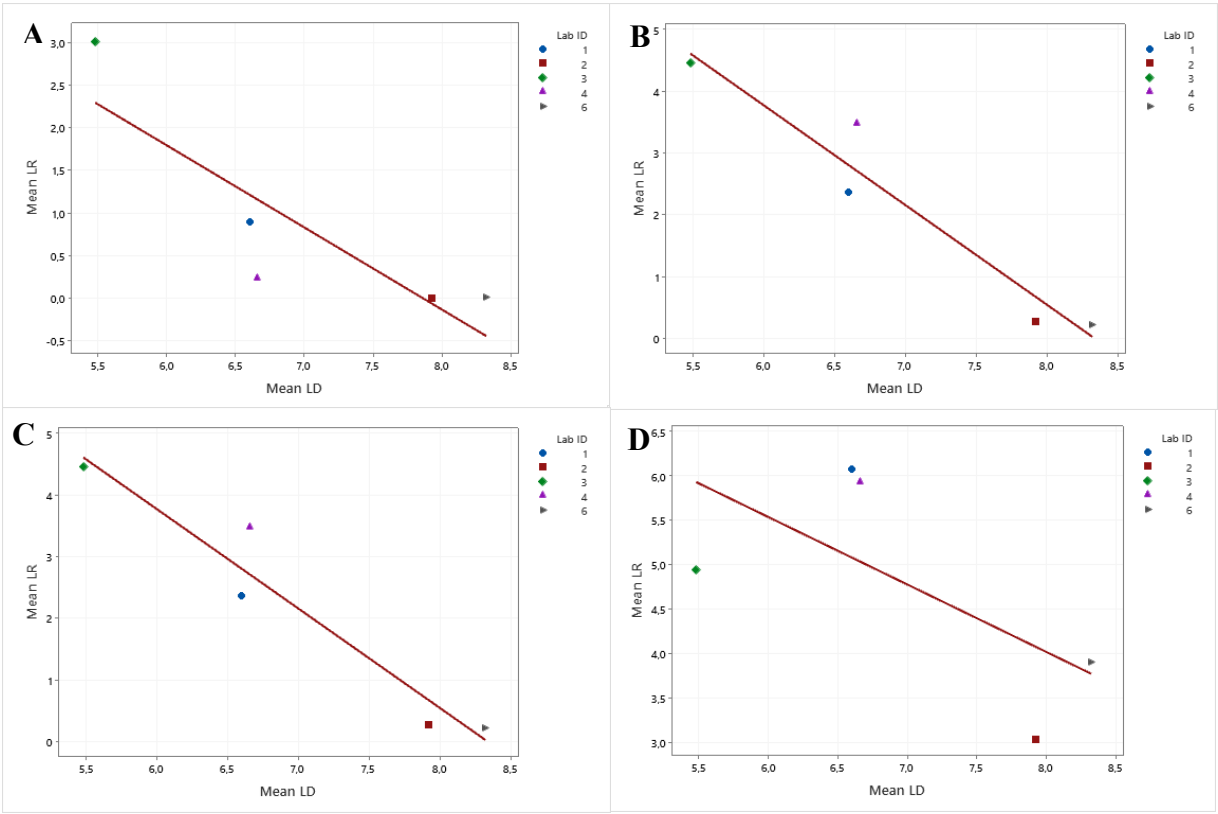


Figure S4.5.8. Relationship between LR and LD in the treatment experiments for the plate count method. Each data point represents the Mean LR and corresponding Mean LD for each lab at different concentrations of NaOCl: **A.** 10 mg/L **B.** 100 mg/L **C.** 500 mg/L **D.** 1000 mg/L

