

Opinion

Biofilms vs. cities and humans vs. aliens – a tale of reproducibility in biofilms

Nuno F. Azevedo,^{1,*} Jontana Allkja,¹ and Darla M. Goeres²

Biofilms are complex and dynamic structures that include many more components than just viable cells. Therefore, the apparently simple goal of 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 particular 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 article 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 expand on the 'cities of microbes' analogy for biofilms by imagining that a new civilization has reached the Earth's outskirts and starts studying the Earth's cities. This will provide a better sense of scale and illustrate how small details can impact profoundly on the growth and behavior of a biofilm and our understanding of reproducibility.

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., [3,4]). 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 USA, at a cost of approximately US\$28 billion/year, is not reproducible [5]. There is also a scientific cost as subsequent research is often based on conclusions from preclinical studies, and 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 antivaccination 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

Highlights

In recent decades the scientific community has started to appreciate that most microorganisms live in complex 3D structures composed of cells, polysaccharides, and other components such as proteins, extracellular (e)DNA, and lipids. These structures are commonly designated 'biofilms'.

Similar to other areas of research, biofilm studies have been affected by a lack of reproducibility. In this article, we propose a new scheme on how to classify the level of reproducibility in biofilms. This consists of four different levels: level 1, no reproducibility; level 2, standard reproducibility; level 3, potential standard reproducibility; and level 4, total reproducibility.

Some methods aim to improve reproducibility by focusing on biofilm growth reactors, while others focus on biofilm characterization methods. Moreover, initiatives such as minimum information guidelines and biofilm-centered databases offer alternative strategies to tackle the reproducibility problem. The path to total reproducibility is certainly complex, but novel experimental and computational strategies are bringing us closer to achieving this goal.

¹LEPABE – Laboratory for Process Engineering, Environment, Biotechnology, and Energy, Faculty of Engineering, University of Porto, Rua Dr Roberto Frias, 4200-465 Porto, Portugal
²Montana State University, Center for Biofilm Engineering, 366 Barnard Hall, Bozeman, MT 59717, USA

*Correspondence: nazevedo@fe.up.pt (N.F. Azevedo).

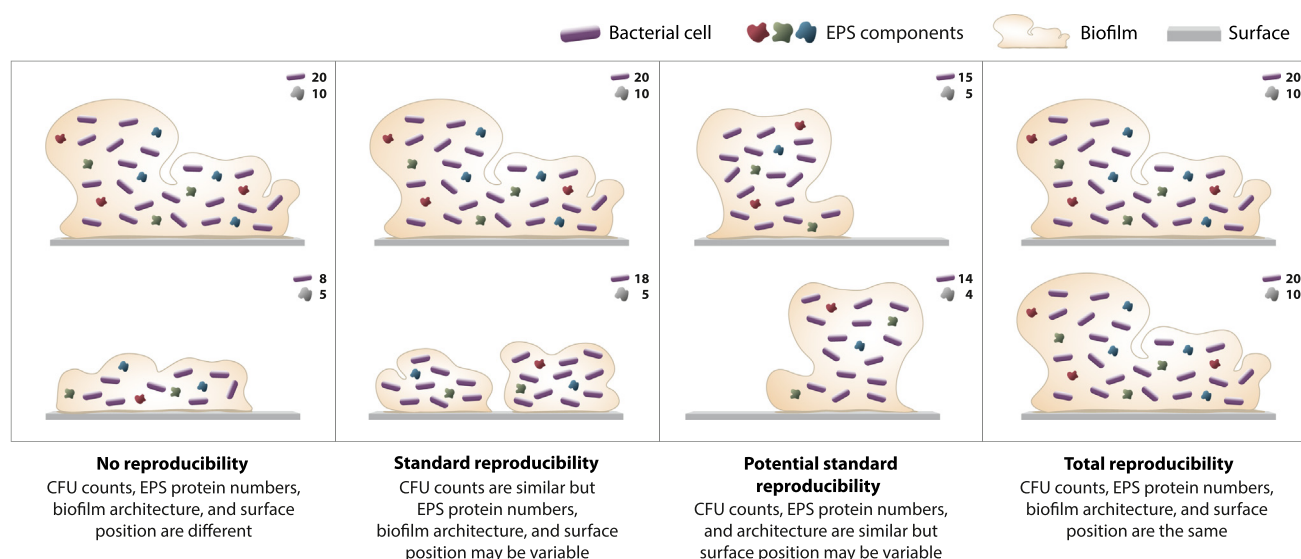


achieved with the different ASTM (American Society for Testing and Materials)-approved methods is appropriate [10,11]. Furthermore, in the case of biofilms involved in wastewater treatment processes, the most important feature is their metabolism which is needed to keep the water parameters at the outlet of the reactor below a certain threshold [12]. Hence, it is important to define reproducibility in the context of the biofilm experiment.

