

BUILDING 3D PRINTED SYNTHETIC BIOLOGY TO EXPLORE BIOFILM LIFESTYLE

by

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DEDICATION

Dedicated to my ancestors and to all the relatives I will not have the chance to meet.

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ABSTRACT

A predominant proportion of the microbial biosphere has been naturally selected to live and grow as biofilms. Biofilms are communities of microorganisms where cells live attached to surfaces and/or other cells and are embedded in self-produced extracellular polymeric substances. Biofilms are spatially structured, often in three dimensions, and small-scale heterogeneity has important consequences for community-level behavior and ecology. However, biofilms are usually studied as self-assembled systems, and the physical forces that drive biofilm structural development are difficult to manipulate at small scales. 3D hydrogel bioprinting is an emerging technology directly addressing the need for small-scale control over biofilm structure and has been used to great effect for building synthetic biofilms with desirable functional characteristics. However, the selective environment of a hydrogel matrix is often different from where biofilms were naturally selected to grow. The intrinsic behavioral responses of microorganisms to encapsulation in hydrogel matrices are not well understood. To effectively design synthetic biofilms for diverse functional purposes, predicting and understanding encapsulated microbial behavior are salient goals. We integrated 3D bioprinting with quantitative microscopy and flow cell reactors to observe spatiotemporal population dynamics in synthetic biofilms. We varied initial population density, varied external transport and nutrient conditions, and compared population growth of synthetic and self-assembled biofilms among *Pseudomonas* isolates. Results suggest that (i) length scale of biovolume development can be controlled by altering initial population density, (ii) hydrogel carrying capacity can be altered by changing external fluid velocity but not initial population density, (iii) growth and behavior of self-assembled biofilms in fluctuating transport and nutrient environments does not predict growth and behavior of synthetic biofilms, (iv) encapsulation in a hydrogel matrix acts as a selection bottleneck where only a subset of the initial population is detectable with fluorescence microscopy, and (v) encapsulation in the hydrogel matrix homogenizes growth rate averages compared to self-assembled biofilms under identical fluid velocity conditions. Altogether, spatiotemporal population dynamics of synthetic 3D printed biofilms were distinct from self-assembled biofilms. Integrating observations of hydrogel-encapsulated cell behavior with synthetic biofilm design will aid efforts in developing biofilm-based biotechnology and aid *in vitro* structural-functional mimicry of natural biofilms.

INTRODUCTION

1.1. Mutual Dependence of Structure and Function in Biofilms

Biofilms are microbial communities embedded within self-produced polymeric substances that live attached to surfaces and/or other cells. At the microscopic scale, biofilms are extraordinary examples of the intricate structure-function relationship in all living communities. Biofilm structures are informed by the conditions of the selective environment both inside and outside the biofilm. On the inside, biofilms are characterized by their polymer production and excretion ¹, balance between immigration and emigration ², biofilm thickness and topography ³, cell differentiation ⁴, and physiological diversity ⁵. These internal conditions are also influenced by external factors such as nutrient diffusion into the biofilm's polymeric matrix ⁶, mass transport by bulk fluid flow ⁷, and environmental challenges such as antibiotic exposure or rapidly fluctuating perturbations ⁸. Over evolutionary time, biofilms have adapted diverse three-dimensional structures in response to their environmental conditions and the intricate interplay of internal and external influences; the diversity of biofilm structure and function resulting from this evolution is incalculable.

On Earth, biofilms are ubiquitous and can be found in diverse environments such as rumen microbiomes ⁹, thermal hot springs ¹⁰, and deep ocean sediment ¹¹. A review article published by Flemming et al., places the percentage of all prokaryotes living in biofilm between 40-80% globally ¹². This is a wide margin, but regardless of the exact amount of microbial life that lives as a biofilm, a significant fraction of the microbial biosphere has been naturally selected to grow and thrive these self-organized and spatially structured systems. Biofilms have shaped the natural

history of Earth and continue to influence worldwide ecosystems, both as essential ecological foundations of trophic networks ¹³, and as severe threats to human welfare in medical and industrial spaces ^{14,15}.

Biofilms can pose serious problems for human health and infrastructure. In the 1980s, biofilms were discovered on cardiac pacemakers and can cause recurring infections that are resistant to antibiotic treatment ¹⁶. Urinary tract infections (UTI)s are common infections causing an estimated 150 million deaths per year. Of these 150 million deaths, biofilms are a significant cause owing to their persistence in the face of treatment and their persistent colonization of catheters ¹⁷. Contact lenses can also develop potentially infectious biofilms ¹⁸. In an industrial context, biofilms foul equipment and food products ¹⁹, corrode surfaces ²⁰, and occlude pipes ²¹. Thus, a deep understanding of biofilm function and development will be needed to address these problems that biofilms present through their colonization and growth.

Knowledge on microbial growth and evolution, stress tolerance and response, and population dynamics has been gained historically on planktonic cells that do not reside in structured biofilms. Certainly, research in the context of planktonic cells is valuable, productive, and has led to fundamental insights on growth and evolution that underpin our understanding of individual and community microbial behavior. However, microbial behavior and evolution in the context of a biofilm constitutes a major difference in selective conditions compared to conditions surrounding free-living planktonic cells. Patterns of growth, stress response, ecological relationships, and evolution observed in planktonic populations may not meaningfully translate to biofilms because biofilm properties generally emerge as a consequence of cells self-organizing and attaining three-dimensional structure ⁸.

Physiological and evolutionary emergent properties of biofilms are numerous and fundamentally connected to their macroscopic structure. These emergent properties can include bolstered antimicrobial resistance due to mass transport limitation through dense extracellular polymeric matrices²², horizontal gene transfer hotspots between cell lines living in close proximity²³, and selection for greater phenotypic diversity in biofilms compared to planktonic populations²⁴. Due to mass transport constraints imposed by biofilm structure, biofilms typically contain heterogeneities in nutrient (i.e., carbon, nitrogen, electron donors/acceptors) availability. The development of small-scale nutrient heterogeneity as a consequence of three-dimensional biofilm structure can sustain ecotype and metabolic diversity to a greater extent than free-swimming populations²⁴. One common example is that biofilms in the human oral microbiome can develop densely populated outer layers which consume oxygen before it can diffuse throughout the biofilm²⁵. Diffusion limitation of oxygen constrains the growth of obligate aerobic populations in the biofilm interior while providing a niche for anaerobic populations.

Individual variability in cell physiology is a key factor that contributes to biofilm complexity. Even in a relatively simple case of a single-population biofilm grown in a laboratory, individual cells can be heterogeneous at small spatial scales²⁶. Such subtle complexities can exist at the micron scale in biofilms and can be predictive of community-level ecology and outcomes of mutualism, competition, and cross-feeding interactions²⁷. In contrast, at least in well-mixed planktonic populations studied in a laboratory, it is generally assumed that all individual cells are exposed to the same environmental conditions. This is not to say it is impossible for any structure and heterogeneity to develop among planktonic populations. For example, an average parameter such as a growth rate constant does not account for variability known to exist even among a single

planktonic population ²⁸, However, structure and variability is typically more complex and consequential in a biofilm context.

Biofilm structure in nature is extremely complex and heterogeneous. To understand fundamentals of biofilm structural development, there is a growing research effort aimed at developing methods for systematically controlling the physical structure of biofilms via 3D bioprinting. As the cost of 3D printing has decreased and development of biocompatible polymer matrices has advanced, 3D printing has been integrated with biology. Knowing that the structure of an organ is intimately tied to its function, researchers have combined mammalian cells with biocompatible materials to print synthetic mimics of human skin ²⁹, cartilage tissue ³⁰, and simplified livers and hearts ³¹. These 3D bioprinting methods have more recently been extended to building synthetic biofilms by controlling structure at scales ranging from centimeters to micrometers. Current literature surrounding microbial 3D bioprinting will be reviewed in detail in chapter 2. Briefly, the bulk of the recent microbial 3D bioprinting literature is focused on the design and fabrication of materials capable of sustaining cell populations within; these materials are referred to as engineered living materials. Cutting-edge research to develop 3D bioprinting technology capable of fabricating synthetic biofilm as a living material is ongoing, however, there is still very little understanding of how cells intrinsically respond to selective constraints of synthetic matrices.

The research presented in this dissertation will examine the population dynamics and behavior of *Pseudomonas fluorescens* N2E2 and five isolates of *Pseudomonas putida* encapsulated in a 3D printed hydrogel matrix. Chapter 1 is a discussion on fundamentals of biofilm growth and development, the influence of mass transport on biofilm structure and physiology, viewing biofilm

structure through a sociobiological lens, and a perspective piece about the current major themes in microbial 3D bioprinting literature and an outlook on the field's future development. Chapter two is a manuscript examining the growth and development of hydrogel-encapsulated *P. fluorescens* when inoculum density is systematically altered over three orders of magnitude. Chapter three is a manuscript integrating 3D bioprinting with flow cell microscopy to investigate how *P. fluorescens* populations encapsulated in 3D printed biofilms respond to systematically altered fluid velocity and nutrient conditions. In chapter four, several related strains of *Pseudomonas putida* are encapsulated in synthetic 3D printed biofilms, and differences in basic physiological metrics are examined and compared to self-assembled *P. putida* biofilms. Chapters two, three, and four detail methodology used to fabricate and observe 3D printed synthetic biofilms, including their design, printing, growth, imaging, image analysis, and additional follow-up characterizations. Finally, chapter five synthesizes general conclusions regarding bacterial population dynamics and physiology in the context of 3D printed biofilms.

1.1.1. Fundamentals of Biofilm Growth and Development

Bacteria reproduce asexually through binary fission. A mother cell produces two daughter cells through replication and polar separation of the mother cell's chromosome, replication of auxiliary genetic elements, and the formation of a dividing septum. There are deviations from this simple model – examples of asymmetric reproduction exist even within the paradigm of asexual reproduction. For example, *Caulobacter crescentus* division produces an adhesive stalk cell and a motile swarmer cell³². Even without obvious physiological differentiation, binary fission produces variability between daughter cells through mutation³³ or inheritance of cytoplasmic asymmetries³⁴. These microevolutionary processes can all have population-level consequences over

evolutionary timescales. However, when developing a simple and generalizable growth model to describe the reproductive behavior of an entire population, it is not usually possible to take individual variabilities into account. Generally, when describing bacterial growth at the population level, measuring biomass doubling is the most convenient model.

Growth Kinetics of Binary Fission. Bacterial population growth is often described using an exponential growth function. Binary fission and biomass doubling might suggest a model of the form $N(t) = N_0 2^{\mu t}$ where $N(t)$ is biomass at a defined time, N_0 is initial biomass, and μ is the instantaneous growth rate constant. However, this assumes synchronous division across the population, a condition rarely met in real biological systems given that multiple physiological states and precise replication timings exist simultaneously. In practice, cell division is inherently asynchronous, making it more appropriate to model population growth using a continuous exponential, as described in the fourth edition of *The Physiology and Biochemistry of Prokaryotes*³⁵. The instantaneous growth rate of the population (dN/dt) is defined as the current population ($N(t)$) multiplied by the growth rate constant μ :

$$\frac{dN(t)}{dt} = \mu N(t) \quad \text{eq.1}$$

Equation 1 captures the cumulative effect of asynchronous cell division, observed at the population level, and provides a robust framework for analyzing growth dynamics. While the differential rate law may be useful for understanding instantaneous growth behavior, the integrated rate law provides a direct link between the growth dynamics and the observable changes in population size over time. This can be derived as follows:

$$\frac{dN}{dt} = \mu N$$

$$\frac{dN}{N} = \mu dt$$

$$\int_{N_0}^N \frac{dN}{N} = \int_0^t \mu dt$$

$$\ln(N) - \ln(N_0) = \mu t$$

$$N(t) = N_0 e^{\mu t} \quad \text{eq.2}$$

Equation 2, also described in *Bacterial Growth: Constant Obsession with dN/dt* , describes an autocatalytic process: the birth of new organisms accelerates overall population growth³⁶. N_0 represents initial biomass, $N(t)$ represents biomass after elapsed time t , and μt is the growth rate constant, μ , multiplied again by elapsed time.

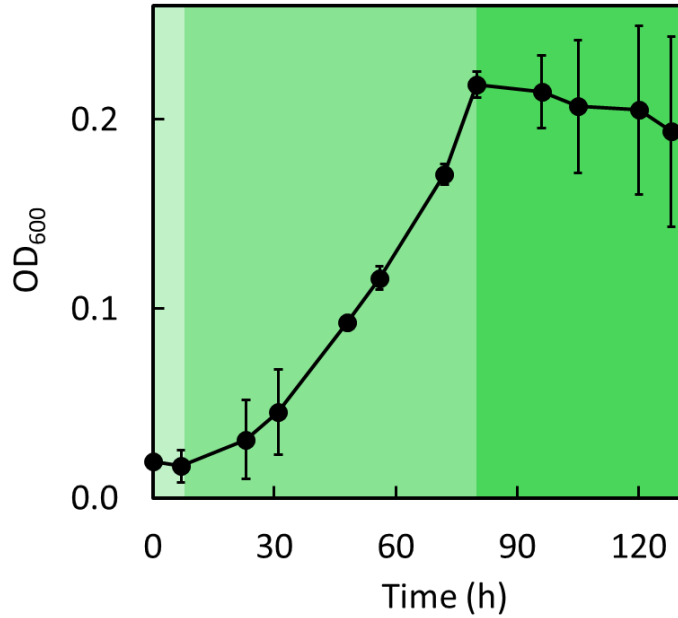


Figure 1.1. Bacterial growth curve plotted on a linear scale versus time. The light green shading represents the lag portion of the growth curve, the medium green represents exponential phase, and the dark green represents stationary phase.

A bacterial population observed in the laboratory will often grow in four distinct phases: i) lag phase; ii) exponential phase; iii) stationary phase; and iv) death phase. **Figure 1.1** illustrates different growth phases of a bacterial population. Lag phase is a period of adaptation to a new environment; for example, a facultative anaerobe will usually need time to up- and down-regulate relevant respiration genes if transferred from an aerobic environment to an anaerobic environment. Similarly, a colony picked off an agar plate and placed in a tube with liquid culture media will adjust to the new aqueous environment for a period. The exponential phase constitutes the period of biomass accumulation through population doubling each generation. Stationary phase is reached when a nutrient becomes limited, and the division and birth of new cells is approximately equal to the amount of cell death. During death phase, cell death outpaces cell replication, resulting in overall population decline. Population-level physiological changes accompany each growth phase. For example, the stringent response is characterized by shifts in transcriptional regulation impacting cell functions such as RNA polymerase activity during stationary phase as a response to stress and nutrient deprivation³⁷.

Population growth in figure 1.1 is measured with optical density (O.D.), one of the most common bacterial growth metrics. O.D. is generally easy to measure in a monodispersed population, and cell population is directly proportional to O.D., though this relationship deviates from linear at very high cell densities. In essence, the higher the population of a cell culture, the less light is transmitted through the culture due to scattering or absorption. This relationship is described by the Beer-Lambert law:

$$O.D. = -\text{Log}\left(\frac{I}{I_0}\right) = xl \quad \text{eq.3}$$

Where I_0 is the light incident on a sample, I is transmitted light, l refers to the path length which light takes through a sample, and x is the bacterial population density. While optical density is not a direct cell count and is only a meaningful growth metric when cells are distributed homogeneously in solution, it provides a quick and robust method to determine population density under the appropriate circumstances. These optical measurements are a common indirect measurement of growth when bacteria are grown in batch culture where volume remains constant over the relevant time. However, O.D. is not a viable strategy for measuring biofilm growth. A meaningful O.D. measurement of a biofilm would require its destruction and homogenization, and achieving resuspension of each individual cell is unreliable. Colony forming unit (CFU) enumeration and flow cytometry also require homogenization of a biofilm prior to quantification³⁸.

Biofilm Quantification through Imaging. Quantifying metrics of biofilm growth, i.e., biomass accumulation and positive correlations to biomass accumulation, can be achieved with a range of analysis methods including dry mass, protein content, ATP bioluminescence, total organic carbon, and crystal violet staining³⁸, as well as advanced methods such as Raman microscopy/spectroscopy³⁹. Microscopy coupled with image analysis are widespread tools for biofilm quantification, and brightfield and fluorescence microscopy are the most accessible imaging techniques. Fluorescence imaging requires autofluorescence or fluorochrome integration through genetic modification or staining, along with appropriate excitation and emission detection systems.