Chapter 5. Evaluation of multispecies biofilm methods

S5.1. Selective media optimisation

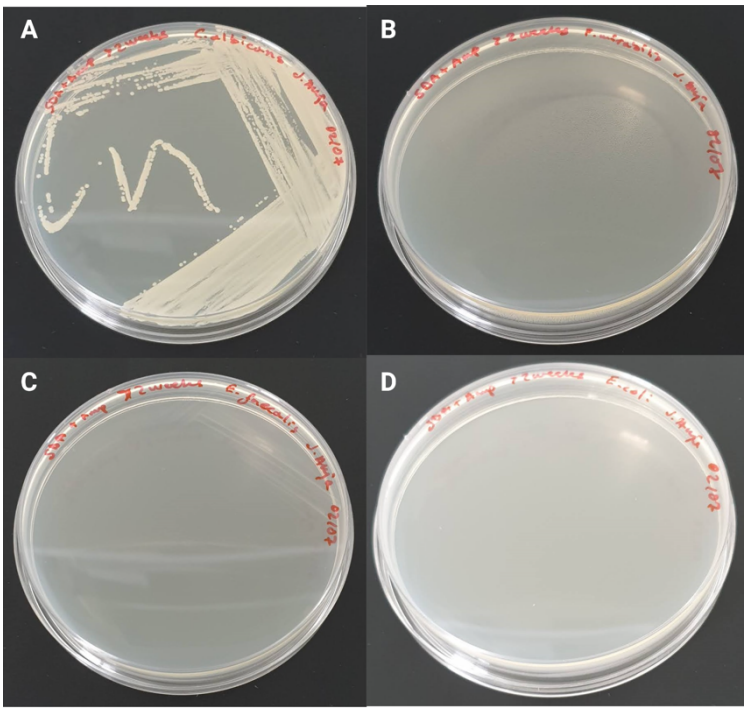


Figure S5.1.1. Selective growth of C. albicans in SDA with 100 µg/mL of ampicillin. A. *C. albicans* **B.** *P. mirabilis* **C.** *E. faecalis* **D.** *E. coli*

Table S5.1.1. *C. albicans* plate count comparison in SDA with and without ampicillin

Media	**Log CFU/mL**
SDA	**6.34**
SDA + Amp	**6.30**

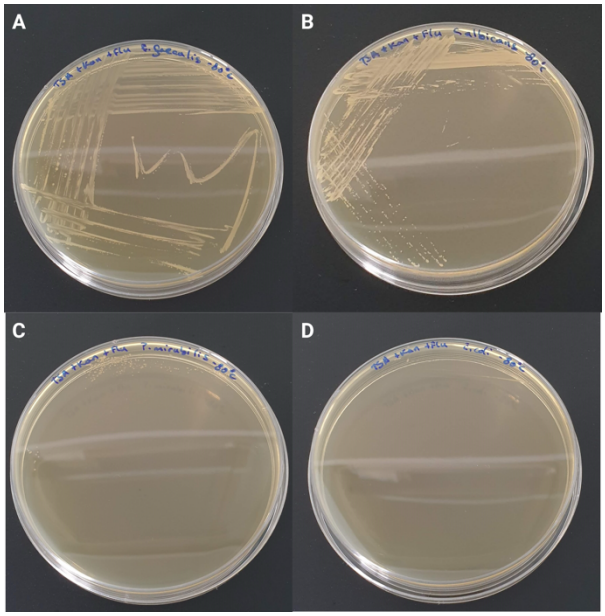


Figure S5.1.2. Selective growth of *E. faecalis* in TSA with 50 µg/mL of kanamycin and 150 µg/mL of fluconazole. A. *E. faecalis* **B.** *C. albicans* **C.** *P. mirabilis* **D.** *E. coli*