In this article, we categorize reproducibility into four different levels (Figure 1). Level 1 represents no reproducibility, meaning that the biofilm varies significantly in the number of cells, structure, and extracellular polymeric substance (EPS) components between experiments and among different laboratories. 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 in which the same experiment is performed across different laboratories and the reproducibility standard deviation is calculated. If this standard deviation is deemed acceptable by organizations such as ASTM and ISO (International Organization for Standardization), then the method is approved as a Standard Test Method (STM). Regulatory agencies reference STMs in their guidance documents to define the pathway that companies must follow in order to get one of the following label claims on their product: kills, removes, or prevents biofilm.

Level 3 represents a 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



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Figure 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 (with their sizes enlarged for easier visualization). Illustration created by Jill Story (Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA). Abbreviation: EPS, extracellular polymeric substance.

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 to 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, that is, overlapping coordinates of biofilm components, as an extra requirement when compared with level 3. Hence, the biofilms not only need to have a similar shape and architecture but their components must also have the same 3D spatial location within the biofilm each time. As with level 3 reproducibility, this has not been achieved experimentally.

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 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 go beyond the analogy of biofilms and cities and ask for the help of friends from outer space.

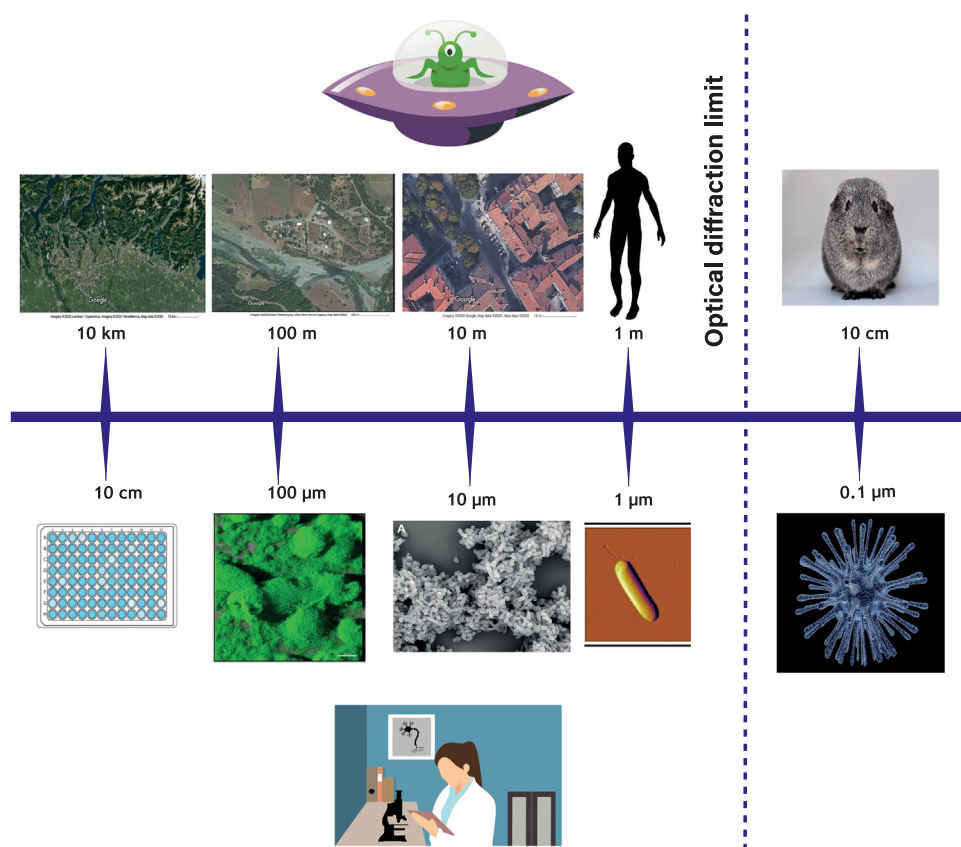
Cities and aliens

Let us imagine that a new civilization has reached the outskirts of the Earth and the members of this civilization start looking at the surface of our planet using magnifying equipment for visualization. For the sake of argument, that magnifying equipment would allow them to have a resolution of roughly 0.1 meter, that is, only structures/features over 0.1 meter would be discriminated (Figure 2). This would allow aliens to identify individual humans (complex 3D structures), but no individual features. 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.

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. When we observe a biofilm using an optical or epifluorescence microscope we get a sense of the general structure of the biofilm (Figure 2). This can be improved using electron microscopy or super-resolution imaging, but generally at the expense of some biofilm features [15].

Regardless of these limitations, 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



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Figure 2. Comparison between different visualization scales for biofilms and Earth. The scale for visualizing 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 visualization of both the Earth and the biofilm. At 10 cm/10 km no details are visible; at 100 μm /100 m the general shape and architecture can be assessed; at 10 μm /10 m the internal structure/organization can be assessed; at 1 μm /1 m the individual cells/humans can be assessed; at 0.1 μm /10 cm the minute components can be visualized. Microscopy images were repurposed under the creative commons attribution license: scanning electron microscopy (SEM) [69]; atomic force microscopy (AFM) [70]. Confocal laser scanning microscopy (CLSM) image courtesy of Kelli Buckingham-Meyer (Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA). Map images obtained from Google Maps.