When paired with continuous culture, biofilm quantification enables study of how mass transport (i.e., nutrient flow and shear forces) influences biofilm structure and function.

Continuous culture is achieved through consistent nutrient delivery from a reservoir. The culture chamber is kept at a constant volume in a reactor called a chemostat. Unlike batch culture, continuous culture may introduce significant advective and fluid shear forces which impact biofilm attachment, detachment, and nutrient delivery to a biofilm. Continuous culture also enables cell culture under steady-state nutrient conditions by allowing the operator to control nutrient delivery and precisely introduce environmental perturbations. Finally, in continuous culture, a population's growth rate is determined by the dilution rate of a limiting nutrient. If the dilution rate exceeds the maximum growth rate, washout of the planktonic population is expected.

Chemostats vary widely in their scale, composition, and purpose. The CDC biofilm reactor 40 is designed for continuous stirring and pump-driven advection. Drip-flow reactors are smaller in scale, and fluid flow gravitationally drips through material coupons ⁴⁰. Both systems are designed to enable quantitative analyses such as protein/dry mass, biofilm homogenization and enumeration, staining and imaging macro-scale biofilm structure, and bulk 'omics analysis. The principle of continuous culture can also be extended to the microfluidic scale. Microfluidic flow cells are used to grow and image biofilms in real-time, interrogating fundamentals of their growth, physical characteristics of biofilm structure, and responses to environmental perturbation. Methodological details pertaining to flow cell microscopy and image analysis will be expanded on in future chapters. For now, an example measurement of biovolume and calculation of μ from microfluidic flow cell microscopy of *P. fluorescens* biofilms is presented in **Figure 1.2**.

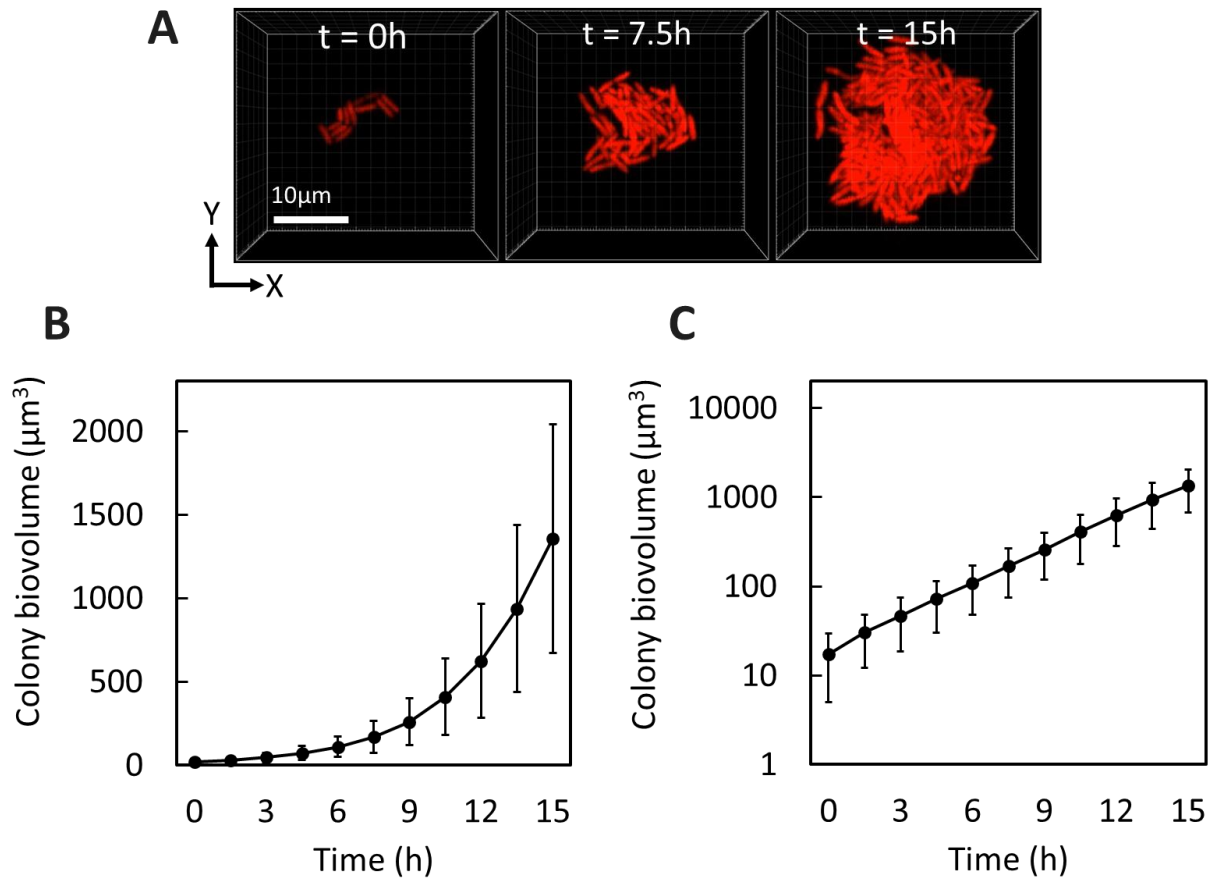


Figure 1.2. A) *Pseudomonas fluorescens* colonies expressing mCherry grown in a microfluidic flow cell and imaged with fluorescence microscopy. The development of individual colonies was observed for fifteen hours, with photos of each colony taken every 1.5 hours. B) Biovolume quantification of nine colonies plotted versus time on a linear scale, and C) on a semilog scale.

Defining Biofilm Developmental Life Cycles. **Figure 1.2** illustrates how a measurement that corresponds positively to growth can be used to represent growth when constructing a growth curve. Bacterial growth curves are likened to a population-level life cycle where stages (lag, exponential, stationary, death) are delineated on the lines of both gene expression and growth kinetic differences. In **Figure 1.2** the biofilm exits lag phase and enters exponential growth over a fifteen-hour interval. The term “life cycle” in the context of biofilm is not meant to be analogous to coordinated, long-range developmental behavior, such as that of a multicellular organism.

Rather, “life cycle” refers to reproducible biofilm development with defined stages, which vary widely between microbial species. Like planktonic growth curves, as biofilms move through stages of development, distinctions can often be made in growth kinetics, gene regulation, and phenotype, with the added complexity of three-dimensional spatial structure.

Our understanding of biofilm life cycles is largely the result of detailed observation and experimentation on a few model organisms. Some of the most well characterized biofilm life cycles are from *Pseudomonas aeruginosa* clinical isolates, which are opportunistic human pathogens and model organisms for biofilm development. Biofilm formation of *P. aeruginosa* isolates begins with attachment of single cells or small groups of cells, followed by formation of microcolonies which exhibit three-dimensional structure. There is often a distinctive reversible attachment, where cells attach to a surface on their poles or appendages then their longitudinal axis, and irreversible attachment, with down-regulation of flagella and expression of EPS (extracellular polymeric substances) ⁴¹. Differences in gene expression regulating motility and transporter-mediated protein secretion constitute evidence for this distinction under laboratory conditions ⁴². Following attachment are stages of maturation, detachment of some or all of the population, and potential re-attachment to an available surface ⁴¹.

It is critical to note that there is no singular definition of a biofilm life cycle. Detailed in vitro observations of *Pseudomonas* biofilm growth on a surface, while valuable, do not meaningfully represent development of biofilms from all organisms. This is a truism but representing biofilm life cycles as a linear process of attachment, maturation, and dispersal is pervasive in literature. For example, delineating a difference between reversible and irreversible attachment is not a meaningful categorization for all biofilms. For example, bacteria that do not

possess motility appendages may instead express adhesins independent of whether a surface is present ⁴³. In these cases, the role of motility up- or down-regulation in a biofilm life cycle is irrelevant.

Dividing the stages of biofilm development into a five-step model of reversible attachment, irreversible attachment, maturation I and II, and dispersal is not generalizable. A 2022 review on conceptually representing biofilm formation advocates for broadening biofilm life cycle models as follows: include non-surface attached aggregate biofilms, reduce specificity of models to mushroom-shaped *Pseudomonas* biofilms, and simplify a general developmental model to three stages: aggregation, growth, and (potential) disaggregation ⁴⁴. Practically, it can still be useful to define a general beginning to the process of biofilm formation. The onset of EPS production fulfills this role well, as EPS production begets 3D structure and acts as a medium for shared space and resources between cells ¹. The synthesis and aggregation of biofilm EPS enables an internal environment where micron-scale resource gradients and population-level physiological heterogeneity can emerge in biofilms ¹¹⁷. From the perspective of treating infectious biofilms, internal heterogeneity poses a challenge: an antibiotic that targets an active physiological state will be less effective at eliminating cells if dormant cells persist in a biofilm, and large antimicrobial molecules may not effectively diffuse through biofilm EPS ⁸. Resource gradients in biofilms can stratify microbial populations and species and contribute to diversification and division of labor ⁵⁵, and Jo et. al. argued that it is crucial to integrate our knowledge of microbial physiology governed by molecular genetics with our understanding of the physical/chemical constraints that form biofilm structure and topology ⁸¹.

Biofilm complexity is observable across scales ranging from single molecules and gene expression profiles between individual cells to population-level life cycles and developmental patterns. To understand, predict, and control biofilms, it is necessary to understand environmental forces that exert powerful selective pressure on biofilm structure and emergent complexity. Looking toward the future of biofilm research, understanding how spatial and physiological heterogeneity and diversity arise in biofilms throughout development will require continued integration of microbial genetics and physiology, growth fundamentals, evolutionary and ecological theory, and mass transport within and outside of biofilms.

1.1.2. Mass Transport is a Powerful Selective Pressure on Biofilm Structure and Function

Biofilms differ from planktonic cells not only in their physiology, phenotype, and life cycle, but also in how their internal conditions are shaped by mass transport processes. As discussed in the introductory paragraphs, biofilms are usually more heterogeneous than planktonic counterparts, regarding both the physiological state of individual cells and internal environmental conditions. This can be attributed in large part to mass transport constraints which produce variability in nutrient concentrations at small scales, and the impact of this variability on biofilm structural development and community function cannot be overstated. Two complementary transport mechanisms that shape biofilms in nature are diffusion and advection.

Diffusion is defined as the movement of matter, such as a group of particles, from an area of high to low concentration. Fick's laws of diffusion were developed by Adolf Fick in the 1800s to describe solute diffusion in fluids. Of Fick's two laws, the first law which relates particle

movement (flux) to a particle's concentration gradient in a defined space is directly relevant to mass transport in biofilms ⁴⁵:

$$J = -D \frac{dC}{dx} \quad \text{eq.4}$$

J is flux in mol/m², D is the diffusion coefficient proportionality constant in units m²/s, and dC/dx is the particle concentration gradient. This holds true when the material medium through which particles are diffusing is homogeneous (i.e., at steady state) and does not change over the relevant measurement timescale. Perhaps the most important aspect of understanding diffusion behavior in biofilms is defining a diffusion coefficient, also known as diffusivity. Fick's first law is used to calculate diffusion coefficients in biological samples with confocal microscopy of inert tracers diffusing through a biofilm ⁴⁶, modelled based on experimental measurements of particle concentration and flux ⁴⁷, and measured with microelectrodes specialized for detecting a specific molecule ⁴⁸. In biofilms, effective diffusivity (*i.e.*, diffusivity changes in the biofilm compared to the aqueous solution) of molecules is affected by density and porosity of the cell population(s) and extracellular polymeric substances ¹¹⁸. Generally, from the diffusing molecule's frame of reference, effective diffusion coefficients decrease with increasing molecule size ¹¹⁹. Wilking et. al. described biofilms as complex fluids where cells resemble colloids and extracellular matrices resemble a cross-linked gel, where heterogeneous matrix composition affects nutrient accessibility and local mechanical properties ¹²⁰.

A 1997 review on effective diffusion in biofilms outlines diffusion coefficient estimation and modelling different solute categories (large, small, and ionic), using known effective diffusion values of biologically relevant solutes in water ⁴⁹. This review contains further mathematical details regarding modeling diffusion across length scales ranging from single cells to biofilms

embedded in EPS. A similar commentary outlines mathematical examples of how biofilm heterogeneity can emerge from mass transport constraints ⁴⁹. It is important to note that for some biofilms, the homogeneous material assumption may be violated, as cell volume fraction can change throughout biofilm depth, thus changing the material properties of the diffusive medium. This is a limitation of the assumptions inherent to Fick's first law. Simple models of diffusion do not explain all behavior and heterogeneity observed in biofilms, but when synthesized with knowledge of cell physiology and biofilm chemical and mechanical properties, can be powerfully predictive of emergent biofilm properties.

Stewart et. al. developed reaction-diffusion models of bacterial biofilms using experimental data from multiple biofilms from in chronic infection environments ¹²¹ and found that reaction-diffusion theory was sufficient to explain biofilm hypoxia observed in chronic biofilm infections. Hypoxia can contribute to the physiological heterogeneity that enables antibiotic tolerance in a biofilm ¹²³, so understanding the constraints that reaction and diffusion of electron donors, acceptors, and other nutrients impose on biofilms is immediately applicable towards understanding chronic infection phenotypes. Advective-diffusion, in combination with cell motility, has been shown to influence development of *C. crescentus* biofilms by affecting morphological assembly patterns and the extent of lineage mixing ¹²². Mass transport dynamics are thus demonstrably connected to surface attachment and downstream spatial patterns of biofilm development

Figure 1.3 illustrates the influence of diffusion on biofilm population structure of a hypothetical biofilm. Biofilms of different thicknesses, and consisting of a facultative and strict anaerobe, are exposed to an oxygenated atmosphere. If the penetration depth of oxygen (which is

a function of both oxygen diffusion and oxygen consumption) is 15 microns, niche space will remain for the strict anaerobe in the thicker biofilm, but this niche space will be wiped out in the thinner biofilm. This principle can be applied to multiple nutrients or electron donors/acceptors simultaneously. A complex picture emerges of biofilms as living collections of cells and extracellular material with biofilm structure and mass transport limitations mutually influencing one another at small spatial scales.

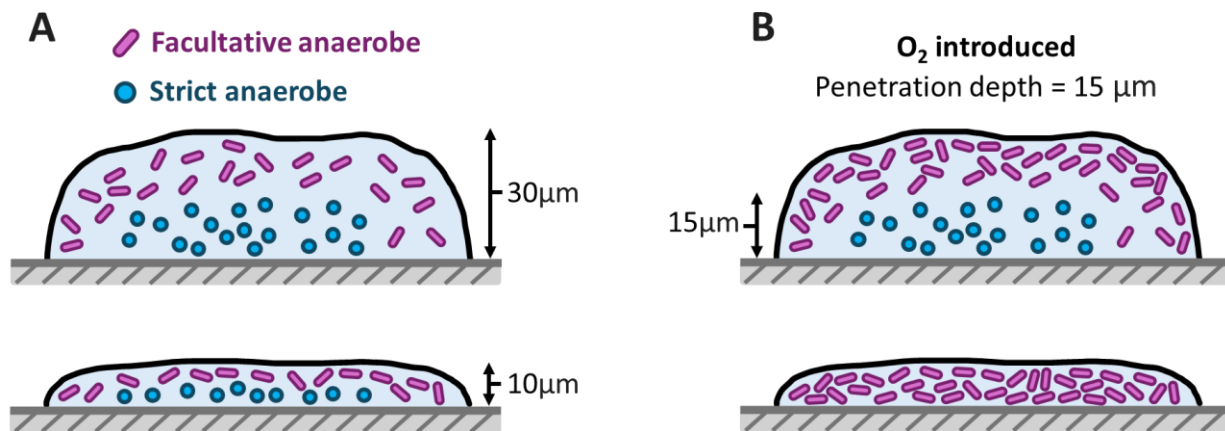


Figure 1.3. A hypothetical representation of a biofilm cross-section composed of a facultative and strict anaerobe with separate spatial niches. A) Two examples of this biofilm with different thicknesses, 30µm and 10µm. B) O₂ is introduced into the headspace and penetrates 15µm deep into the biofilm. The thicker biofilm is left with niche space for the strict anaerobe, while in the thinner biofilm, strict anaerobic niche space is wiped out.

There are cases when the influence of diffusion on biofilm growth and development is outweighed by other mass transport processes. Many biofilms live in environments where material is transported by fluid flow in the natural and built world¹³. Advection is the transport of matter, such as particles suspended in solution, with the bulk motion of fluid in a wide range of environments⁵⁰. This is a transport process by which, for example, upstream sand or silt is moved downstream. If, in this same stream, a biofilm attached to a rock is submerged in the water, nutrients required for its growth will also be transported via advection.