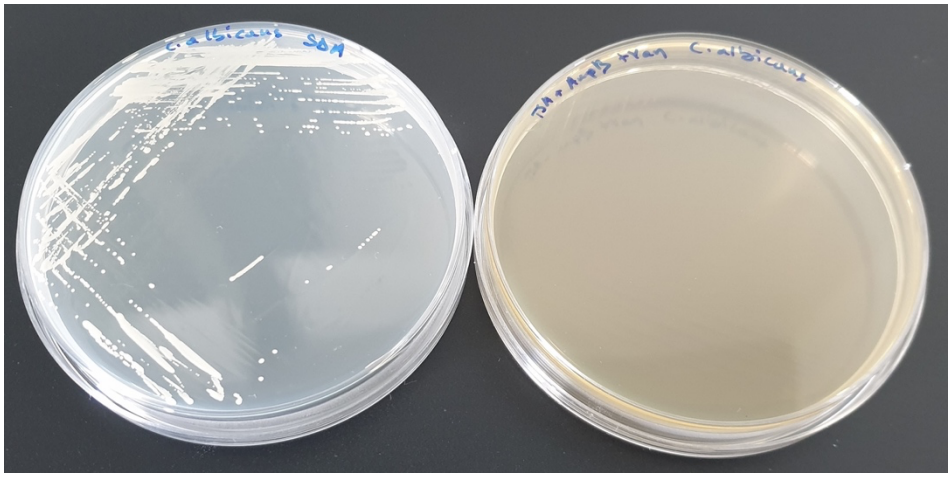


Figure S5.1.3. Inhibition of *C. albicans* growth in TSA with 10 µg/mL of Amphotericin B

Table S5.1.2. *E. faecalis* plate count comparison in TSA with and without kanamycin and Amphotericin B

Media	**Log CFU/mL**
TSA	**7.70**
TSA + Kan + AmpB	**7.64**

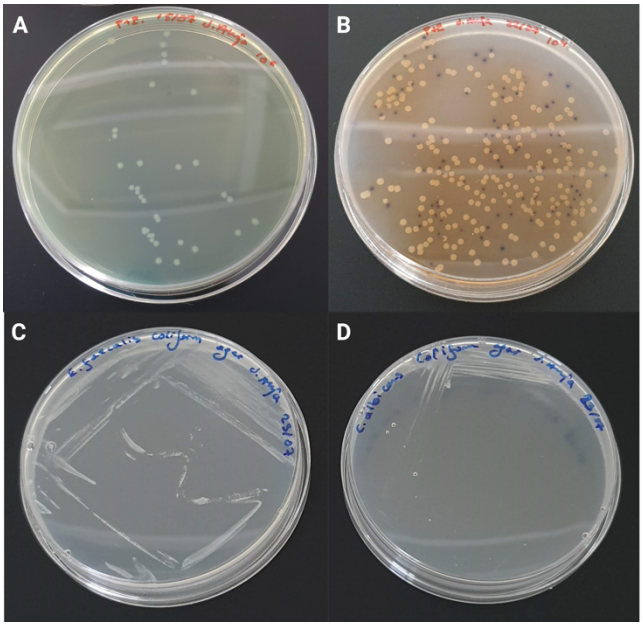


Figure S5.1.4. Selective growth of *E. coli and P. mirabilis* A. *E. coli and P. mirabilis* mixed suspension in CLED agar **B.** *E. coli and P. mirabilis* mixed suspension in coliform agar **C.** *E. faecalis* in coliform agar **D.** *C. albicans* in coliform agar

Table S5.1.3. *E. coli and P. mirabilis* plate count comparison in TSA, CLED and coliform agar

Media	Species	Log CFU/mL	Mean Log Difference
TSA	***E. coli***	**7.17**	**0.05**
TSA	***E. coli***	**7.20**	
Coliform	***E. coli***	**7.21**	
Coliform	***E. coli***	**7.25**	
CLED	***P. mirabilis***	**7.89**	**0.19**
CLED	***P. mirabilis***	**7.83**	
Coliform	***P. mirabilis***	**8.06**	
Coliform	***P. mirabilis***	**8.05**	