thickness [16]. 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 microorganism) has on the overall functioning of a multispecies biofilm [17], and how communication between different cells occurs [18]. Hence, in terms of reproducibility, it is important that we not only assess the log density of biofilms but also other structural and behavioral characteristics.

Humans and aliens

If aliens had arrived in 1931 they would be puzzled by 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, for example, ± 1 log difference in cell counts or different architecture[†]. These results are not (solely) due to experimental errors – they are also due to minute but unavoidable differences in the initial cellular contents, physiology, and location, as well as variations in the substrate/growth surface. While generally these differences are considered for log density numbers in level 2 devices during ruggedness testing[†], 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.

Considering level 3 reproducibility, achieving a reproducible biofilm would mean that cells form similar structures in a similar fashion. While scientists do control for different experimental conditions, such as flow rate, temperature, microbe strains, and the type of material used to promote reproducible growth [19], 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 100 m 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, for example, 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 [20]. These differences in roughness can affect bacterial adhesion which, in turn, affects biofilm formation and leads to different experimental outcomes [21].

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 multiwell plates – may lead to variability [22]. While standard laboratory practices are obviously followed, researchers should consider recording more variables and employing additional analytical methods to ensure that variability is kept to a minimum.

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, that is, cellular noise [23]. 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 (Box 1).

One approach to improve reproducibility is to focus on biofilm growth reactors. Most reactors achieve only level 2 reproducibility (Figure 1). Many of them already have been validated for STMs by ASTM for specific microorganisms and applications, most recently, the drip flow reactor. Data generated in a ten-laboratory ring trial using this device resulted in a reproducibility standard deviation (SD) = 0.27 for the growth of *Pseudomonas aeruginosa* biofilms [10,24]. 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

Box 1. Improving biofilm methods

Biofilm formation methods

Level 1 devices have generally not been tested for standard methods validation, meaning that their reproducibility, that is, among laboratory variability, is unknown; however, their repeatability, that is, within laboratory variability, has been assessed. For example, the simplest and most widely used of these methods relies on multiwell reactors or microtiter plates where the biofilm is grown in a batch system, meaning that there is no flow of nutrients into the system [35]. Their repeatability was assessed for different characterization methods in two separate studies, but the overall observation was that the microtiter plate was not robust [19]. Flow cell reactors, which grow biofilm under a continuous flow of nutrients, is another example of a level 1 device. Its repeatability has been investigated by Heydorn *et al.* where they assessed the repeatability of biofilm thickness for different *P. aeruginosa* strains across different coupons on different experimental days [36].