Bulk fluid flow can insulate a biofilm from mass transport via diffusion by flowing around, but not always through, a biofilm ⁵¹, and can impact surface colonization behavior. *C. crescentus* biofilms in controlled microfluidic channels display different patterns of early surface colonization, both in terms of colonization speed and spatial distribution of clusters, depending on the fluid velocity the population is exposed to ⁵². Also in a microfluidic context, pore channel angle relative to the direction of flow in an agarose-embedded *Pseudomonas aeruginosa* influences oxygen delivery to the biofilm as well as the relative importance of diffusion versus advection in delivering nutrients to cells ⁵⁰.

As biofilms build themselves using the nutrients delivered by advection, they can alter these very same mass transport conditions. Coyte et al. showed that rapidly growing *E. coli* biofilms in a porous media microfluidic flow cell could divert flow and nutrients to a channel inoculated with a slower-growing *E. coli* population ⁵³. This effect was dependent on flow rate: in strong flow, the rapidly growing biofilm did not occlude its channel. The authors also considered the relationship between fluid flow on growth rate evolution and integrated these findings with game theory analysis. They predicted an evolutionarily stable state (ESS) would emerge due to faster-growing biofilms eventually occluding their own pore space and diverting resources to an ESS population, and slower-growing biofilms would disperse too few cells to invade an ESS population. When investigating relationships among and between biofilms and making inferences to real-world environments, it is important to consider realistic physical contexts in which biofilms are naturally found. Mass transport is a crucial component of the physical phenomena that shape biofilm structure and function in environments everywhere on Earth.

1.1.3. Social Behavior and Evolution through the Context of Biofilm Structure

With key physical processes that shape biofilm architecture described, the following section will examine how a biofilm's structure mutually influences microbial social behavior and evolution. Sociobiology is a field primarily concerned with understanding social behavior through the processes of evolution and the theoretical lens of natural selection. This field was first outlined in the book *Sociobiology: The New Synthesis* ⁵⁴ which synthesized observations of social behavior with principles of Mendelian genetics and natural selection, with a focus on interpreting the behavior of animals. For historical context, sociobiology is an extension of the 20th century Modern Synthesis and is a body of work characterized by the convergence of Gregor Mendel's experimentation on inheritance and Charles Darwin's theory of natural selection. More recently, evolutionary biologists have begun exploring the social complexity of the microbiological world and examining the applicability of sociobiology towards microorganisms.

Determining what constitutes social phenotypes and behavior is challenging. A principal difficulty is that, in biofilms, the appearance of coordinated behavior can occur without a defined developmental plan ⁵⁵ given that biofilms are multicellular *assemblies*, but not multicellular evolutionary *individuals* ⁵⁶. For example, heterogeneities in population distribution throughout the depth of a biofilm are likely the result of selection for survival in a local environment (i.e., what substrates and electron acceptors are available) rather than programmed behavioral coordination for optimization of community productivity ⁵⁷. Given the more parsimonious view that a phenotype simply incidentally impacts others, how is it determined whether a social phenotype was selected precisely because of the phenotype's effect on other individuals?

Evolutionary biologists studying animal behavior were the first to describe features of social phenotypes and selective conditions which make their evolution possible ⁵⁴. First, a social phenotype is usually costly to express and may not be expressed in the absence of a recipient due to this cost ⁵⁸. Second, social phenotype expression is intertwined with the spatial structure and population density of a community. Finally, there is consideration of genetic relatedness between an actor and recipient of a social phenotype. Altruistic social phenotypes (which, by definition, decrease the reproductive fitness of the actor) are predicted to emerge when altruism is directed towards genetically related individuals, also known as kin selection/inclusive fitness theory, where relatedness is required for the selection of altruism ⁵⁹.

A close examination of biofilm structure and cell-cell communication through molecular signaling reveals emergence of social phenotypes in microbial communities. Some microorganisms have evolved quorum sensing two-component systems to facilitate group-level behavior. The expression of quorum sensing and the behavioral changes it precipitates depend intrinsically on population density ⁶⁰, suggesting quorum sensing was selected at least in part due to impact on non-focal individuals. Though quorum sensing does not always regulate biofilm formation, and biofilm formation can occur without quorum sensing, some bacteria including strains of *Pseudomonas aeruginosa* pair quorum sensing systems with EPS production ⁶¹. The EPS matrix is complex and modular with a multitude of fitness benefits for a biofilm. EPS is generally viscoelastic and predominantly composed of protein, extracellular DNA, lipids, and polysaccharides ⁶². It confers mechanical stability, shelter from predation and antimicrobial attack, can act as a reservoir for nutrients derived from lysed cells, and is a structured environment where cohesive and adhesive forces can help anchor a biofilm to a surface.

EPS production is sometimes referred to as “public goods” production and interpreted as coordinated cooperation among bacteria in a biofilm. In this view, EPS production is a trait that can be exploited by non-EPS producing populations ⁶³. Notably, however, EPS expression is not necessarily a cooperative social phenotype, depending on the frame of reference relating a focal individual to a group. Xavier et al. conducted a simulation of EPS production which suggested that EPS can suffocate cells adjacent to a focal, EPS-producing strain, while pushing genetically related cells up towards oxygen in an aerobically respiring model population ⁶⁴. The authors described this interaction as analogous to trees growing vertically and competing for access to light, with competition being facilitated by the structure of the community. EPS secretion has social fitness consequences, is essential for biofilm emergent properties, and this social phenotype can powerfully influence a biofilm’s physical structure as it grows.

The impact of social relationships between populations residing in a biofilm can be influenced by spatial structure throughout development. If spatial structure is not disturbed, the likelihood that social phenotypes such as public goods secretion can be directed toward related individuals may increase, providing inclusive fitness benefits ⁶⁵. This is a key predictor for the evolution and fixation of sociality: natural selection in a spatially structured environment is predicted to promote the emergence of social cooperation when cells are surrounded by identical genotypes at relevant loci ⁶⁶. In this view, biofilms may harbor more potential as a reservoir for genetic diversity (such as the emergence and fixation of social phenotypes) compared to planktonic populations, which are more commonly characterized by selective sweeps of a small number of successful mutations which purge diversity. Differences in selective constraints and maintenance of diversity between planktonic and biofilm populations is a consistent finding of microbial

experimental evolution, demonstrated by the greater abundance of mutations maintained in biofilm
24.

Biofilms exemplify the connection between social and structural complexity. Returning to the overarching theme of mutual dependence between structure and function, metabolic interactions between different populations at the onset of biofilm formation can drastically affect a biofilm's developing structure. Ciccarese et al. examined the range expansion behavior of *Pseudomonas stutzeri* biofilms with two different strains, each expressing a different fluorescent reporter⁶⁷. One biofilm consisted of two *P. stutzeri* strains that each completely reduce nitrate to nitrogen gas, thus competing for access to nitrate to fuel dissimilatory metabolism (the complete reducer biofilm). The other biofilm consisted of one *P. stutzeri* strain with *nirS* loss-of-function preventing nitrite reduction, and one *P. stutzeri* strain with *narG* loss-of-function preventing nitrate reduction, meaning the two populations do not compete for nitrate (the producer-consumer biofilm). Electron acceptor competition in the complete reducer biofilm produced a pattern of spatial organization where each population was segregated along the range expansion front. In contrast, the producer-consumer biofilm produced a pattern of spatial mixing as the biofilm grew and expanded. In this simple model system, different spatial organizations emerged from initially mixed cultures due to imposed changes on cell metabolism which either facilitated or removed competition for nitrate.

The former example illustrates the structure-function connection between metabolic competition and biofilm development by manipulating the metabolic relationships of two different populations. To explore the inverse condition, i.e., the outcomes of competition changing as a function of initial spatial arrangement, 3D bioprinting can offer structural control over microbial

spatial mixing to enable experimentation on biofilm structure-function relationships at sub-millimeter spatial scales. An elegant example of this approach was developed by Kumar et al., who developed a droplet-based extrusion bioprinting system and built synthetic biofilm by controlling the spatial mixing of different strains of *E. coli* ⁶⁸. These different strains were either susceptible to bacteriocin toxins, resistant to them, or expressed different bacteriocins with variable toxicity. When bacteriocin-producing and bacteriocin-susceptible populations of *E. coli* were arranged with no spatial mixing, an ecological refuge effect was created where a subset of the susceptible population persisted. However, when populations were mixed, the susceptible cells were largely wiped out. This is a clear demonstration that micron-scale changes in initial spatial structure within a biofilm can generate drastically different outcomes of competition between populations.

The effects of micron-scale structural manipulation may be different than larger-scale structural manipulation would predict. Kumar et. al. discuss a similar manuscript examining the effects of competition via bacteriocin production, but with colonies of the two populations spanning several millimeters in diameter ⁶⁹ in contrast to arranging genotypes via bioprinting in colonies approximately 600 microns in diameter. At larger scales, two bacteriocin-producing strains were able to persist despite the other's competitive attack, while at the smaller scale, both populations were wiped out. This highlights the need to understand and predict bacterial behavior at small spatial scales, and the need to further develop microfabrication methodology to finely control biofilm structure. The following section will examine the state of the microbial 3D bioprinting literature in detail and evaluate microfabrication with 3D bioprinting as a tool for building biofilms with useful function and for understanding fundamentals of microbial behavior.

1.2. Controlling Small-Scale Biofilm Structure with 3D Bioprinting

Research on controlling biofilms is typically in the context of biofilm elimination or harm reduction given the massive economic and health threats that biofilms can pose to society. Biofilm control in the context of designing physical structure and community composition is a newer concept enabled by technological advancements in microfabrication and small-scale biofilm reactor design. Controlling self-assembled biofilm structure, particularly biofilm thickness, has provided insight into biofilm diversity and development from a homogeneous planktonic inoculum. Fowler et. al. controlled the maximum thickness of nitrifying biofilms using a physical growth barrier, resulting in biofilms 50 to 500 microns thick ⁷⁰. They found that thicker biofilms consistently supported the development of a more diverse community from a planktonic inoculum, compared to the thinnest 50-micron biofilms which were more phylogenetically homogeneous. In a previous related manuscript, the authors found that controlling biofilm thickness resulted in a functional tradeoff. Thicker biofilms captured and degraded pollutants more effectively than thinner biofilms, but thinner biofilms performed more efficient nitrification ⁷¹. Suarez et. al. compared diversity of 50- and 400-micron thick nitrifying biofilms grown in the same reactor ⁷². They found that changing thickness causes changes in community composition, and FISH-stained cryosections of each biofilm revealed that the thicker biofilms supported greater diversity of nitrifying ecotypes.

The three studies above utilized commercially available AnoxK™ Z-carriers which have a grid-covered surface that sets an upper limit on thickness as a biofilm grows in a reactor. These carriers can control biofilm thickness in steps of 100 microns, up to 500 microns. Commercially available methods for controlling biofilm structure are valuable, but do not generally offer the

flexibility for researchers to design bespoke biofilms with modular control over structure in all three spatial dimensions. In addition, the impact of structural manipulation on biofilm function at one scale may not meaningfully predict impacts at other scales ⁶⁸, underscoring the need for fine structural control at multiple scales of resolution. Biofilm microfabrication with 3D bioprinting directly addresses this need.

Given the mutual dependence of structure and function in microbial biofilms, developing experimental methods to finely control biofilm structure is a salient goal. Microbial 3D bioprinting has grown out of the tissue engineering literature, where 3D bioprinting was recognized for its potential to fabricate simplified mimics of human tissue in three-dimensional space ⁷³. 3D bioprinting repurposes existing 3D printing technology to design and construct biological materials with a living component, such as a tissue type or microbial population, and a non-living component, such as a synthetic or natural polymer. The living and non-living components, when combined, are called bioinks. Bioinks are usually designed with channels and/or hydrogel properties that permit nutrient exchange with the outside environment but with pore sizes small enough to provide cell encapsulation and immobilization in the materials ⁷⁴. Bioinks have versatile rheological properties and metabolic capabilities, much like natural biofilms, although bioinks differ from natural biofilms in that cell populations are held captive inside of synthetic bioink matrices. When encapsulated in a polymerized bioink, cells may not be able to engage in a biofilm developmental life cycle, being deprived of the opportunity to disperse and re-attach.

Microbial 3D bioprinting is performed with commercially available printers ⁷⁵, modified commercially available printers ⁷⁶, and entirely home-built printers ⁷⁷. Most bioprinting is assisted by computer-aided design, and printing is typically done in a layer-by-layer fashion ⁷⁸. Though

there is wide methodological variability, three broad categories of microbial 3D bioprinting are dominant in the literature: extrusion bioprinting, droplet printing, and laser-assisted vat polymerization ^{79,80}. Each method has unique advantages and drawbacks regarding biocompatibility, spatial resolution, printing time, and ease of use. Extrusion 3D bioprinters utilize a printhead which moves in the x-, y-, and z-directions. The printhead deposits a fluid bioink fed mechanically or pneumatically through a nozzle onto a solid surface or into a cross-linking bath ⁸¹. The bioink is then solidified into a hydrogel. Resolution depends predominantly on the size of the extrusion nozzle and is currently limited to about 100 microns in three spatial dimensions ⁸². Extrusion bioprinting is possible with a wide range of bioinks. The main constraint on compatibility is whether the bioink polymer can be deposited in a liquid state through the printhead nozzle without killing the cell population due to shear or temperature stress ⁸¹. Polymers used with extrusion bioprinting include alginate, polyethylene glycol, collagen, gelatin, fibrin, chitosan, agarose, and more ⁸².

Droplet printing is a printing technique where, like extrusion printing, controlled droplet volumes are deposited through a nozzle ⁸³. Cells become encapsulated within printed droplets when the bioink is deposited. Droplets can be isolated, deposited in small groups, or part of a larger complex shape. As with extrusion printing, droplet printing resolution is limited by nozzle size ⁸⁴. Advantages of this technique are the precision of material deposition, both in location and volume, and general versatility. Drawbacks include shear stress-induced cell death ⁸⁵, which becomes more problematic as the nozzle size is reduced, creating a trade-off between cell viability and spatial resolution. Several polymers are also compatible with droplet printing: gelatin, polyethylene glycol, collagen, alginate, agarose, and fibrin ⁸³.

Finally, vat polymerization is a 3D printing technique where liquid photopolymerizable resin is cured via laser exposure. Prints are assembled layer-by-layer. Resin consists of, at minimum, a monomer and a photoinitiator. The photoinitiator absorbs light at a specific wavelength and is promoted to the excited state, beginning a free-radical polymerization reaction. Photoblockers such as chlorophyllin or tartrazine have a concentration-dependent effect on cure depth reduction via absorption when added to the resin, which improves depth resolution to 10s of microns⁸⁶. Laser wavelength for vat polymerization is typically UV or near-UV. Stereolithography (SLA) printers utilize a raster-scanning laser in the *xy* plane to cure resin into a desired shape, while digital light processing (DLP) printers direct the laser into the resin vat with a series of micromirrors to selectively cure resin at the desired locations⁸⁷. Vat polymerization is faster than extrusion and droplet printing methods and has improved spatial resolution. However, a major disadvantage is reduced compatibility of resin polymers compared to extrusion and droplet printing, as the monomer must be photopolymerizable. Alginate, for example, must be functionalized to cross-link via photoexcitation.

The following perspective explores three unifying themes present in the nascent microbial 3D bioprinting literature: designing microbial biofilms for medical and environmental research, printing biofilms for fundamental behavioral research in the context of biofilm spatial structure, and the fabrication of functionalized, engineered living materials with bioprinting. The themes discussed here are distinct but are not mutually exclusive and span both fundamental and applied research. Bioprinting, broadly, is a vast field when eukaryotic tissue engineering is considered, but the focus of this perspective is restricted to recent advancements in microbial 3D bioprinting. The

breadth of research in each category will be discussed, and a synthesis and outlook on the future of microbial 3D bioprinting will be explored.

1.2.1. Designing *in vitro* Microbial Habitat

Natural microbial habitats can be complex and variable at very small spatial scales. Generally, microbial biogeography is focused on how microbial communities disperse, develop, and function while integrating the spatial context of respective, natural environments. Microbial behavior is connected to the physical context of natural environments and of internal biofilm structure, but examining this connection is challenging, as replication of environmental complexity *in vitro* requires small-scale control over the relevant spatial characteristics (porosity, surface texture, interface with host cell types, fluid transport, etc.) of a microbial habitat. Researchers are using microbial 3D bioprinting to achieve necessary spatial control to construct microbial niches and design biofilms *in vitro* for environmentally and medically focused research.