S5.2. Probe testing

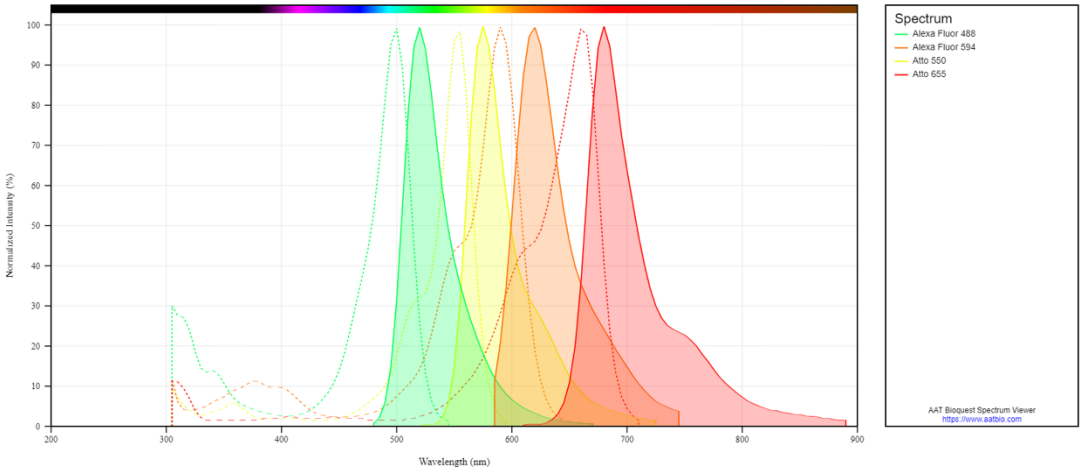


Figure S5.2.1. Spectral overlap of selected fluorochromes. Generated with AAT Biorequest Spectra Viewer tool.

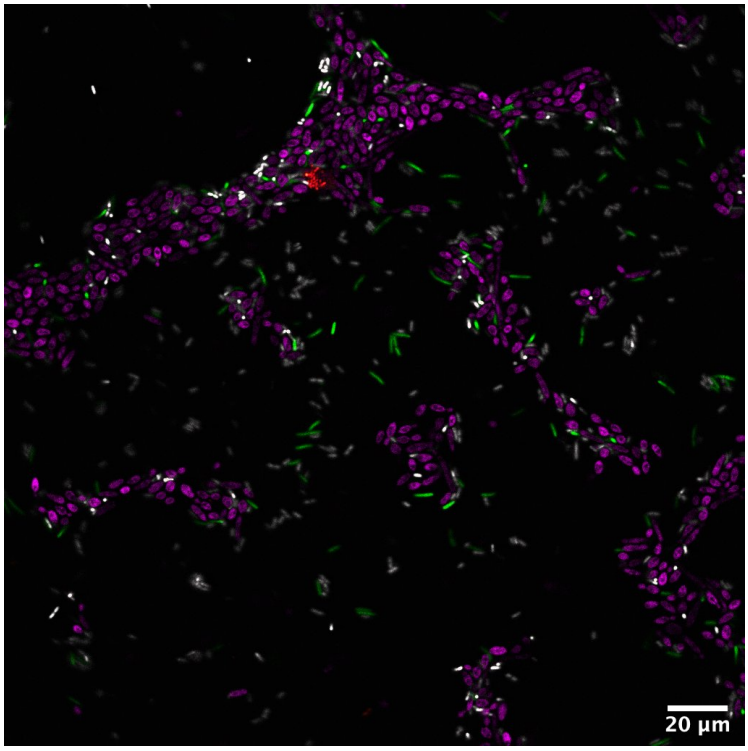


Figure S5.2.2. FISH in mixed suspension of *E. coli, C. albicans, P. mirabilis and E. faecalis*. *E. coli* cells tagged green. *C. albicans* cells tagged purple. *P. mirabilis* cells tagged white. *E. faecalis* cells tagged red. Image acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6.