Reproducibility of level 2 devices has been validated through a ring trial. The simplest of these is an alternative to the microtiter plate, the Calgary or minimum biofilm eradication concentration (MBEC) device, where the microtiter lid has been modified to contain pegs [37]. The reproducibility of the MBEC system has been evaluated for disinfectant efficacy testing on *P. aeruginosa* biofilm using the CFU count method through an eight-laboratory collaborative (ring trial) study [38]. The Center for Disease Control (CDC) biofilm reactor and drip flow biofilm reactor grow biofilm under continuous flow [24,39]. Both of these reactors have been approved by ASTM as standard biofilm methods [10,11].

While no level 3 reproducibility has yet been achieved, microfluidics devices show promise [40]. These are low-volume flow reactors (10^{-2} – 10^{11} μ l) with a very small adhesion surface area (10 mm²– 10^{-6} mm²). They allow investigation of the effect of fluid dynamics in a more controllable manner as experimental conditions (temperature, flow rate, pressure, pH, O₂ level) can be better regulated compared with existing techniques, but their application is limited to early stages of biofilm formation [41].

In all the reactors mentioned herein, advances in nano-surface technology allow us to model the topography (roughness, porosity) of the surface to the micro and nanoscale. This could help to improve reproducibility for all devices previously mentioned; surfaces could be modified or modeled to inhibit or stimulate adhesion, or to direct cell growth in a specific direction [26]. To go a step further, droplet printing of micropatterned bacterial communities was recently achieved by Kumar *et al.* Their work demonstrated the impact that micro-scale spatial location and structure differences can have on biofilm structure and the interactions within it [42].

Biofilm characterization methods

Level 1 characterization methods widely rely on dyes such as crystal violet and resazurin, which are generally used on microtiter plate grown biofilms, and rely on an optical density or fluorescence-emission reading [35]. Additionally, omics-based characterization methods have risen in popularity in recent years. Through next-generation sequencing techniques it is possible to run single- or multi-omics analysis –such as metagenomic, metabolomic, proteomic, transcriptomic, and lipidomic – on biofilm samples [43,44]. These methods provide information on the ecology of biofilms and the behavior of different species within it.

The most widely used level 2 characterization method is the CFU count method, which is the method of choice in the ASTM approved biofilm methods [10,39]. Guidelines exist for acceptable reproducibility standard deviations for viable plate counts^v.

In order to go past level 2 reproducibility, the ability to investigate the location and spatial organization of the biofilm and its components is crucial. Microscopy provides information on the spatial organization of biofilms and the individual bacteria within them [17]. They can also provide information on the behavior of the biofilm and the social interactions between different bacterial species in mixed biofilms [45]. Additionally, microscopy can be used to quantify biofilm structures by measuring areal porosity, fractal dimensions, and average diffusion distances [46].

Light-based imaging, using epifluorescence and confocal laser scanning microscopy (CLSM), remains the most widely used approach in the field [47]. However, the use of more advanced microscopy aiming at surpassing the optical diffraction limit is becoming increasingly popular. Examples include scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM) and super-resolution microscopy techniques [48]. The latter are an advancement in the field of light-based microscopy and allow imaging at resolutions past the optical diffraction limit (~50 nm or less) [49]. Moreover, the use of the Mesolens microscope to image *Escherichia coli* biofilms made possible the imaging of intracolony microchannels and tracking of the uptake of fluorescently labeled microspheres, hinting at the possible role of these microchannels in biofilm formation and stability [50].

Additionally, microscopy techniques mentioned here require a labeling method in order to be able to image the biofilm and its various elements. Some labeling methods could be used to label the organisms, for example, CLASI-FISH (combinatorial labeling and spectral imaging fluorescence *in situ* hybridization) and GFP [51,52]. More advanced methods allowing for single-molecule labeling are also available, for example, BIFC-PALM (photoactivated localization microscopy with bimolecular fluorescence complementation) and STORM (stochastic optical reconstruction microscopy) [53,54].

Alternatively, label-free imaging techniques, such as Raman and mass spectrometry imaging (MS-I), can also be used to image biofilms [55,56]. Another promising label-free technology for use in biofilms is X-ray imaging, reaching resolutions as low as 0.01 nm [57].

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 to reach level 3 [25]. 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 [26].