Smercina et. al.⁷⁵ fabricated soil habitats at small spatial scales by 3D bioprinting porous soil aggregates and investigated how physical habitat structure affects bacterial/fungal co-culture biogeography. The authors used a commercially available printer to fabricate aggregates with encapsulated bacteria and/or fungi with a mean pore size of 18.5 μm . Following habitat fabrication, community growth was measured with a combination of fluorescence microscopy, diffusion kinetics, and multi-omics characterization of community physiology. Similarly, biomimetic coral habitats were 3D printed by Wangpraseurt et. al.⁸⁸ using vat polymerization to mimic coral spatial microenvironments and build an experimentally tractable coral-algal interaction model system. The data suggested that coral skeletal and cavity structures replicated natural diffusion processes at the coral tissue diffusive boundary layer. Symbiotic microalgae were encapsulated in the coral

tissue and growth was imaged with fluorescence microscopy. The authors stated that fabricating accurate coral microenvironment structures with 3D bioprinting may have long-term positive impacts on natural coral reef restoration. Liu et. al.⁸⁹ printed combinations of alginate-gelatin hydrogels to intentionally produce a stable denitrifier niche. The authors characterized the rheological properties and viscoelastic behavior of bioinks with variable gelatin ratios, and encapsulated cell growth was visualized with fluorescence microscopy. The denitrification performance was comparable to free-swimming bacteria; however, longevity of activity was not assessed between the gel-based and planktonic cells.

Ceballos-González et. al.⁷⁷ used a simple extrusion method to print hydrogel fibers with multiple interwoven bioinks. The interface surface area between bioinks containing different populations could be controlled, and more shared interface between *Lacotbacillus* and *E. coli* resulted in greater *E. coli* growth inhibition. The authors proposed 3D bioprinting as technology to test predictions of microbial consortia behavior and modeling host/microbial interactions. In subsequent work, hydrogel fiber bioprinting was used to fabricate 3-population hydrogels with spatial resolution at 10s of microns⁹⁰. The populations were either well-mixed in the printed hydrogel or spatially separated. Formation of anoxic niches in the hydrogel facilitated growth of both facultative and strict anaerobic populations, and growth of two of the three populations in the consortia improved when spatially separated.

These examples suggest that 3D printing could further refine the ability to utilize engineering control points for desired outputs with biofilms due to the added control at small scales. Typical control points with engineered biological systems include the (i) division of labor, (ii) spatial/temporal organization, and (iii) emergent properties from interactions⁹¹. Division of

labor (i) among community members mitigates constraints imposed by tradeoffs, assuming the pursuit of one objective is realized at the cost of an alternative objective ⁹². Spatial/temporal organization (ii) within nature allows the incorporation of incompatible members or processes through spatial and/or temporal segregation. The design of artificial systems (*e.g.*, 3D printed biofilms) that incorporate spatial and/or temporal segregation will allow for rational engineering of system stability while mitigating competition and/or promoting cooperation. Emergent properties (iii) result from interactions at various scales that lead to system-level behavior that is more than the sum of the parts^{93, 94}. Ultimately, biofilms (and biology in general) appear to self-organize into complementary units or modules, and engineering principles with 3D printing could be used to design and control biological, modular systems for desired outputs.

As natural biofilms mature, altered phenotypes develop, for example, related to increased antibiotic resistance or tolerance more effectively than planktonic cells. Tolerance or resistance can be conferred through mass transport limitation in the biofilm interior as 3D structure develops, or through development of ecological niches inside a biofilm. Physiological diversity of cells in a biofilm can result in a wide range of phenotypic responses to antibiotics ²², often resulting in incomplete killing of the biofilm. The utility of 3D bioprinting for investigating the biofilms antibiotics resistance was demonstrated clearly by Ning et. al. ⁹⁵. Extrusion bioprinting was used to print *E. coli*, *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA), and *P. aeruginosa* bioinks with an alginate polymer. The synthetic biofilms were printed with varying porosity and thickness, with thickness ranging from 0.25 mm to 4 mm, and the films were cultured under batch conditions for 28 days. Organisms encapsulated in the printed hydrogels (3D) displayed improved antibiotic resistance compared to biofilm monolayers (2D) cultured in a well plate, and *E. coli*

cells in 2mm thick hydrogels survived tetracycline treatment more than *E. coli* cells in 1mm thick hydrogels. While an elegant demonstration of mimicking the structure of natural biofilms *in vitro*, it is not well understood whether synthetic hydrogel-encapsulated cells are physiologically similar to an *in vivo* biofilm infection at the level of gene transcription and general response to environmental conditions, and this question is grounds for productive future work in the microbial 3D bioprinting field.

Medical applications of 3D bioprinting extend to studying host-pathogen interactions by building environments that meaningfully mimic biofilm growth on host tissue. Aliyazdi et. al. developed a gelatin/alginate bioink with encapsulated *E. coli* to print hydrogels with a simple extrusion method ⁹⁶. Biofilm cells were pre-grown before mixing and printing with the bioink polymer and then the printed hydrogels were tested for antibiotic susceptibility. Printed biofilms experienced similar log reductions in CFU/mL counts to self-assembled biofilms for *E. coli*. Metabolomic analysis showed that self-assembled and printed biofilm metabolic profiles were approximately 70% similar in measured metabolites. Finally, the authors printed *E. coli* biofilms on top of human bronchial epithelial cells and chemically cross-linked the printed biofilms to retain shapes on epithelial cells for at least 24 hours without meaningful cytotoxic effects on encapsulated cells. Cheng et. al ⁹⁷ printed a matrix with an open channel, seeded gut epithelial cells on the matrix, and inoculated the epithelial channel model with *Salmonella* and *Lactobacillus*. Anaerobic conditions were maintained in the channel and showed that *Salmonella* infection physiologically mimics *in vivo* infection (though at a short timescale, as *Salmonella* killed all epithelial cells within 12 hours), while the benign *Lactobacillus* had no meaningful negative effect on the epithelial cells.

Results indicate that the authors developed a simplified but relevant *in vitro* spatial and physiological mimic of an anaerobic gut environment.

Graf et. al. printed hydrogels with encapsulated human macrophages and assessed how macrophages are affected by changes in material stiffness and structure, concentrating on measuring acute immune responses ⁹⁸. When challenged with *P. aeruginosa* invasion into the bioprinted matrix, possible due to the estimated pore size of about 10 microns, macrophages could phagocytose the bacteria. Phagocytosis was slightly more effective in the lower-stiffness hydrogel, though still observed in both gels. The authors note that it is crucial to develop experimental systems that represent the physical context of an infection microenvironment to understand how both the host and pathogen are behaving, and to develop effective treatments.

The scales at which biofilm spatial structure was controlled in the studies examined here range from 10s-100s of micrometers to millimeters. *In vitro* microbial niche construction and environment modeling is currently a small subset, albeit diverse, of all 3D bioprinting research. In principle, 3D bioprinting can be used to build a wide range of *in vitro* microbial environments, if the printing methodology has sufficient resolution to fabricate relevant environmental characteristics such as porosity or topology of multiple microenvironments. Across wide-ranging subjects from fabricating synthetic coral microenvironments to developing specific infection models, this research is united by identifying the most important physical characteristics of microbial environments that can be controlled and systematically tested.

1.2.2. Fundamentals of Behavior and Sociomicrobiology

Social relationships between microbial populations in biofilms are deeply intertwined with spatial structure, and the inherent structure can affect the overall functions of the bio-system that

ultimately have consequential impacts on the overall system of interest. Recognizing this, there is a small but fascinating literature using 3D bioprinting to build biofilms, perturb spatial structure or mixing, and examine the impact on microbial behavior and relationships. These studies explore the fundamental nature of the structure-function relationships for biofilms that provide insight for any system where biofilms play a part, including human health, environmental systems, water distribution, soil health, and industrial processing. Integrated into the context of physiological heterogeneity, microbial behavior is impacted by sociomicrobiology where different microorganisms interact in overall neutral, positive, or negative ways. 3D bioprinting for fundamental microbial behavioral research is currently the least explored area of the topics included in this perspective. However, this fundamental research overlaps with the other three categories, given that any living system (i.e., an engineered living material or *in vitro* bioprinted microbial habitat) is subject to the conditions and constraints of natural selection in the presence of other cells and/or populations. Kumar et. al. reviewed the microbial 3D bioprinting literature from the perspective of modelling and understanding microbiomes⁹⁹. The authors discuss that the ability to precisely arrange microbial populations in three-dimensional space opens tremendous potential to understand the relationships between initial and subsequent spatial arrangements and the associated microbial behavior. Thus, the 3D printing technology allows researchers to test predictions of ecological theory at small spatial scales. Perhaps, not unlike investigating seed dispersal and forest propagation for trees, biofilm seeding and dispersal for different environments could be explored with the control enabled by 3D bioprinting.

In a research article demonstrating the utility of 3D bioprinting for fundamental research, Kumar et. al. used a custom-built agarose droplet printer to arrange different genotypes of *E. coli*

at the sub-millimeter scale ⁶⁸. Different genotypes were accurately patterned with complete mixing, complete separation, and degrees of mixing/separation in between. The bulk of the study examined interference competition between the tested *E. coli* genotypes producing colicin toxins of variable potency and a colicin-susceptible *E. coli* strain. The susceptible population was either reduced in population or wiped out by colicin toxins when it was completely mixed with colicin producers, depending on the potency of the colicin. However, for all but the most potent colicin, the susceptible population was more resistant to the toxin when spatial mixing decreased. Initial spatial mixing, controlled with 3D bioprinting, directly affected the outcomes of ecological competition between *E. coli* strains. Observations such as these have been reported previously where ‘self-assembled’ biofilms had varying degrees of population intermixing based upon extents of cooperation or competition ^{100,94}. Bioprinting methods now facilitate the ability to spatially position different structures and elucidate subsequent functions and ecologies.

3D bioprinting was used to spatiotemporally examine syntrophic metabolite sharing between *Salmonella enterica* and *E. coli* in a manuscript by Hynes et. al ⁷⁶. The authors used a microarray printer to arrange the two populations at increasing colony center-to-center spatial distances ranging from 1 mm to 3.5 mm, examined the consequences of this distance variability on growth, and compared the results to simulations of biomass accumulation. Increased distance between the syntrophic consortium decreased biomass at 96 and 144 hours of elapsed time, which aligned with model predictions. When a diffusion barrier of wild-type *S. enterica* was printed between the two populations, growth of the syntrophic consortia was substantially arrested. The authors (along with others) demonstrated the value of 3D bioprinting for testing predictions of microbial growth and behavior modeling at small spatial scales.

The two studies examined above are united by an exploration of how initial biofilm spatial structure, controlled with 3D bioprinting, impacts microbial relationships through outcomes of competition and syntrophy. Along related themes, Müller et. al studied *E. coli* chemotaxis, growth, and communication via quorum sensing inside a 3D printed alginate/agarose hydrogel, examining the fundamental characteristics of the encapsulated environment ¹⁰¹. The authors printed hydrogels up to 10 mm thick, characterized rheological properties of the bioink, and introduced inducer molecules to control gene expression of the encapsulated bacteria. Following this, a quorum sensing sender-receiver pair was 3D printed in a spiral shape, with some trials producing differentiation in the response timing of the receiver strain based upon spatial position. Finally, nonmotile cell-laden hydrogel was printed into a soft agar plate, and the authors demonstrated that the hydrogel acted as a barrier for the chemotactic migration of a motile strain. The authors stated that 3D bioprinting could enable rational engineering of cooperative co-cultures, given that bacterial interactions are dependent on spatial positioning, advance the study of bacterial ecology under controlled experimental conditions, and enable fabrication of soft living biomaterials capable of information processing.

1.2.3. Engineering Living Materials

Engineered living materials (ELMs) are a class of materials containing living cells and a polymeric scaffold that are metabolically active, interactive with the environment, and are usually fabricated for specific purposes such as sensing, chemical synthesis, waste processing, living textile production, or wound repair ¹⁰². Biofilms, with inherent diversity of material properties, diversity of constituent organisms, and self-healing properties, have been used in a natural or modified form to construct ELMs ¹⁰³. In fact, microbial biofilms could be viewed as some of the

first and most-evolved biomaterials known on the planet, in which microbial populations and/or communities are self-organized and encased in a matrix of polymeric materials. Most of the microbial 3D bioprinting literature is either focused on, or references, fabricating ELMs. As with fundamental behavioral research, ELM fabrication underpins the entire microbial bioprinting literature, since any printed material made from microbial bioink fits the definition of an ELM. What distinguishes the research reviewed in this section from the previous sections is concentration on the specific, functional design and purpose of the respective bioprinted material. The living component of the ELM is generally treated as a means to the functional end of the material. The design space of ELM fabrication for microbial bioprinting is vast: several, though not all, focal areas will be highlighted here.

Gantenbein et. al. 3D printed a mycelium/agar-based living material and shaped mycelial behavior by controlling nutrient availability to the printed constructs ¹⁰⁴. An agar hydrogel was mixed and inoculated with fungal mycelium to create a bulk material referred to as the feedstock ink. The feedstock was then extrusion printed to create complex shapes. Altering the malt extract concentration in the bioink changed the spatial expansion of the fungal hyphae within the material, and a malt extract concentration was established which allowed hyphae to bridge air gaps of 2.5 mm. Mycelia were able to bridge a deliberate cut in the material and cover the gap over a period of six days. The authors characterize mechanical properties of the mycelia-laden hydrogel and measure mycelial growth over time by dry mass, showing a nearly five-fold increase in biomass at 10 days of growth. The behavior of the ELM was tunable with simple environmental parameters and was used as protective skin allowing a simple robot to move underwater. Balasubramanian et. al. developed flexible photosynthetic materials with 3D bioprinting by encapsulating microalgae

in an alginate matrix and demonstrated both structural robustness of the living material and reusability with entirely biodegradable bioink components ¹⁰⁵.

Emerging areas of synthesis between ELM fabrication and synthetic biology are inducible bacterial biosensors and genetically programmable synthetic biofilms. Using stereolithography, Dubbin et. al. bioprinted *E. coli* as a proof-of-concept for engineering multiple *E. coli* strains with complex spatial geometry ¹⁰⁵. They fabricated ELMs with two different PEG polymers of different molecular weights and found that the higher molecular weight polymer blend produced a hydrogel with higher diffusivity and supported more biomass accumulation. After characterizing material properties, the authors printed *Caulobacter crescentus* hydrogels with a flat cube and a porous grid geometry and exposed the hydrogels to neodymium to test absorption of this rare earth element. The grid geometry showed more rapid absorption compared to the cube. Finally, a genetically engineered *C. crescentus* strain was incorporated into printed hydrogels for uranium sensing. When exposed to 2.5 μM and 10 μM dissolved uranium, fluorescence intensity of the printed *C. crescentus* hydrogel increased compared to a no-uranium control. Liu et. al. used an extrusion printer to fabricate ELM sensors with 30 μm resolution ¹⁰⁶. They designed their living material to respond to an AHL (a quorum sensing molecule) by expressing a GFP reporter through genetically engineered sensing in *E. coli*. The authors demonstrated construction of Boolean logic gates, turning GFP signal on or off depending on the structures printed, and translated this printing technology to a living flexible tattoo onto human skin. The printed tattoo was able to sense and activate GFP reporter expression in response to rhamnose, IPTG, and the AHL smeared on skin.

González et. al. printed *Bacillus subtilis* spores in an agarose scaffold via extrusion printing and showed extreme resiliency of the resulting living material to environmental stressors such as

desiccation, UV light and radiation, pH and osmolarity fluctuation, and solvents¹⁰⁷. Printed ELMs could be dehydrated and rehydrated, with *B. subtilis* surviving this process and germinating in the agarose hydrogel. Cutting the hydrogel also induced germination at the cut site. IPTG, xylose, vanillic acid, and cuminic acid-inducible genetically encoded sensors were responsive in the living material, showing GFP signal discernable from background fluorescence 12 hours after induction. The authors printed a hydrogel to cover a millimeter-scale wound model. The hydrogel contained *B. subtilis* strains that could sense *S. aureus* quorum signals and synthesize a *S. aureus*-clearing antibiotic in response.