S5.3. FISH in biofilm

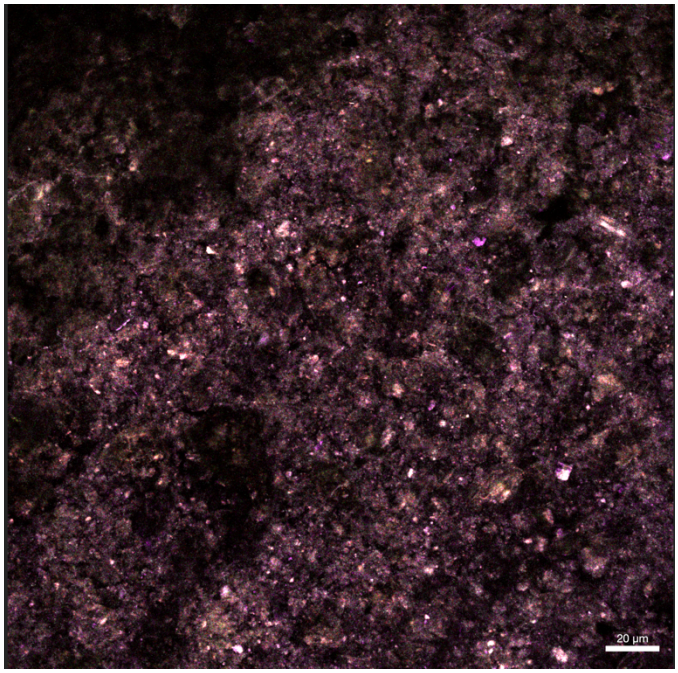


Figure S5.3.1. Negative control FISH in C11 combination. Image acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6.

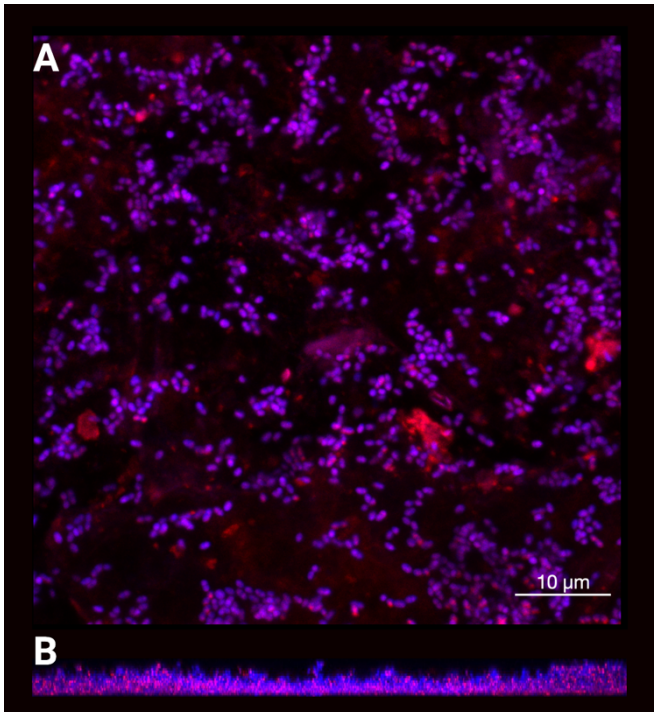


Figure S5.3.2. DAPI counterstain *E. faecalis* biofilm. A. Widefield view of central slice, zoom factor: 4. **B.** Orthogonal projection of XZ plane, zoom factor:1. Image acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; line average: 6. Orthogonal projection generated in LEICA Application Suite X (LASX).