Another approach is to focus on biofilm characterization methods. While some of these methods enable quantification of the biofilm as a single entity, others look at one specific element of the biofilm. The former can reach only level 2 reproducibility, as they provide no information on the composition and spatial location or arrangement of components within the biofilm. Furthermore, being able to characterize and quantify specific elements of the biofilm, coupled with microscopy, could help us to 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. This has allowed for the visualization of cell ultrastructure to unprecedented details, down to the visualization of individual proteins, their location within this structure, and how they interact with the different organelles and each other [27].

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 (Box 2). Moreover, while advances in microscopy have allowed us to visualize 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 a recent work by Shi *et al.*, an algorithm was developed which could extract each cell's directional information, within the biofilm, in relation to neighboring ones. This allowed them to calculate association matrices, a form of quantification for the spatial organization of the biofilm that can be compared across different biofilms [28].

Box 2. Bioinformatics tools and modeling strategies

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 analyzed through different software. For image analysis, with each new application and acquisition method, new image analysis protocols need to be validated and developed [46]. Tools such as BiofilmQ have been recently introduced; they can be used to quantify shape, volume, biomass, cell coordinates, and more [58,59].

Similarly, the data analysis issue cannot be ignored for omics methods. Many 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 [60], CLC Genomics Workbench [61], MetaboAnalyst [62], and many others. Issues with primer bias and lack of complete and accurate gene libraries for biofilms need to be addressed in order to improve reproducibility and data analysis [63].

Biofilm modeling

Mathematical modeling uses mathematical formulas and concepts to describe a system based on existing experimental evidence and make predictions accordingly. They can be applied to investigate the dynamics of biofilm formation [64]. D'Acunto *et al.* developed a mathematical model to run simulations on the dispersal of bacterial cells from biofilms. The model looks at the motility of dispersed cells as different environmental cues are applied and can predict dispersal occurrence at different locations of the biofilm [65].

There are different types of computer modeling, such as individual-, agent-, and atomistic- based modeling. Individual-based modeling accounts for the differences between specific individuals in the biofilm community and may be used to predict all the different outcomes of an experiment. Individual models can be combined with population models to simulate all the different stages of biofilm formation and behavioral patterns in a specific model [29].

Agent-based modeling simulates the interactions and behavior of autonomous agents within a model system. It is often either used in conjunction with, or mistaken for, individual-based modeling due to the similarities. This type of modeling can help simulate the behavior of a biological system such as biofilms at the molecular level [66].

Atomistic modeling is a very novel type of computer modeling. It can model interactions at the atomic level and look at interatomic forces in materials [67]. Moreover, machine-learning and deep-learning methods can help in the processing of high-throughput data, such as microscopy and omics data, more reliably and detect patterns within these large datasets. They can also speed up the process through automation [68].

Currently, no laboratory method can reach level 3 or 4 reproducibility. The application of mathematical and computer modeling 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 variables [29]. 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 behavior. 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 [30]. Moreover, since these models are computationally demanding and time-consuming, it is not feasible to model large-scale systems. Hence, most models analyze a chosen subset of the biofilm which does not account for everything in the system [29]. Examples of different modeling systems can be found in [Box 2](#).

Alternative approaches to tackling reproducibility

Advances in the methodologies mentioned earlier are not the only solution to the problem of reproducibility. Alternatives are available to address other issues pertaining to reproducibility, such as poor reporting and unavailability of raw (untreated) experimental data [3]. For instance, existing initiatives, such as the BioOmics database, can be used to store raw data from various biofilm studies, thereby improving accessibility [31].

Furthermore, initiatives such as the minimum information about a biofilm experiment (MIABIE)ⁱⁱ guidelines can help to improve the reporting of biofilm experiments in the literature [32,33]. To improve compliance with the guidelines, the information that MIABIE asks researchers to report is easily obtained by most laboratories 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 *et al.*, material composition and synthesis descriptions are provided in detail [34]. Additionally, the new ontology initiative by the National Biofilms Innovation Centre (NBIC)ⁱⁱⁱ can help in standardizing the terminology used across different biofilm-related publications. This will help to avoid ambiguity in the vocabulary used, thus increasing the effectiveness of minimum information guidelines.

Concluding remarks

Biofilms exhibit a chaotic behavior 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 (see [Outstanding questions](#)). In the meantime, we can strive to minimize sources of variation and rely on statistical methods that enable us to quantify the chaotic behavior of biofilms.