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Engineered living materials with genetically engineered fibers were fabricated using *E. coli* as the constituent organism by Duraj-Thatte et. al¹⁰⁷. In this study, the bioink was entirely derived from genetically engineered *E. coli* culture which produced recombinant nanofibers made from fibrin domains and curli nanofibers. Three inks with three different engineered nanofibers were

produced and printed via extrusion bioprinting, rheological properties were characterized, and printing was demonstrated up to 21 layers. Three different applications of the functional living material were demonstrated: IPTG-inducible release of an anticancer drug, sequestration of BPA in the printed structure by genetically programmed production of BPA-binding peptide, and population regulation by induction of a cell growth-arresting exoribonuclease to control population size within the material.

A 3D bioreactor was printed by Cui et. al. in order to efficiently produce hyaluronic acid, integrating microbial biosynthesis with 3D bioprinting ¹⁰⁸. The researchers sought to improve the harvesting and purification process of hyaluronic acid by immobilizing cells in the printed matrix while the product diffused out. They used a GelMA matrix with hyaluronic acid-producing *Streptococcus zooepidemicus* and showed that cells encapsulated in the bioprinted grid structure produced hyaluronic acid more efficiently than a solid structure or structure-free fermentation in the culture media. Building on this, grid geometry was systematically altered by changing layer orientation and inter-filament distance. The former had minor effects on hyaluronic acid production, while the latter was more pronounced: an intermediate filament distance improved yield by almost 20%.

Applications for ELM fabrication using 3D bioprinting have also been extended into electrochemistry. Bajracharya et. al. 3D printed a bioink made of alginate and *Sporomusa ovata*, an organism capable of producing acetate from carbon dioxide and electrolytically produced hydrogen, onto copper, stainless steel, and titanium meshes ¹¹⁰. They aimed to improve hydrogen accessibility to the organism by printing directly onto metal cathodes and demonstrated microbial electrosynthesis on each material, but the bioprinted matrix improved acetate production only on

the titanium mesh, possible due to biocompatibility issues with the former two materials. Krige et. al. also used extrusion bioprinting to print a living material matrix of *S. ovata* directly onto a carbon cathode to attempt to improve electron transfer to the microbial population ¹¹¹. They demonstrated substantially higher acetate production when cells were 3D printed directly onto the cathode compared to a suspended cell inoculum control.

Engineered living materials are an area of heavy focus in microbial 3D bioprinting literature. Scales of interest regarding ELM fabrication range from micrometers to nearly a meter, demonstrating the versatility of 3D bioprinting. Applications of 3D printed ELMs are broad: the diverse metabolic capabilities of microorganisms have been utilized to fabricate materials capable of environmental sensing, fermentation, photosynthesis, and regeneration. An important consideration of living material fabrication is the ability to scale up for practical applications, which is a constraint that fundamental research does not often have. The transdisciplinary nature of ELMs requires synthesizing materials science, soft-matter physics, microbiology, and engineering and has resulted in a growing field that explores the capabilities of microorganisms in human-defined contexts.

1.2.4. Microbial 3D Bioprinting Outlook

Microbial 3D bioprinting is a diverse field integrating microbiology, additive manufacturing, and materials science. Recent advances in biofilm and soft materials research have facilitated the development and building of living microbial materials for a wide range of functions. Coupling 3D bioprinting techniques with traditional methods in biofilm research and analysis such as microscopy, cell enumeration, antimicrobial tolerance and resistance testing, genetic programming, and 'omics has enabled researchers to define a synthetic biofilm design

landscape serving both fundamental and applied research (**Figure 1.4.**). There is still much to explore in synthetic biofilm design and application towards the growing bioeconomy, and a few lines of inquiry will be examined to conclude.

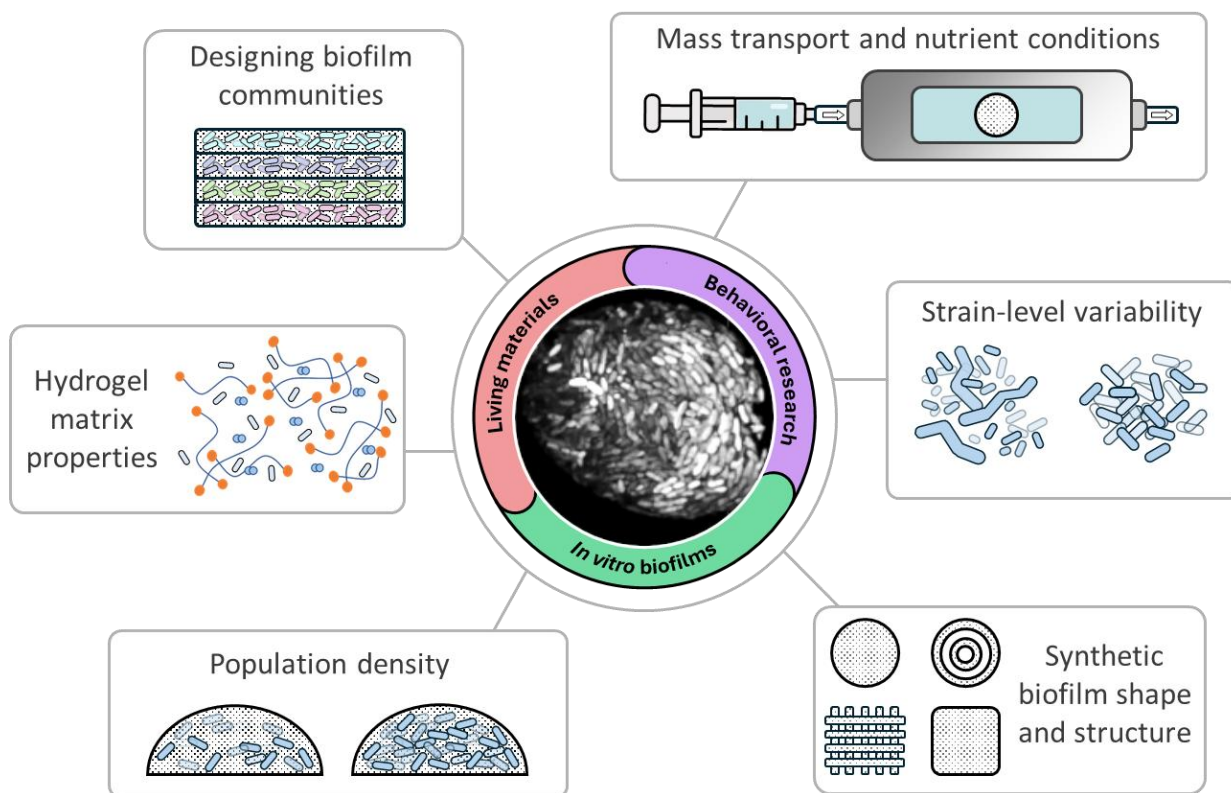


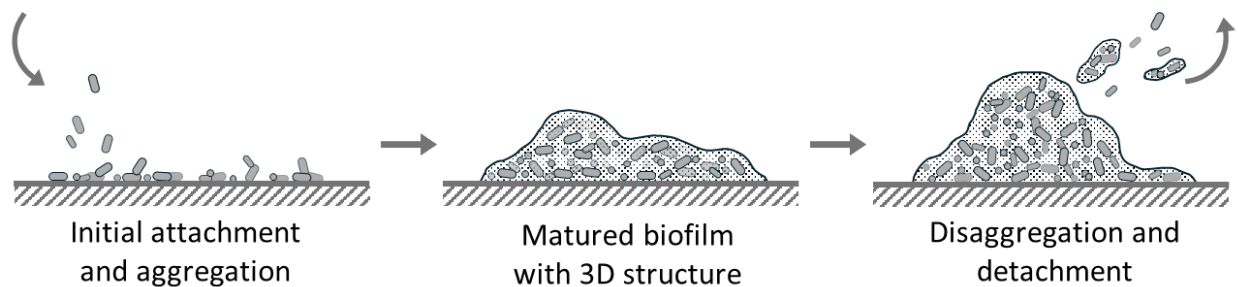
Figure 1.4. Conceptualization of the synthetic biofilm design landscape. Controllable variables (i.e., population density, matrix properties) all potentially influence the design, structure, and overall function of synthetic 3D printed biofilms in each of the three identified literature themes.

First, we will consider the material environment of printed hydrogels. There are several instances of researchers characterizing hydrogel rheological properties during and after printing^{101,107} to optimize bioink printability and retention of intended structure. Material characterizations are important for printing replicable structures with desirable hydrogel properties and these properties may change during biological development. Improved understanding of the

living material environment will be necessary to predict and control microbial behavior within different materials. Measuring and modelling effective diffusion coefficients as well as surface boundary conditions for synthetic printed biofilms can help determine if synthetic biofilms have similar mass transport properties to self-assembled biofilms. Overall, determining what selective constraints cells experience when encapsulated in a synthetic material, and whether these constraints differ meaningfully from a natural context at different scales (cell, population, community), will allow researchers to identify key controls governing synthetic biofilm growth and development and inform understanding about fundamentals of biological organization.

In nature, biofilms grow persistently at interfaces exposed to fluid and/or gas flow, such as interfaces in a stream, pipe, or skin wound. Mass transport via fluid flow delivers nutrients to biofilms, and in some species, increasing shear stress can change biofilm attachment and physiology on a given surface ¹⁰⁹. There is a body of literature dedicated to categorizing, simulating, and predicting microbial responses to fluid flow which is relevant for any biofilms in the respective environments and include fate and transport in the subsurface ¹¹⁰, biofilm attachment and detachment ¹¹¹, and understanding diffusion of substrates into biofilms ⁴⁸. It is not well known how changing mass transport conditions, in terms of nutrient flux, internal diffusion, fluid shear, osmotic pressure, and fluid velocity, affect cells encapsulated in 3D printed matrices. Additionally, given that cells are typically immobilized inside 3D printed biofilms, population immigration and emigration due to fluid shear may not be relevant to the life cycle of a synthetic printed biofilm. If dispersal is an important part of the biofilm life cycle, encapsulation in a printed hydrogel may have unpredictable long-range effects in terms of biofilm behavior (**figure 1.5**). That said, the biofilm life cycle was recently revised to better recognize the importance of aggregates ⁴⁴, and

aggregates are cohesive, distinct, small micro-colonies of microbial cells with similar extracellular materials and phenotypes has larger biofilms. Depending upon the organism(s), cell density, printing, and growth conditions, aggregate growth (especially aggregates embedded in another natural material) may represent aspects of synthetic biofilm growth, and further work is needed to fully explore microbial responses to the multitude of conditions and gradients within bioprinted materials, including fundamental aspects of biofilm behavior that could now be controlled via engineering approaches via 3D bioprinting.



Self-assembled biofilm

Synthetic biofilm

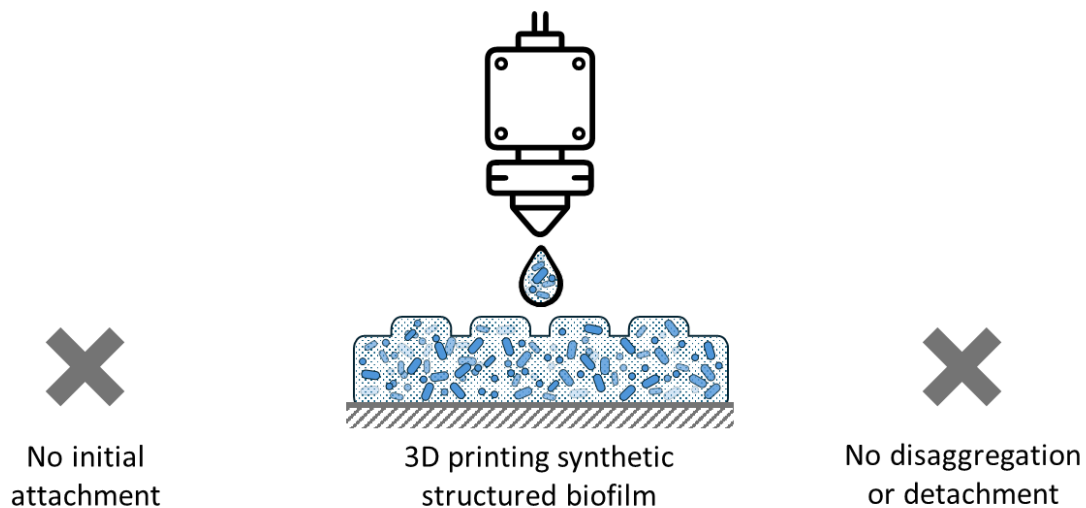


Figure 1.5. 3D bioprinting can be used to build biofilms not beholden to the process of biofilm attachment, structural maturation, and detachment. The long-term consequences of life cycle restriction in a synthetic matrix on microorganisms are unknown.

While a biofilm life cycle in a 3D printed structure will likely not proceed exactly to natural, self-assembled biofilms, bioprinted synthetic biofilms can provide advantages in the context of application. Through the process of 3D printing, microorganisms become trapped in a polymer matrix and deprived of the opportunity to disperse and re-attach to other surfaces. Trapping becomes a control measure that allows bioaccumulation that is an inherent property to natural biofilms and usually desirable for practical applications of ELMs to prevent accidental release of microorganisms and retain functional capabilities. However, Kalairaj et. al. demonstrated the utility of controlled release of organisms from a hydrogel ¹¹², and thus, further work is needed to understand the relationships between growth, function, and sustainable maintenance of 3D bioprinted ELMs. In addition, the long-term phenotypic and evolutionary effects of containing a population or community in a synthetic matrix where cells are both mechanically constrained and cannot engage in motility/dispersal are unknown. Currently to the authors knowledge, there are no research studies evaluating patterns of adaptation and/or diversification over evolutionary time in a 3D printed structure, and these potential impacts would obviously be dependent upon the time-scales relevant to the designed purpose of the ELM. Groundwork exists for pursuing these questions, for example, high-quality RNA purification has been achieved from eukaryotic cells grown in PEG scaffolds ¹¹³, and cell culture in 3D printed hydrogels has been demonstrated for multiple weeks, approaching one month ^{95, 114}. The capability to perform spatially resolved, single-cell transcriptomics and metabolomics will provide much insight into the local environments of cells and cell aggregates in synthetic biofilms over time.

Fundamentals of microbial behavior in synthetic 3D printed biofilms are relatively underexplored. Understanding how a cell will behave in a synthetic structure is an interesting topic in and of itself, but also serves applied research in ELMs, *in vitro* microbial habitat fabrication, and *in vitro* biofilm disease modeling. For example, it is well known that in *Pseudomonas aeruginosa* biofilms, physical properties can vary with depth¹¹⁵, and physiological heterogeneity can depend on availability of oxygen and other electron acceptors which are also depth-dependent^{116, 22}. It is not clear if an organism encapsulated in a synthetic 3D printed biofilm would respond to oxygen limitation with similar physiological variability, and on what other factors this variability might depend. In addition, it may be possible to use 3D bioprinting to precisely, and simultaneously, control biofilm thickness, pore size, and population density to achieve a desired physiological state. Spatially resolved measurements of behavioral metrics, such as transcriptomics and metabolomics, would provide a wealth of information about microbial behavior as a function of spatial position in 3D printed biofilms. The utility of spatially resolved measurements applies to microscopy as well; fluorescence microscopy is a common and effective method of characterizing 3D printed biofilms across all three research categories described here. Applied research in material development and understanding microbial behavior in living materials are both served by spatially resolved microbial population dynamics.

There are some limitations inherent to the process of 3D bioprinting that constrain synthetic biofilm design. By definition, a hydrogel must be hydrated to retain its intended shape, structure, and material properties. Only natural biofilms existing at a liquid interface can be accurately replicated *in vitro* with hydrogel printing, and functional engineered living materials are mostly constrained to operating in a hydrated environment. Some hydrogel compositions are also fragile

and cannot be moved without destroying the hydrogel's structure, so must be printed in place, for example, with direct-to-glass printing. Particularly for extrusion bioprinting, there is a trade-off between spatial resolution (dictated by nozzle size) and mechanical stress experienced by cells. A narrow nozzle is expected to increase the shear stress on cells in the bioink and potentially reduce viable population size through cell lysis ⁸¹.