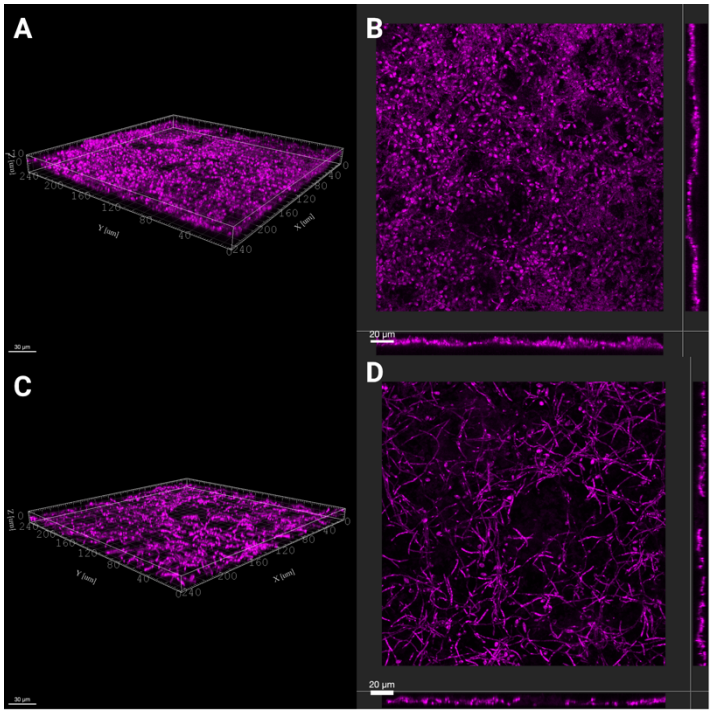


Figure S5.3.3. Differences in *C. albicans* biofilms between experimental days. A. Day 1- 3D projection **B.** Day 1 – Orthogonal views. **C.** Day 2- 3D projection **D.** Day 2 – Orthogonal views. For orthogonal views: Central view shows XY plane, bottom view shows XZ plane and Right-side view represents YZ plane. Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. Projections generated with IMARIS viewer 9.7.2.

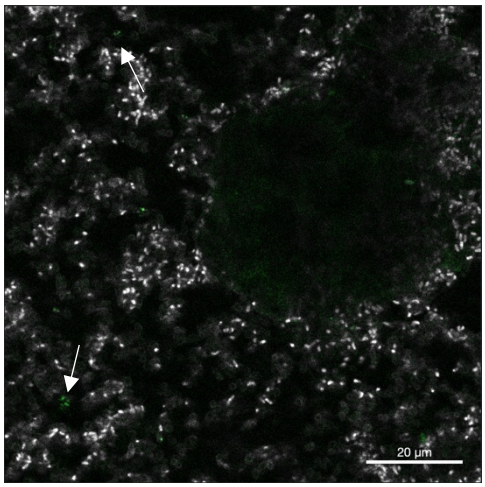


Figure S5.3.4. Widefield view of C5 biofilm. Each species was tagged with a specific colour: *E. coli* (green) and *P. mirabilis* (white). *E. coli* cells highlighted by arrows. Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 4; line average: 6.