Acknowledgments

This work was supported by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 722467, Print-Aid project and Base Funding – UIDB/00511/2020 of the Laboratory for Process Engineering, Environment, Biotechnology and Energy – LEPABE – funded by national funds through the FCT/MCTES (PIDDAC).

Outstanding questions

Is the lack of reproducibility more heavily reliant on the biofilm growth reactor used, characterization method, or data analysis approach? How can we assess the specific impact each of them has on reproducibility?

Which type of biofilm reactors will be able to provide level 3 reproducible biofilms?

How does the study's aim determine what level of reproducibility is acceptable? Is level 4 always the goal, or for some applications will level 2 always be acceptable?

Are spatial statistics sufficient to assess reproducibility of microscopy data? What other statistical tools need to be developed to assess reproducibility from different types of analytical methods?

So far, total reproducibility is only feasible using computer modeling. Is it possible to reach in wet laboratory biofilm experiments?

Declaration of interests

There are no interests to declare.

Resources

ⁱ<https://biofilm.montana.edu/documents/KSA-SM-03.pdf> (2010)

ⁱⁱwww.miabie.org/

ⁱⁱⁱwww.biofilms.ac.uk/biofilm-ontology/

^{iv}https://biofilm.montana.edu/documents/KSA-SM-11_rev_103011.pdf (2011)