Microbial 3D bioprinting is at an exciting stage, despite current technological limitations. Biofilm research has revealed the significance of spatial structure in microbial communities, and advances in 3D bioprinting allow researchers to experimentally control important aspects of biofilm structure and life cycle. Through transdisciplinary approaches, researchers have designed creative engineered living materials, pushed the boundaries of *in vitro* habitat modeling for medical and environmental research, and designed experimental systems to unravel the dependence of ecological interactions on small-scale structure. There is a deep appreciation in the 3D bioprinting literature of the structure-function relationship in biofilms, and a recognition of the value in accurately representing the physical characteristics in an environment that impacts biofilm *in vivo* structure. Future work in microbial 3D bioprinting will certainly extend beyond the topics of this outlook, and continued integration of engineering, materials science, and the biological sciences will advance our understanding of structure-function relationships at even the smallest scales of life.

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CHAPTER TWO

CHARACTERIZING SPATIOTEMPORAL MICROBIAL
COLONY DISTRIBUTIONS IN PRINTED PEG-DA
HYDROGEL FILMS

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2.1. Abstract

Biofilms are surface-attached microbial communities that play vital roles in natural ecosystems and contribute to persistent problems in medicine and industry. These communities exhibit heterogeneous chemical, physical, and physiological properties, which are governed by reciprocal structure–function relationships. Linking structure to function is crucial for understanding biofilm physiology but remains challenging due to the structural complexity of naturally formed biofilms. Bioprinting offers exquisite control over biofilm structure and holds potential for systematically exploring these relationships; however, the microscale colony distributions that emerge within hydrogel-based print resins remain unexplored. To address this, we use light-based bioprinting to create single-layer hydrogel films containing homogeneously dispersed *Pseudomonas fluorescens* bacteria and characterize the spatiotemporal distribution of colonies that develop within these films. We systematically vary the concentration of bacteria over nearly three orders of magnitude, track colony growth using microscopy, and quantify structural features with image analysis. We observe empirical relationships between initial cell concentration and key structural features: colony size, colony volume, total biovolume, and characteristic gradient length scale. This knowledge can be used to print microbial communities with well-defined features, is readily applicable to more complex three-dimensional shapes, and provides a tool for advancing our understanding of microbial communities.

2.2. Introduction

Many microorganisms have been naturally selected for growth in multicellular communities known as biofilms ¹. These communities are implicated in a host of medical ²⁻⁴,

industrial, and environmental challenges, including global nutrient cycling⁵ and waste remediation⁶. Biofilms typically form on surfaces/interfaces and consist of cells embedded in a polymeric, self-produced extracellular matrix. Within these films, fluid flow is suppressed, and diffusion is the primary means of transport⁷. Consequently, nutrient consumption and waste production lead to the development of chemical gradients, which, in turn, generate heterogeneities in physiological processes, such as metabolic activity, gene expression, and protein production⁸⁻¹¹. These heterogeneities contribute to the physiological complexity of biofilms and influence important functions such as stress resistance^{10,12}, community-level resource partitioning, and metabolism¹³.

Biofilm structure plays a key role in the development of chemical gradients and the resulting biological heterogeneities. For example, film thickness and cell density influence local nutrient concentrations and metabolic activity^{7,13-15}. Conversely, biological processes such as cell growth, matrix production, and phenotypic differentiation can affect biofilm structure¹⁶⁻¹⁸. Knowledge of these reciprocal structure–function relationships is essential to advances in biofilm control, bioremediation, and microbial ecology as well as engineered living materials^{1,2,13,18,19}. However, elucidating these relationships is challenging because self-assembled biofilms, whether formed naturally or *in vitro*, develop complex, heterogeneous structures^{17,20-23}.

3D bioprinting offers exquisite control over the structure of living materials²⁴⁻²⁷. Printed materials typically consist of cells embedded in a soft, noncytotoxic hydrogel, which provides structural integrity and partially recapitulates key physicochemical properties of the physiological environment. Hydrogels containing microorganisms have been printed for diverse applications, including living material synthesis²⁸⁻³², bioprocessing³³, electrosynthesis³⁴, biosensors³⁵, synthetic soil aggregate models³⁶, microbial quorum sensing and chemotaxis models³⁷,

antimicrobial resistance models ³⁸, and probiotic bone scaffolds ³⁹. However, while printing provides precise control over material structure, little is known regarding the colony structures that develop within these materials. Microbial cells embedded in hydrogel typically grow within several hundred microns of the gel surface, where nutrient consumption and diffusion limitations give rise to structural gradients governed by reaction–diffusion processes ⁴⁰. Yet, the spatiotemporal distributions of colonies that form within printed materials, particularly those fabricated by photopolymerization, have not been characterized. Such knowledge is needed to understand fundamental structure–function relationships and to engineer living materials with well-defined functional behaviors.

Here, we use light-based bioprinting to create single-layer hydrogel films containing homogeneously dispersed bacteria (*Pseudomonas fluorescens*) and characterize the spatiotemporal distribution of colonies within the films using fluorescence microscopy. We systematically vary the initial concentration of bacteria over nearly three orders of magnitude, track colony growth over six days, and quantify colony structural features using image analysis. We observe the following: (i) colony number at long times is directly proportional to the initial concentration of cells, (ii) average colony volume is inversely proportional to the initial concentration, (iii) all conditions approach the same biovolume independent of initial cell concentration, and (iv) the characteristic length scale of the primary structural gradient decreases logarithmically with increasing initial cell concentration. These empirical relationships between initial cell concentration and resulting colony distribution can be used to print planar microbial films with predictable, well-defined structural features. Importantly, these relationships are directly applicable to microbial colony distributions in three-dimensional hydrogels, which—regardless of

structural complexity—are still governed by near-surface reaction–diffusion processes. Consequently, our approach offers a valuable tool for investigating structure–function relationships in printed microbial biofilms.

2.3. Materials & Methods

2.3.1. Preparation of Recombinant Cells and Growth Media

The *P. fluorescens* N2E2 strain harboring the red fluorescent protein (RFP)-expressing pGinger suite plasmid was obtained from Dr A. Deutschbauer (Lawrence Berkeley National Laboratory). The pGinger plasmid in N2E2 was developed under kanamycin selection and constitutive expression via conjugation from *E. coli* WM3064TSB as previously described ⁴¹. Growth differences between the wild-type and pGinger strains were not observed in the tested growth medium. Tryptic soy broth (TSB) medium was prepared by dissolving 15 g of TSB powder (MP Biomedicals LLC) in 500 ml Milli-Q® water and autoclaving for 20 min at 121 °C. TSB medium was supplemented with 7.5 g agar to prepare Tryptic soy agar (TSA) plates at 1.5% w v⁻¹ agar concentration. Before pouring plates, kanamycin sulfate (Sigma-Aldrich) was added to a final concentration of 50 µg ml⁻¹ to prepare antibiotic selection plates for growth of recombinant N2E2. The kanamycin stock solution was prepared at 50 mg ml⁻¹ by dissolving in Milli-Q® water and filter-sterilizing through a 0.2 µm polycarbonate filter. Kanamycin stock was stored in Falcon tubes at 4 °C as per manufacturer’s recommendation.

2.3.2. Preparation of Growth Media

All experiments were conducted with a defined groundwater media recipe as previously described, excluding supplemental metals and antibiotics (**Supplemental Tables 2.1.–2.5.**) under

ambient atmosphere ⁴². Media was prepared by mixing the salt stock solution, mineral stock solution, sodium bicarbonate, and Milli-Q® water and adjusting pH to 7.2 with 2 N HCl in a 1 l round bottom flask. The vitamin stock solution was added to a final dilution of 1× along with 1 M monobasic sodium phosphate stock. The solution was filter-sterilized into sterile 50 ml conical tubes through a 0.2 µm polycarbonate filter. Sodium acetate and sodium nitrate were added to a final concentration of 20 mM and 0.1 mM, respectively. Sodium acetate, sodium nitrate, and monobasic sodium phosphate (Sigma-Aldrich) stock solutions were prepared at 1 M by dissolving into Milli-Q® water and filter sterilizing through a 0.2 µm polycarbonate filter. Stock solutions were stored at 4 °C.

2.3.3. Growth of Inoculum for Printing Experiments

Cells were stored in 2 ml cryogenic vials (Corning) at −80 °C in 1 ml of 20% v/v glycerol, prepared by mixing 500 µl cell culture grown in TSB at an OD₆₀₀ of 0.8 with 500 µl 40% v/v sterile glycerol. Streak plates were prepared from N2E2 glycerol stocks by streaking cells onto TSA plates under 50 µg ml^{−1} kanamycin selection using a sterile inoculating loop and incubating in the dark at 30 °C for 36 h. To prepare cells for printing experiments, a single N2E2 colony from a TSB agar plate was inoculated into 50 ml of TSB medium containing 50 µg ml^{−1} kanamycin in a 250 ml Pyrex® culture shake flask. Flasks were incubated aerobically at 30 °C, 130 RPM, until an OD₆₀₀ of 0.7 was reached (corresponding to approximately 3 × 10⁸ CFU ml^{−1}). Where applicable, ten- and hundred-fold population dilutions were prepared by a two-step serial dilution of cells into sterile TSB medium. Ten-fold and approximate hundred-fold population concentrations were prepared by transferring 50 ml of N2E2 culture at an OD₆₀₀ of 0.7 into a sterile Falcon tube and centrifuging at 10 000 g for 20 min at room temperature. Supernatant was poured off and the cell pellet was

resuspended in 5 ml fresh TSB medium for ten-fold concentration, and 0.5 ml fresh TSB medium for the highest concentration. Cells were stored in Falcon tubes at room temperature prior to printing for no more than one hour.

2.3.4. Preparation of Photoactive Resin

The non-cytotoxic photoactive resin was prepared before each experiment. The photoinitiator, lithium phenyl-2,4,6-trimethyl-benzoylphosphinate (LAP) (Sigma Aldrich), was dissolved in PBS at 60 °C to a final concentration of 0.1% (w/v). Poly(ethylene glycol) diacrylate (PEG-DA) oligomers (Sigma Aldrich PEG-DA Mn 700) were added at a 10% (v/v) final concentration. A yellow azo dye, tartrazine (Alfa Aesar) acted as a photoabsorber and was incorporated at concentrations between 0.01% and 0.05% (w/v). Photoabsorber concentration and light exposures were tuned based on desired cure depth as described previously ⁴³. The final solution was filter sterilized and stored away from light before printing. Just before printing, bacteria were concentrated to 10× the desired final cell concentration in culture medium. The printing resin was then inoculated with the bacterial solution at a 10% (v/v) final PEG-DA concentration before the solution was vortexed and protected from ambient light immediately pre-printing.

2.3.5. Printing Hydrogels Containing Bacteria

Print geometries were designed with computer-aided design software (Autodesk, Fusion 360), exported as standard triangle language files, and uploaded to the openFL slicing program (Formlabs). Custom laser profiles with control over laser power, speed, and number of passes were created to achieve the desired cure depth, as described previously ⁴³. Resin was prepared containing

cells at volume fractions, $\varphi_0 = 0.0003, 0.002, 0.035, 0.055$, and 0.119 . Liquid resin was added to a depth of approximately 4 mm to individual wells of a glass bottom well plate (Cellvis 6-well, P06-1.5 H-N), and the plate was mounted in the SLA printer (Form 1+, Formlabs) using a custom-built insert. We note very minor increases in cell density at the cover glass-gel interface (**Supplemental Figure 2.2.**). We find that manually agitating the resin with cells before loading the well plate and similar manual agitation of the well plate before printing minimizes this effect. To reduce bacteria residence time in resin before printing and minimize cell sedimentation, care was taken to print quickly (< 5 min) following the addition of bacteria to the resin. Following printing, the well plate was immediately removed from the printer and any non-polymerized resin was extracted from each well. The gels were then rinsed three times in sterile PBS, followed by a one-time rinse in ethanol (70% v/v) for ~ 10 s. Gels were again rinsed three times in sterile PBS before filling the wells with culture medium. The ethanol rinse effectively eliminates bacterial growth in the medium and on the gel surface over the duration of the experiment. If this rinse step is not performed, the culture medium becomes turbid from cells growing outside the hydrogel.

2.3.6. Hydrogel incubation and media replacement

Following the rinse sequence, 5 ml of groundwater culture medium was added to each well, fully submerging the gels. The plates were then incubated at 30°C under ambient atmosphere. Culture medium was replaced every 24 h for up to 6 d. For each exchange, old medium was extracted, the gel was rinsed in PBS, and 5 ml of fresh groundwater medium added. Following each exchange, the plates were immediately returned to 30°C incubation. While the inoculum was prepared under antibiotic selection as previously described, antibiotics were not added to the

culture medium during hydrogel incubation. *P. fluorescens* is a facultative anaerobe, so nitrate was restricted to limit anaerobic respiration.

Confocal imaging of printed hydrogels

Printed hydrogels were imaged with fluorescence time-lapse confocal microscopy (inverted Stellaris 8, Leica Microsystems). RFP expression was imaged using the Leica mCherry preset (ex/em: 587 nm 610 nm⁻¹). A 10× dry objective (HC PL APO CS2 10×/0.40 Dry, Leica Microsystems) enabled full thickness imaging of each hydrogel. Laser power was set to roughly half the dynamic range at the beginning of each experiment to allow for increased fluorescence intensity over the course of the experiment. Glass bottom well plates were used for optimal imaging. Two independent, six-day time-lapse experiments consisting of up to six different conditions each were performed. For each experiment, hydrogels corresponding to specific conditions were printed in individual wells in a multi-well dish. Image volumes of each gel were collected once per time point. Image volumes were captured near the very center of the gel to avoid edge effects caused by light attenuation and nutrient diffusion. Image volumes consisted of a *z*-stack with a wide *xy* field of view (775 μm × 775 μm) and a *z*-depth sufficient to capture the entire thickness of the gel (step size ≈ 2 μm; 60–200 images). Each imaged volume was then divided (in the *xy* plane) into nine equal subfields for analysis to generate a statistical assessment of variation within the imaged volume. For time points >20 h, the multiwell dish was removed from the confocal and incubated for 24 h between each time point; thus, each time series represents multiple different imaged locations for a given gel.

2.3.7. Biomass Quantification

We use image analysis software (Imaris v9.9, Oxford Instruments) to process and analyze image data. A Gaussian blur was used to preprocess images to reduce noise for thresholding. We then use the surface tool to automatically threshold the images and produce 3D reconstructions of the bacterial colonies (e.g. top row of **Figure 2.2**). Automated thresholding selects appropriate values for each condition and time point, with seed splitting applied to high density prints to enhance segmentation accuracy. Tabulated data detailing colony location and size at each timepoint is exported from Imapris. We use custom MATLAB scripts for data quantification and visualization. To assess variability and calculate averages, each field of view is divided into nine equal sections. For quantification of z -dependent parameters, each section is further divided along the z axis into bins based on the number of colonies within each section.

2.3.8. Statistics and Data Analysis

To quantify variability and spatial distribution within the biofilms, each field of view was divided into nine subfields (3×3) of equal size, each representing one-ninth of the xy area of the field of view. These subfields include all colonies whose central xyz coordinates fell within the subfield boundaries and are used to calculate averages and assess variability for whole-system properties such as volume fraction and colony count. The constraints of the experimental observation, including imaging throughput and field of view size, necessitate this subfield division. While this approach captures variability within the central region of the gel, it may limit the ability to fully assess whole-gel variability.

For volume fraction, the total biovolume within each subfield was summed and divided by the total volume of the subfield to calculate the subfield volume fraction. The average volume

fraction across all subfields in a gel was plotted, with a shaded region representing the standard deviation to indicate variability within the gel. Colony counts were determined by identifying and counting colonies based on their central coordinates. The average number of colonies across all subfields was calculated and plotted, with standard deviations depicted using markers and shaded regions to illustrate variability. A similar process of finding the average colony size within each subfield, then among the subfields provide the average colony volume and the standard deviation.

For biovolume gradient analysis, each subfield was further divided along the z -axis into bins. The number of bins was determined as the square root of the number of colonies within the subfield. Colony biovolumes within each z -bin were summed, and the distributions were plotted for individual subfields as dashed lines. For the lowest volume fraction condition, the number of colonies was insufficient to support this binning procedure due to the low number of colonies per bin and subfield. Instead, four subfields were plotted for this condition. Additionally, the data for this condition did not support fitting an exponential decay due to the lack of data points and the similarity of the distribution to a horizontal line. The average biovolume distribution across all subfields was represented by a solid black line, with shaded regions indicating the standard deviation.