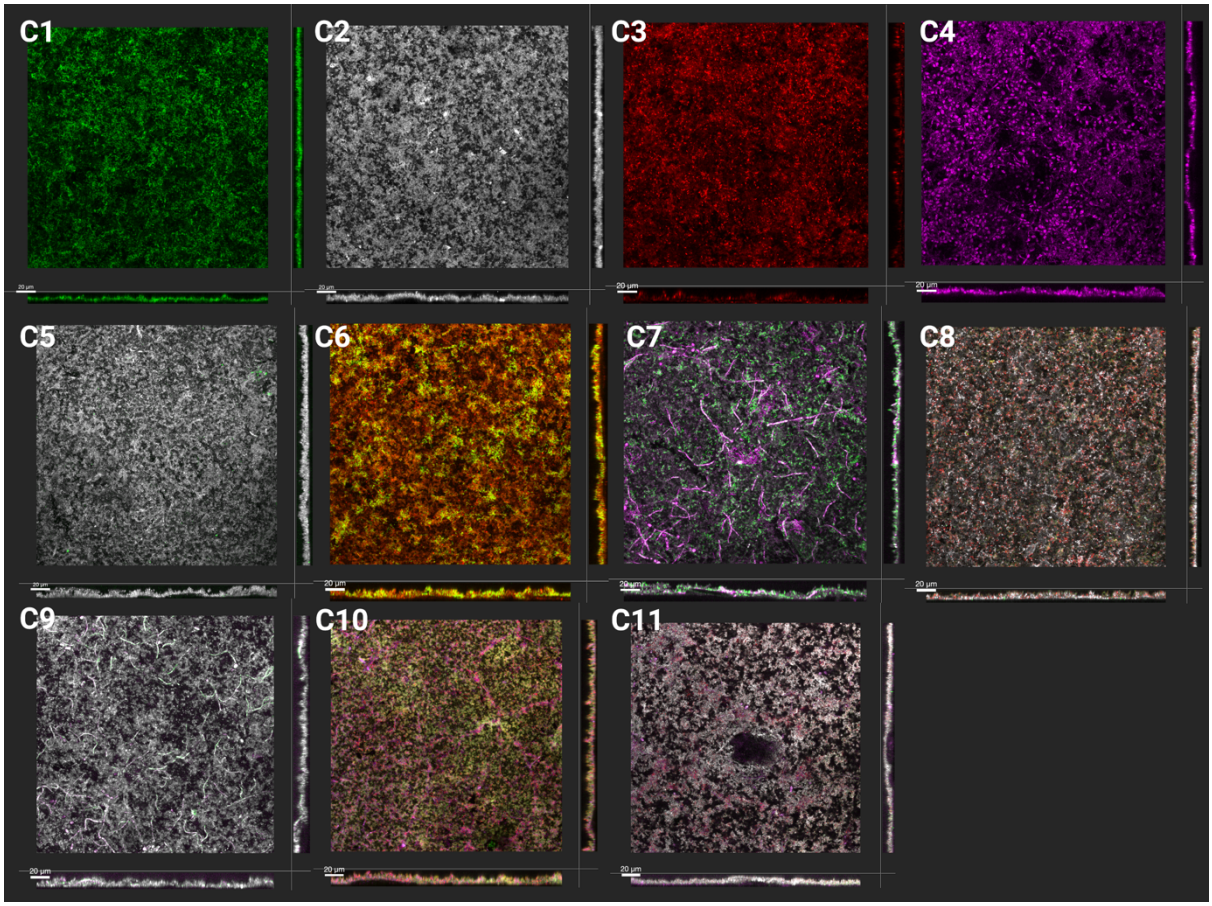


Figure S5.3.5. Orthogonal views of each biofilm per combination. Central view shows XY plane, bottom view shows XZ plane and Right-side view represents YZ plane. Each species was tagged with a specific colour: *E. coli* (green), *P. mirabilis* (white), *E. faecalis* (red) and *C. albicans* (purple). Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 1; line average: 6. Orthogonal projections generated with IMARIS viewer 9.7.2.