References

- Watnick, P. and Kolter, R. (2000) Biofilm, city of microbes. *J. Bacteriol.* 182, 2675–2679
- Paula, A.J. *et al.* (2020) Dynamics of bacterial population growth in biofilms resemble spatial and structural aspects of urbanization. *Nat. Commun.* 11, 1354
- Baker, M. (2016) Reproducibility crisis? *Nature* 533, 26
- Schloss, P.D. (2018) Identifying and overcoming threats to reproducibility, replicability, robustness, and generalizability in microbiome research. *mBio* 9, e00525-18
- Freedman, L.P. *et al.* (2015) The economics of reproducibility in preclinical research. *PLoS Biol.* 13, e1002165
- Goodman, S.N. *et al.* (2016) What does research reproducibility mean? *Sci. Transl. Med.* 8, 341ps12
- Van Acker, H. and Coenye, T. (2017) The role of reactive oxygen species in antibiotic-mediated killing of bacteria. *Trends Microbiol.* 25, 456–466
- Grimes, D.R. *et al.* (2018) Modelling science trustworthiness under publish or perish pressure. *R. Soc. Open Sci.* 5, 171511
- Rosenau, J. (2012) Science denial: a guide for scientists. *Trends Microbiol.* 20, 567–569
- ASTM E2647-20 (2020) Standard test method for quantification of a *Pseudomonas aeruginosa* biofilm grown using a drip flow biofilm reactor with low shear and continuous flow. ASTM International, Published online May 15, 2020. <https://www.astm.org/Standards/E2647.htm>
- ASTM E3161 (2018) Standard practice for preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilm using the CDC biofilm reactor. ASTM International, Published online July 3, 2018. <https://www.astm.org/Standards/E3161.htm>
- Pérez, J. *et al.* (2020) The role of the external mass transfer resistance in nitrite oxidizing bacteria repression in biofilm-based partial nitrification/anammox reactors. *Water Res.* 186, 116348
- Jackson, G. *et al.* (2001) Growing reproducible biofilms with respect to structure and viable cell counts. *J. Microbiol. Methods* 47, 1–10
- Andersson, E. (2006) Urban landscapes and sustainable cities. *Ecol. Soc.* 11, 34
- Hufnagel, L. and Pepperkok, R. (2016) Let there be light – at the right place. *Nat. Biotechnol.* 34, 1246
- Harrison, J.J. *et al.* (2006) 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
- Nadell, C.D. *et al.* (2016) Spatial structure, cooperation and competition in biofilms. *Nat. Rev. Microbiol.* 14, 589
- McDougald, D. *et al.* (2012) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat. Rev. Microbiol.* 10, 39
- Kragh, K.N. *et al.* (2019) Into the well – a close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm* 1, 100006
- Zeiger, A.S. *et al.* (2013) Why the dish makes a difference: quantitative comparison of polystyrene culture surfaces. *Acta Biomater.* 9, 7354–7361
- Encinas, N. *et al.* (2020) Submicrometer-sized roughness suppresses bacteria adhesion. *ACS Appl. Mater. Interfaces* 12, 21192–21200
- Grosch, J.-H. *et al.* (2016) Enzyme activity deviates due to spatial and temporal temperature profiles in commercial microtiter plate readers. *Biotechnol. J.* 11, 519–529
- Eldar, A. and Elowitz, M.B. (2010) Functional roles for noise in genetic circuits. *Nature* 467, 167–173
- Goeres, D.M. *et al.* (2020) Drip flow reactor method exhibits excellent reproducibility based on a 10-laboratory collaborative study. *J. Microbiol. Methods* 174, 105963
- Aryasomayajula, A. *et al.* (2017) Microfluidic devices and their applications. In *Springer Handbook of Nanotechnology* (Bhushan, B., ed.), pp. 487–536, Springer
- Hu, Y. *et al.* (2016) Micropatterned hydrogel surface with high-aspect-ratio features for cell guidance and tissue growth. *ACS Appl. Mater. Interfaces* 8, 21939–21945
- Hoffman, D.P. *et al.* (2020) Correlative three-dimensional super-resolution and block-face electron microscopy of whole vitreously frozen cells. *Science* 367, eaaz5357
- Shi, H. *et al.* (2020) Highly multiplexed spatial mapping of microbial communities. *Nature* 588, 676–681
- Hellweger, F.L. *et al.* (2016) Advancing microbial sciences by individual-based modelling. *Nat. Rev. Microbiol.* 14, nmicro.2016.62
- Yan, J. *et al.* (2019) The ultimate guide to bacterial swarming: an experimental model to study the evolution of cooperative behavior. *Annu. Rev. Microbiol.* 73, 293–312
- Lourenço, A. *et al.* (2012) BiofOmics: a web platform for the systematic and standardized collection of high-throughput biofilm data. *PLoS ONE* 7, e39960
- Lourenço, A. *et al.* (2014) 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
- Alkja, J. *et al.* (2020) Minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates. *Biofilm* 2, 100010
- Borch, T. *et al.* (2008) Evaluation of characterization techniques for iron pipe corrosion products and iron oxide thin films. *J. Environ. Eng.* 134, 835–844
- Peeters, E. *et al.* (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods* 72, 157–165
- Heydorn, A. *et al.* (2000) Experimental reproducibility in flow-chamber biofilms. *Microbiology* 146, 2409–2415
- Ceri, H. *et al.* (1999) The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* 37, 1771–1776
- Parker, A.E. *et al.* (2014) Ruggedness and reproducibility of the MBEC biofilm disinfectant efficacy test. *J. Microbiol. Methods* 102, 55–64
- Goeres, D.M. *et al.* (2019) Development, standardization, and validation of a biofilm efficacy test: The single tube method. *J. Microbiol. Methods* 165, 105694
- Karimi, A. *et al.* (2015) Interplay of physical mechanisms and biofilm processes: review of microfluidic methods. *Lab Chip* 15, 23–42
- Tetala, K.K.R. and Vijayalakshmi, M.A. (2016) A review on recent developments for biomolecule separation at analytical scale using microfluidic devices. *Anal. Chim. Acta* 906, 7–21
- Krishna Kumar, R. *et al.* (2021) Droplet printing reveals the importance of micron-scale structure for bacterial ecology. *Nat. Commun.* 12, 857
- Lassek, C. *et al.* (2015) A metaproteomics approach to elucidate host and pathogen protein expression during catheter-associated urinary tract infections (CAUTIs). *Mol. Cell. Proteom.* 14, 989–1008