2.3.9. Quantification of Biovolume Gradient

To quantify the biovolume gradients in mature synthetic biofilms, we analyze image data at the final timepoint to assess biovolume distributions. We fit an exponential decay function to model the reduction of RFP expressing biovolume along the z -axis as described by $V(z) = V_0 e^{-z/\lambda}$. Here, $V(z)$ represents the biovolume at depth z , where V_0 is the biovolume at the base, and λ is the decay length scale. To determine a characteristic λ for each condition, we fit the volume

distribution between the bottom of the biofilm and the biovolume peak. We average the coefficients of each fit to determine a mean λ and V_0 value for each fitted condition. We plot the mean and standard deviation of both λ and $\log(\varphi_0)$ before fitting a linear regression to the mean (*e.g.* **Figure 2.5.(d)**).

2.3.10. Assessing Impact of Resin and Print Process on Cell Viability

Hydrogels were prepared with identical printer settings and resin preparation as described above. A wild-type culture of N2E2 was grown in TSB without kanamycin to an OD₆₀₀ of 0.7, as the wild-type carries no plasmid expressing mCherry or kanamycin resistance. The wild-type strain was used because convolution of the mCherry and propidium iodide signals would make accurately counting the dead cell fraction challenging. Following the manufacturer's recommended procedure for the Live/Dead Double Staining Kit (Millipore Sigma), the staining solution was prepared by adding 5 μ l of the proprietary Cyto-dye and 5 μ l of propidium iodide to 4,990 μ l of the provided staining buffer. The prepared staining solution (500 μ l) was added to each incubation well, and the hydrogels were incubated with the staining solution in the dark at 37 °C for 30 min. After incubation, the live/dead stain mixture was removed from each well, and the hydrogels were rinsed twice with 0.9% w/v sterile saline solution (1 ml each). The live/dead stain was applied 2, 22, and 48 h after the printing procedures described previously.

Three fields of view were imaged for each hydrogel at each time point with a 20 $\times$ magnification dry objective. As this is an endpoint analysis, different hydrogels were stained and imaged for each time point. The non-active cell fraction was determined by the proportion of cells in each field of view that stain with both live (Cyto) and dead (propidium iodide) stains. A spot

filter was applied in Imaris for each fluorescent stain channel and automatic thresholding filters were applied and then inspected for accuracy. A killed control was included for the purpose of evaluating the efficacy of the live/dead stain. For the killed control, a hydrogel was immersed in 70% ethanol for 30 min before staining and then imaged 2 h post-printing with the methodology described above. Additional details in supplemental text 4.

2.4. Results & Discussion

To fabricate synthetic biofilms, we use a commercially available inverted light-based 3D printer (FormLabs Form 1+) to selectively photopolymerize a single layer of hydrogel directly onto the inner surface of a glass-bottom multi-well plate (**Figure 2.1.**). We use a well-characterized, biologically inert, non-cytotoxic liquid resin containing PEG-DA, which rapidly solidifies when exposed to violet light^{43, 44}. To demonstrate the bioprinting process, we select a facultatively anaerobic, denitrifying bacterial strain *P. fluorescens* N2E2, modified to constitutively express RFP⁴¹. Bacteria are added to the liquid resin at well-defined concentrations before printing.

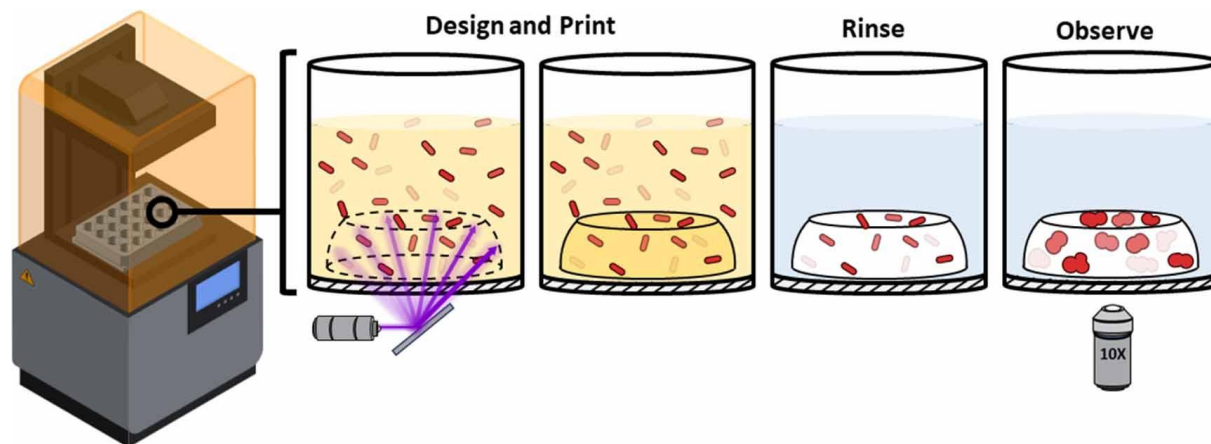


Figure 2.1. Overview of synthetic biofilm printing approach. *Design & Print*: Bacteria are suspended in a photopolymerizable liquid resin. Exposure to violet light (405 nm) induces solidification of liquid into hydrogel. Cells in the print region become trapped in the hydrogel matrix. *Rinse*: Excess resin and cells are removed and exchanged with growth medium. *Observe*: Structure and dynamics of microbial colonies are observed with confocal fluorescence microscopy.

The thickness, h , of the printed film is set to approximately 150 μm by tuning light exposure and photoabsorber concentration⁴³. This thickness is relevant for observing physiological heterogeneity in *Pseudomonas* biofilms^{45, 46}. We print the synthetic biofilms in single uniform layers to avoid the material heterogeneities associated with printing multiple layers. Standardized well plates provide a sterile, accessible printing platform that readily integrates with established microbiology tools, such as inverted confocal microscopy. Additionally, printing directly into a glass-bottom well-plate addresses common vat polymerization issues by avoiding peel forces and printhead/vat misalignment^{47, 48}. The printed gels remain adhered to the bottom of the wells, facilitating microscopy.

We use fluorescence confocal microscopy to monitor microbial growth. Immediately after printing, imaging reveals that individual bacteria are distributed homogeneously throughout the hydrogel (**Supplemental Figures 2.1. and 2.2.**). Because bacteria ($d \approx \mu\text{m}$) are much larger than the gel mesh size ($\xi \approx \text{nm}$; Supplemental Text 1), they are physically constrained and, as they grow, form discrete multicellular colonies consisting of densely packed cells (**Supplemental Figure 2.1.**). To explore the structural evolution of colony distributions within the hydrogel and the influence of initial conditions on population structure and dynamics, we print hydrogels containing different cell concentrations and perform confocal timelapse imaging over six days. We refer to the conditions by their starting cell volume fraction, φ_0 , as determined by image analysis. We print gels containing five different φ_0 , spanning nearly three orders of magnitude ($0.0003 \leq \varphi_0 \leq 0.119$).

While consistent light exposure is used for all prints, we observe an increase in cure depth at high cell densities. As a result, film thickness varies between 100 μm and 250 μm (**Supplemental Figure 2.1., Supplemental Text 2**). For each print, we collect 3D image volumes every two hours for the first 20 h, and once a day for the following five days.

We first consider the colony structures formed at long times, after six days of growth. Using image analysis, we identify the individual positions and volumes of all colonies (see Methods). We observe that gels inoculated with different φ_0 form dramatically different structures, as shown by the image volumes in **Figure 2.2.** (top image series). Systems printed with low φ_0 form a small number of large colonies distributed homogeneously throughout the gel, whereas those printed with high φ_0 form numerous small colonies concentrated near the gel surface. To visually compare these structural differences, for each image volume, we normalize the z -axis by h and plot the xz coordinates of all colonies in 2D (**Figure 2.2.**, bottom). In these plots, the volume of each colony, which is not affected by normalization, is represented by circle size (**Figure 2.2.**, key). We find that for all but the lowest φ_0 , colony size and colony number decrease with increasing depth. At the lowest φ_0 the biovolume appears uniformly distributed in z , indicating that growth is not significantly limited across the depth of the gel. By contrast, higher φ_0 conditions exhibit clear gradients in colony size and colony number density. These structural features are well-known hallmarks of reaction diffusion systems. Preliminary modeling of oxygen consumption and diffusion (not shown) suggests that oxygen is the limiting nutrient.

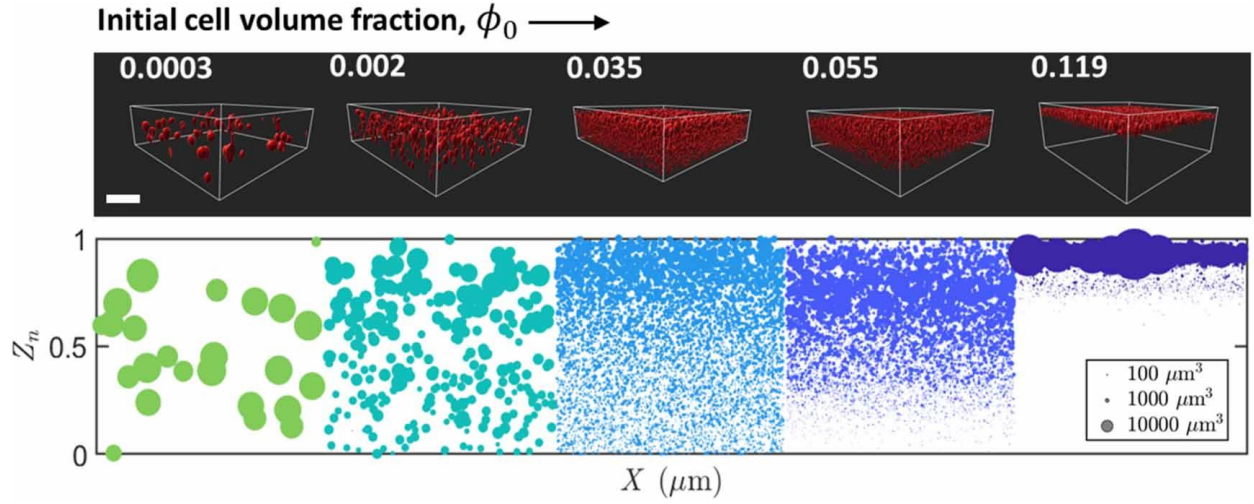


Figure 2.2. Different cell concentrations result in different community structures. (Top row) Three-dimensional renderings based on thresholded confocal microscopy image volumes of *Pseudomonas fluorescens*-containing films printed with varying ϕ_0 over nearly three orders of magnitude reveal dramatically different structures. Biofilms imaged after six days of growth. Scale bar is 200 μm . (Bottom row) Corresponding two-dimensional xz maximum projection plots of the imaged volumes. Here, Z_n is the z position normalized by the thickness of the gel. Each xz projection represents a 400 μm wide section. Circle size corresponds to colony volume, V , as depicted in the legend.

For each ϕ_0 we determine the total number of colonies, n in each image volume as a function of time. We find that for all ϕ_0 , n decreases with time before reaching a steady state, n_s after ~ 48 h (**Figure 2.3.(A)**, log-linear plot). Distinguishing individual colonies at long times for the highest ϕ_0 is challenging due to their proximity and colony merging (see supplemental text 3); so, we include these data but interpret with caution as it underestimates n . Normalizing by $n(t)$ by n_0 (n at $t = 0$), we find that for all but the highest ϕ_0 , $n(t)/n_0$ decreases to $n_s/n_0 \approx 0.44 \pm 0.09$ (**Figure 2.3.(B)**, linear-linear plot). At $t = 0$, n_0 represents individual cells; thus, n_s/n_0 provides a measure of the initial population that remains metabolically active. Dormant (e.g. metabolically inactive) population fractions have been observed in other isogenic⁴⁹ and environmental cultures⁵⁰, but are generally understood to be influenced by resource limitations and other environmental stresses¹².

Our result, observable across more than two and a half orders of magnitude in initial cell concentration ($0.0003 \leq \phi_0 \leq 0.055$) suggests a mechanism that is intrinsic to the cell population (*e.g.*, cell-to-cell physiological heterogeneity) and is not dependent on intercellular signaling or resource limitations. Importantly, the relationship $n_s \approx 0.44n_0$ provides a straightforward control parameter for setting the long-time colony number. We note that for very thin films ($h \approx 10$ s of μm) this relationship breaks down with $n_s/n_0 \rightarrow 0.1$ (preliminary data not shown), with dynamics indicative of colony coarsening, common in non-equilibrium physical and biological systems⁵¹. This warrants further investigation.

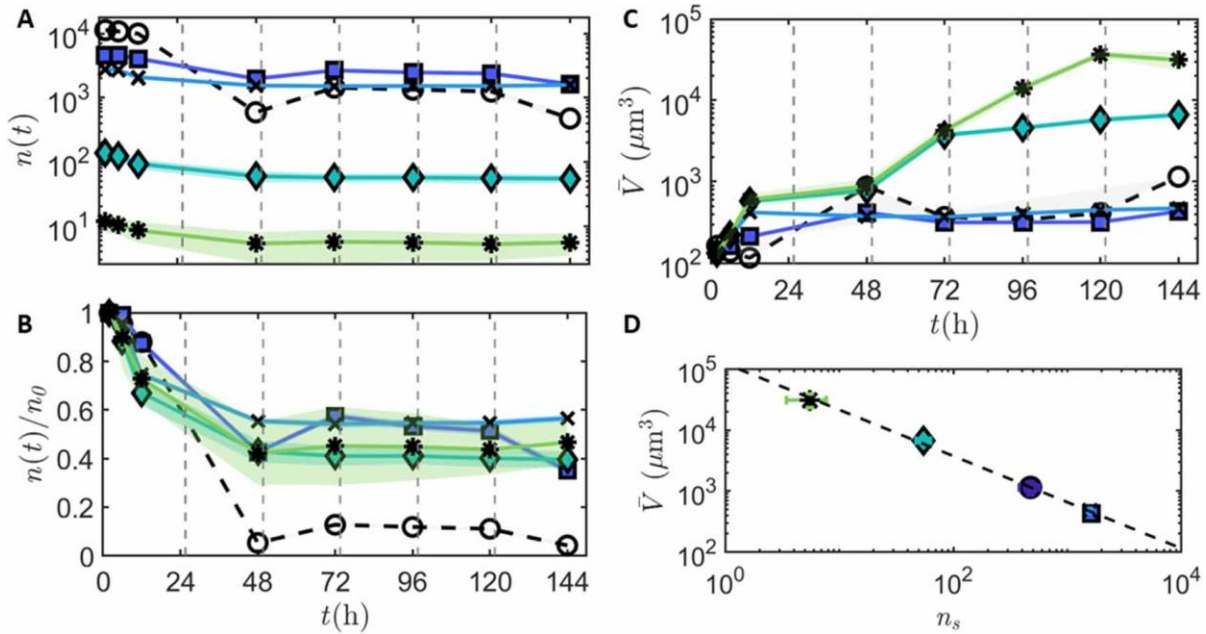


Figure 2.3. Time-dependent analysis of colony number and volume. (A) Total colony number within image volume, $n(t)$, plotted as a function of time for the five conditions shown in Figure 2 ($\phi_0 = 0.0003$ (*), 0.002 (♦), 0.035 (x), 0.055 (▪), and 0.119 (○)). The shaded area around each line represents standard deviation and vertical dashed lines indicate medium exchange. (B) Normalized colony number $n(t)/n_0$ from (A) plotted as a function of time. For all but the highest ϕ_0 , at long times $n(t)/n_0$ approaches $\approx 0.44 \pm 0.09$. (C) Mean colony biovolume, $\bar{V}$, plotted as a function of time. (D) Mean colony biovolume at long times, $\bar{V}_s$ plotted as a function of colony number at long times, n_s is fit well by a power law with exponent, $\alpha = -0.78$ ($R^2 = 0.99$).