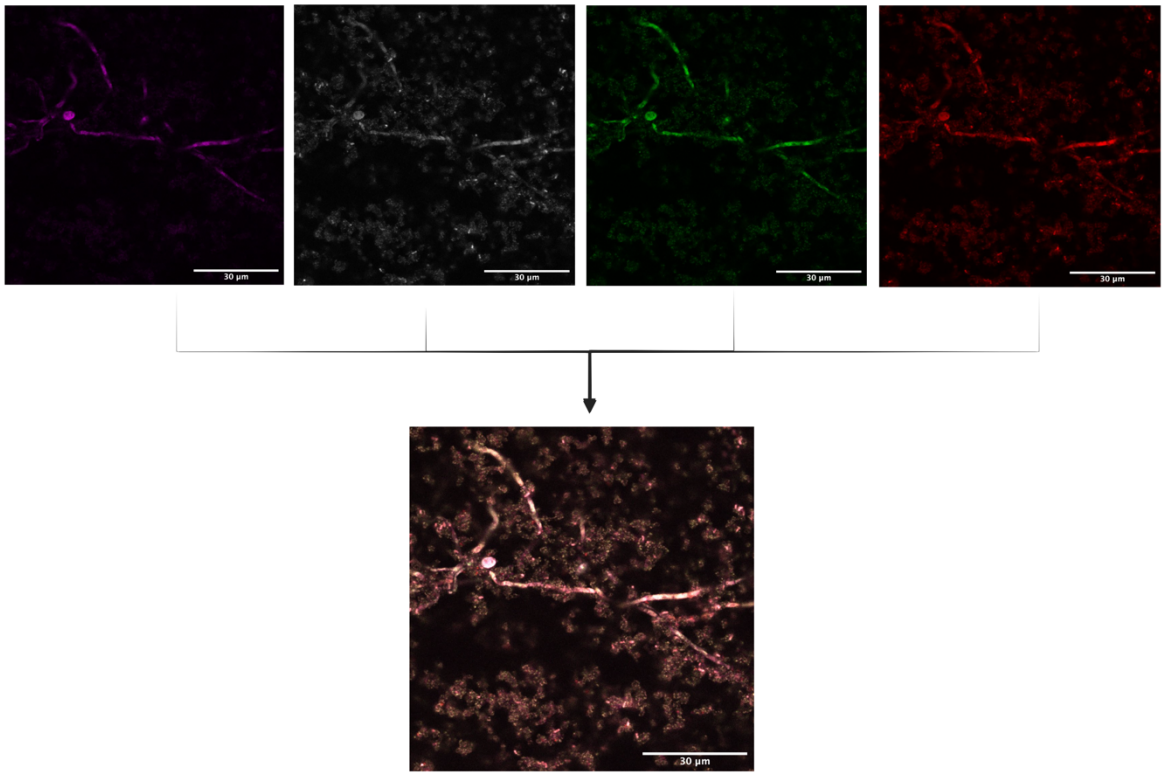


Figure S5.3.6. FISH staining of C11 biofilm separated by channel. Each species was tagged with a specific colour: *E. coli* (green), *P. mirabilis* (white), *E. faecalis* (red) and *C. albicans* (purple). Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 4; line average: 6.

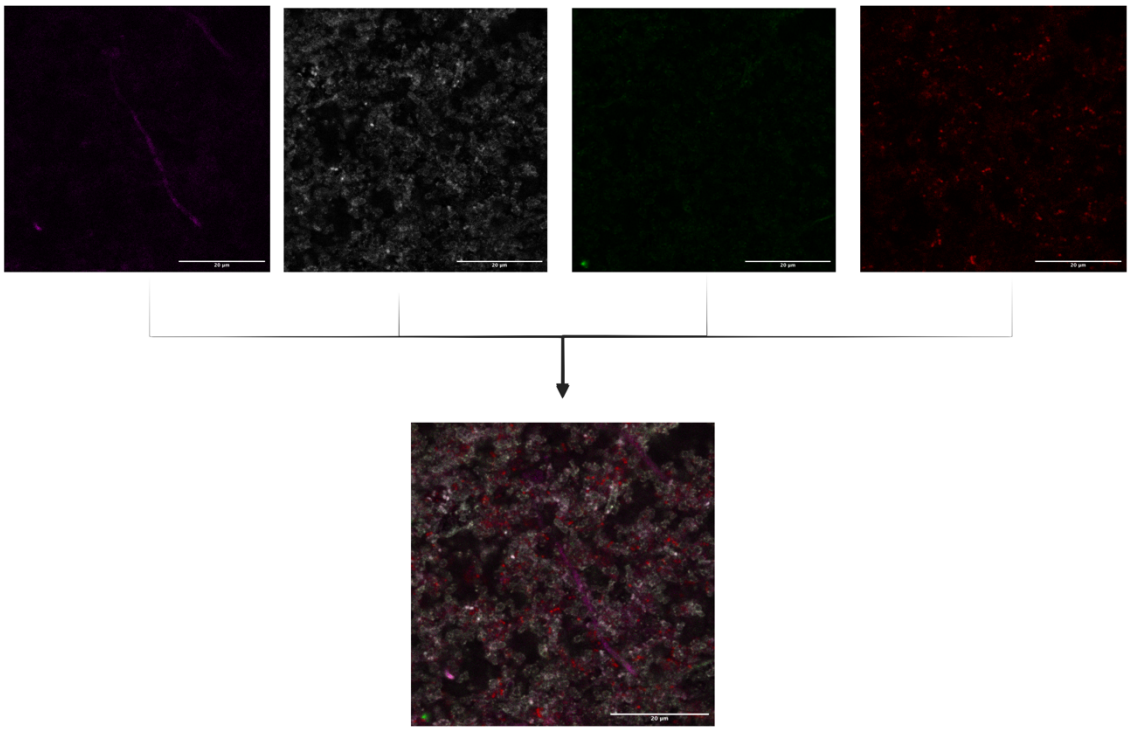


Figure S5.3.7. Manual dye separation of FISH stained C11 biofilm separated by channel. Each species was tagged with a specific colour: *E. coli* (green), *P. mirabilis* (white), *E. faecalis* (red) and *C. albicans* (purple). Images were acquired using Laser Scanning Confocal Leica SP5 microscope, 63x/1.30 glycerol immersion objective. Speed: 400Hz; image format: 1024 x 1024 pixels; zoom factor of 4; line average: 6.