44. Ramos, P.I.P. *et al.* (2018) An integrative, multi-omics approach towards the prioritization of *Klebsiella pneumoniae* drug targets. *Sci. Rep.* 8, 1–19
45. Azevedo, A.S. *et al.* (2016) Impact of *Delftia tsuruhatensis* and *Achromobacter xylosoxidans* on *Escherichia coli* dual-species biofilms treated with antibiotic agents. *Biofouling* 32, 227–241
46. Beyenal, H. *et al.* (2004) Quantifying biofilm structure: facts and fiction. *Biofouling* 20, 1–23
47. Almeida, C. *et al.* (2011) Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH). *PLoS ONE* 6, e14786
48. Weiner, A. and Enninga, J. (2019) The pathogen–host interface in three dimensions: correlative FIB/SEM applications. *Trends Microbiol.* 27, 426–439
49. Wegel, E. *et al.* (2016) Imaging cellular structures in super-resolution with SIM, STED and localisation microscopy: a practical comparison. *Sci. Rep.* 6, 1–13
50. Rooney, L.M. *et al.* (2020) Intra-colony channels in *E. coli* function as a nutrient uptake system. *ISME J.* 14, 2461–2473
51. Schlundt, C. *et al.* (2019) Spatial structure in the ‘plastisphere’: molecular resources for imaging microscopic communities on plastic marine debris. *Mol. Ecol. Resour.* 20, 620–634
52. Hansen, M.F. *et al.* (2019) Fluidic resistance control enables high-throughput establishment of mixed-species biofilms. *BioTechniques* 66, 235–239
53. Nickerson, A. *et al.* (2014) Photoactivated localization microscopy with bimolecular fluorescence complementation (BIFC-PALM) for nanoscale imaging of protein–protein interactions in cells. *PLoS ONE* 9, e100589
54. Xu, K. *et al.* (2012) Dual-objective STORM reveals three-dimensional filament organization in the actin cytoskeleton. *Nat. Methods* 9, 185–188
55. Hu, F. *et al.* (2019) Biological imaging of chemical bonds by stimulated Raman scattering microscopy. *Nat. Methods* 16, 830–842
56. Brockmann, E.U. *et al.* (2019) Advanced methods for MALDI-MS imaging of the chemical communication in microbial communities. *Anal. Chem.* 91, 15081–15089
57. Mino, L. *et al.* (2018) Materials characterization by synchrotron x-ray microprobes and nanoprobe. *Rev. Mod. Phys.* 90, 025007
58. Peschek, N. *et al.* (2020) RNA-mediated control of cell shape modulates antibiotic resistance in *Vibrio cholerae*. *Nat. Commun.* 11, 6067
59. Hartmann, R. *et al.* (2019) BiofilmQ, a software tool for quantitative image analysis of microbial biofilm communities. *bioRxiv* Published online August 15, 2019. <https://doi.org/10.1101/735423>
60. Kumar, S. *et al.* (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549
61. Chacon, J. and Cuajungco, M.P. (2018) Comparative *de novo* transcriptome assembly of *Notophthalmus viridescens* RNA-seq data using two commercial software programs. *Calif. J. Health Promot.* 16, 46–53
62. Chong, J. *et al.* (2018) MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* 46, W486–W494
63. Ogawa, A. *et al.* (2017) Microbiomes of biofilms on decorative siliceous stone: drawbacks and advantages of next generation sequencing. *Curr. Microbiol.* 74, 848–853
64. Ali, Q. and Wahl, L.M. (2017) Mathematical modelling of CRISPR-Cas system effects on biofilm formation. *J. Biol. Dyn.* 11, 264–284
65. D’Acunto, B. *et al.* (2019) Mathematical modeling of dispersal phenomenon in biofilms. *Math. Biosci.* 307, 70–87
66. Pérez-Rodríguez, G. *et al.* (2018) Agent-based model of diffusion of N-acyl homoserine lactones in a multicellular environment of *Pseudomonas aeruginosa* and *Candida albicans*. *Biofouling* 34, 335–345
67. Pun, G.P.P. *et al.* (2019) Physically informed artificial neural networks for atomistic modeling of materials. *Nat. Commun.* 10, 1–10
68. Eraslan, G. *et al.* (2019) Deep learning: new computational modeling techniques for genomics. *Nat. Rev. Genet.* 20, 389–403
69. Dassanayake, R.P. *et al.* (2020) Identification of a reliable fixative solution to preserve the complex architecture of bacterial biofilms for scanning electron microscopy evaluation. *PLoS ONE* 15, e0233973
70. Francius, G. *et al.* (2011) Bacterial surface appendages strongly impact nanomechanical and electrokinetic properties of *Escherichia coli* cells subjected to osmotic stress. *PLoS ONE* 6, e20066