We next determine the individual volume, V of all imaged colonies in each condition and plot the average colony volume, $\bar{V}$ as a function of time (**Figure 2.3.(C)**). For the higher φ_0 conditions (0.035 and 0.055; excluding 0.119) we find $\bar{V}$ that increases by less than an order of magnitude and plateaus after ~ 48 h; whereas at lower φ_0 the colonies continue to grow and $\bar{V}$ plateaus at longer times. This dependence of $\bar{V}$ on φ_0 is consistent with a reaction–diffusion system, in which sparsely distributed colonies have greater access to nutrients for metabolism and growth than more densely packed colonies. In a simplified scenario, where all available nutrients are converted into biomass and V does not influence growth, the average volume at long times, $\bar{V}$, should be inversely proportional to n_s ($\bar{V}_s \propto n_s^{-1}$). Plotting $\bar{V}_s$ at long times ($t = 144$ h) as a function of n_s , we find that $\bar{V}_s \propto n_s^{-0.78}$ (**Figure 2.3.(D)**). This indicates that large, sparse colonies expand less than the simplified case would predict, or that small, concentrated colonies expand more (or a combination of the two). This could be due to a variety of factors. For example, as colonies expand and their surface-to-volume ratio decreases, the exchange of resources and waste changes, which generates internal phenotypic heterogeneities and differences in resource utilization (*e.g.*, anabolic metabolism vs. matrix production). Additionally, the mechanical properties of the hydrogel could affect colony size, with larger colonies experiencing greater compressive stresses than smaller colonies^{52–54}. While compressive stress from the gel is unlikely to directly limit cell growth, as the turgor pressure of Gram-negative bacteria ($\sim 10^5$ Pa) is orders of magnitude greater than the yield stress of the hydrogel ($\sim 10^2$ Pa), compressive stress may influence cellular behavior through known mechanotransduction pathways, by altering the distance between cells, and thus altering intercellular communication, or by altering the spatial organization of cells within the colony^{16, 40, 52, 55}.

In microbial ecology, the carrying capacity, K is the maximum population size that a given system can sustain⁵⁶. In the context of printed microbial systems, it is important to understand how K varies with φ_0 as this parameter is related to total system function and output. Here, it is difficult to resolve individual microorganisms; so, we assume that the biovolume fraction φ , is proportional to population size and plot φ for the five different conditions shown in **Figure 2.2**. We find that $\varphi(t)$ for higher φ_0 remains relatively flat (0.035 and 0.055) and even decreases slightly (0.119). At this highest φ_0 , the decrease occurs as many small, high-density colonies grow near the film surface, but cells deep in the gel become inactive, presumably due to resource depletion (**Figure 2.4**). For the two lowest φ_0 conditions, we observe that φ increases dramatically over 120 h, with large individual colonies growing uniformly throughout the film depth. For these conditions, the slight decrease in φ between 24 h and 48 h is likely due to the initial drop shown in **Figure 2.2**., which momentarily surpasses the increase in colony volume. Remarkably, at long times, all conditions converge to a similar $\varphi \approx 0.04 \pm 0.02$, indicating that the system reaches an equilibrium, independent of initial conditions.

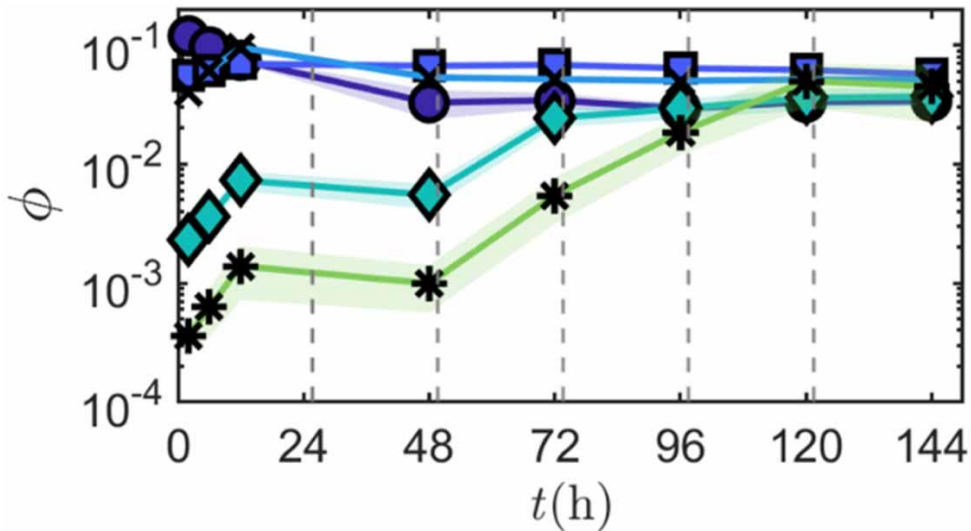


Figure 2.4. Time-dependent analysis of total biovolume fraction. Biovolume fraction, $\phi(t)$ plotted as a function of time for the five conditions shown in Figure 2.2. ($\phi_0 = 0.0003$ (*), 0.002 ($\blacklozenge$), 0.035 (x), 0.055 ($\blacksquare$), and 0.119 ($\circ$)). At long times, ϕ for all conditions approaches 0.040 ± 0.021 .

The bulk quantities n , $\bar{V}$, and ϕ do not capture the spatial gradients apparent in **Figure 2.2**.

To better understand these gradients, we plot the distribution of biovolume, V as a function of z for each condition and at specific times in **Figure 2.5**. At short times, immediately following printing, bacteria are uniformly distributed and $V(z)$ for all conditions is nearly independent of z (**figure 2.5.(A)**), except for boundary effects (see Methods). At long times, $V(z)$ for the three highest ϕ_0 becomes highly dependent on z , exhibiting a sharp decrease with increasing depth (**Figure 2.5.(B)**). The development of this gradient as function of time is shown for $\phi_0 = 0.035$ in **Figure 2.5.(C)**. The gradient is established as cells near the upper surface of the gel (high z) grow more than cells near the bottom (low z), which die or become nonfluorescent. At long times, $V(z)$ for the lowest ϕ_0 does not show a significant spatial gradient (**Figure 2.5.(B)**).

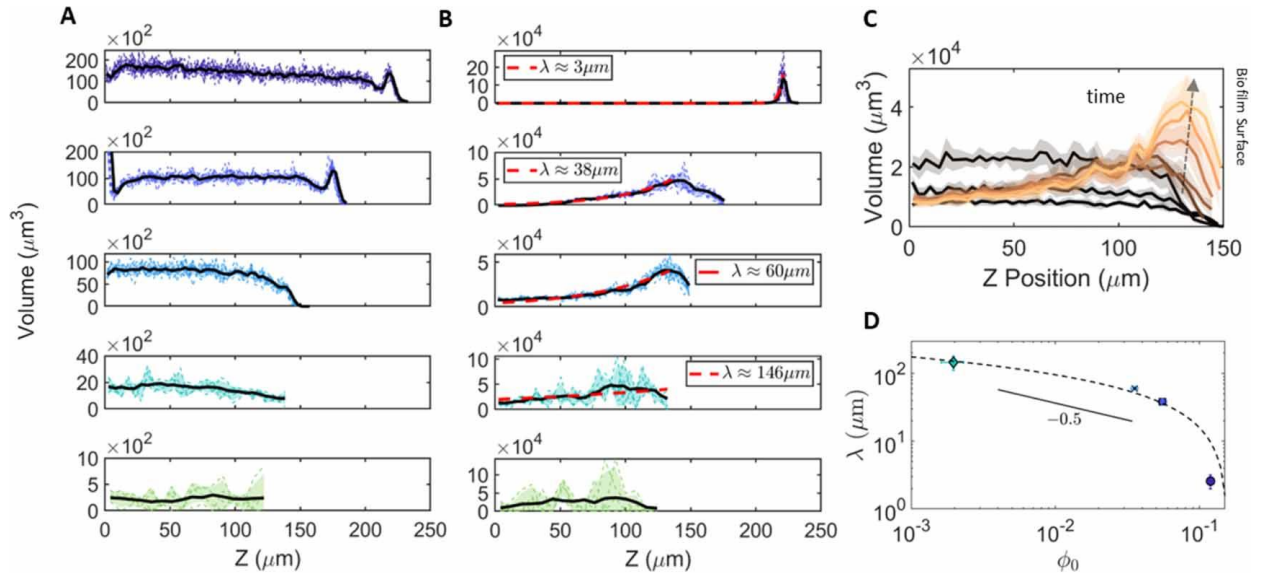


Figure 2.5. Gradient development in synthetic biofilms. Biovolume distributions plotted as a function of z for synthetic biofilms with different ϕ_0 (from top to bottom: 0.0003, 0.002, 0.035, 0.055, and 0.119) at (A) $t = 0$ h and (B) $t = 144$ h. Each plot contains biovolume subvolumes

(dashed lines), mean (black line), and standard deviation (shaded region). At early times (A), bacteria are homogeneously distributed throughout the film. At long times (B) for the highest ϕ_0 sharp gradients have developed, while for the lowest ϕ_0 the distribution appears homogeneous. Fits to an exponential decay (red dashed line) and resulting characteristic length scale, λ , for the four highest are shown. (C) Volume distributions for $\phi_0 = 0.035$ at different times ($t = 2, 6, 12, 48, 72, 96$, and 144 h) show development of gradient. Arrow indicates increasing time. (D) Log-log plot of λ as a function of ϕ_0 . Slope represents 1D reaction-diffusion scaling law estimate $\lambda \sim \phi_0^{-0.5}$. Data fit to the empirical function: $\lambda(\phi_0) = -80 \log(\phi_0) - 64$ (black dashed line; $R^2 = 0.93$). Error bars represent standard deviation.

To quantify these data, we fit the long-time ($t = 144$ h) distributions of the four highest ϕ_0 in **Figure 2.5.(B)** to an exponential decay (red dashed lines). We exclude the lowest ϕ_0 (**Figure 2.5.(B)**, lowest plot) due to insufficient data. The decay length, λ associated with each fit represents the characteristic depth over which the biovolume decreases by a factor of $1/e$ (≈ 0.368). For all fits, λ increases with decreasing ϕ_0 , from $3 \mu\text{m}$ ($\phi_0 = 0.119$) to $146 \mu\text{m}$ ($\phi_0 = 0.002$). At the highest ϕ_0 , biovolume decays to $\sim 5\%$ of its maximum value within $\approx 3\lambda$ ($9 \mu\text{m}$). By contrast, for $\phi_0 = 0.002$, a similar reduction spans $\approx 438 \mu\text{m}$, exceeding the thickness of the gel. To understand this dependence, we consider reaction-diffusion in a simplified model system: an infinitely thick film with a uniform, static colony distribution and first-order nutrient consumption kinetics. The resulting 1D solution predicts a steady-state nutrient gradient with decay length, $\lambda_n = \sqrt{D/kN}$, where D is the nutrient diffusion coefficient, k is the per-cell consumption rate, and N is the cell density. Assuming colony growth is directly proportional to nutrient availability ($\lambda \propto \lambda_n$) and $\phi_0 \propto N$ provides the power law scaling estimate: $\lambda \sim \phi_0^{-0.5}$. Plotting λ as a function of ϕ_0 on a log-log plot (**Figure 2.5.(D)**) we find this scaling (black line) matches surprisingly well with all but the highest ϕ_0 , which deviates significantly from the scaling prediction. This deviation is likely due to high colony density, which effectively lowers the diffusion coefficient, D and reduces λ .

Interestingly, we find that the full φ_0 range is well-fit to a logarithmic function (**Figure 2.5.(D)**, red line). This empirical fit provides a predictive heuristic for synthetic biofilm design.

To determine if swelling or degradation of the gel occurs during experiments, we separately monitor gels ($\varphi_0 = 0, 0.003, 0.055$, and 0.119) using microscopy. We find that h increases only slightly ($\sim 5\%$) over six days, with no dependence on φ_0 (**Supplemental Figure 2.3.**). This indicates that significant degradation does not occur, as degradation typically reduces crosslinking density and results in swelling. These results are consistent with published work on 30% (v/v) PEG-DA hydrogels, which show no signs of degradation over three weeks⁵⁷. Lack of degradation also indicates that the bulk mechanical properties of the gel, like the elastic modulus, G_e (≈ 5 kPa at $t = 0$), do not change significantly, as G_e scales proportionally with the crosslinking density⁵⁸. Nevertheless, local mechanical heterogeneities are expected. For example, regions of high colony number density (e.g. $\varphi_0 = 0.119$ at long times) likely have modified composite properties and colony expansion that likely causes nonlinear deformation and compression of the surrounding gel. These heterogeneities could be quantified using microscopy and micromechanical measurement techniques.

Here, we used a single gel formulation at fixed concentration. Hydrogels with different physical properties (e.g. mechanical properties, porosity, and chemical composition) would likely yield a diverse range of structures and physiological behaviors. For example, adjusting the PEG-DA concentration or molecular weight would change ξ , which can differentially affect the diffusion of small and large molecules. These changes would also alter the gel mechanical properties, influencing cell migration, colony morphology and the activation of mechanotransduction pathways¹⁶. Introducing modified PEG-DA monomers with stimuli-responsive moieties including

protease- and photo-degradation would enable studies of matrix degradation and dynamic remodeling^{59, 60}. Functionalizing PEG-DA monomers with bioactive moieties could enhance adhesion and signaling⁶¹. Additionally, substituting or supplementing PEG-DA with natural or synthetic analogs of biofilm extracellular polymeric substances could produce a range of unexplored biological effects. A wide variety of chemistries and bioinks are available, offering further opportunities to tailor hydrogel properties for specific applications^{33, 62–65}.

The results presented here provide a framework for creating synthetic biofilms with well-defined structures and could be used to better understand structure–function relationships in microbial biofilm communities. The steady state colony number, n_s , colony size, $\bar{V}_s$, and characteristic length scale λ are all dependent on the initial starting cell number, n_0 ; thus, these parameters can be set by controlling n_0 , and, because φ_s is independent of n_0 , the relationship between structure and function (*e.g.* resource utilization and allocation, etc.) could be systematically explored while keeping φ_s fixed⁶⁶. It is likely that the different structures shown in **Figure 2.2.** exhibit significantly different chemical and physiological heterogeneities, resulting in differing functions.

2.5. Conclusion

Here, we use light-based printing to create planar hydrogels containing bacteria and characterize their internal structures using confocal microscopy and image analysis. We find that the long-time, steady state colony number, n_s , colony size, $\bar{V}_s$, and characteristic length scale λ are all dependent on the initial cell volume fraction, φ_0 ($\sim n_0$). We describe these empirical relationships and provide some theoretical basis for their functional forms. We find that K is

independent of φ_0 ; thus, the relationship between structure and function (*e.g.* resource utilization and allocation) could be systematically explored while keeping K fixed ⁶⁶. This work is significant because it provides a new experimental approach and framework for systematically exploring spatiotemporal colony distributions in photopolymerizable, printed materials, which have not been previously described. Because the films created here are flat, uniform, and initially homogeneous, they can be modeled more easily than self-assembled biofilms with more complex structures. This work is immediately applicable to complex 3D-printed shapes, whose internal colony structures will also be governed by surface-mediated reaction–diffusion processes. In future studies, it would be interesting to explore multi-species colony interactions. The use of fluorescent reporters to observe processes like gene expression ⁶⁶, metabolism ⁶⁷, and substrate consumption ⁶⁸, in combination with advanced imaging and modeling techniques, would enable investigations of specific biofilm structure–function relationships ^{7, 14, 66}. It would also be interesting to explore ways to control n_s , $\bar{V}$, and λ independently of one another. For example, in the limit of thin and low φ_0 films where $\lambda \gg h$, preliminary evidence suggests that the functions $n_s \approx 0.44n_0$ and $\bar{V}_s \propto n_s^{-0.78}$ may change. Finally, the use of different gel chemistries and functionalities could result in different colony distributions and physiological behaviors. This integrated engineering approach offers new possibilities for innovation in biofilm research and technology and improved understanding of biofilm fundamentals.

2.6. Supplemental Text

2.6.1. Estimate of Gel Mesh Size/Porosity

Photopolymerized PEG-DA gels are known to be relatively homogenous and lack structural hierarchy. Thus, the porosity of the gels is set by the gel mesh size, ξ . Mesh size measurements of 10% (v/v) PEG-DA for different molecular weights, MW were reported to be: 3,400 Da (4.51 nm); 6,000 Da (6.76 nm); 10,000 Da (7.78 nm), and 20,000 Da (13.09 nm) (3). A linear fit to this data ($\xi = 3.16 + 0.000495 * MW$; $R = 0.99219$) provides an estimate of $\xi \approx 3.5$ nm for our 700 Da molecular weight PEG-DA gels.

2.6.2. Effects of Cell Density on Photopolymerization Cure Depth

For this study we hold all printing parameters constant across the different initial conditions to standardize both the cell light exposure and polymerization of the hydrogel. Strikingly, we observe that cure depth of the polymerized structures increases significantly with an increase in cell density (**Supplemental Figure 2.2.**). This finding contrasts with previous studies on particle-filled photoactive resin systems where an increase in particle volume fraction corresponds with a decrease in cure depth due to light attenuation associated with absorption and scattering ^{4,5}. This surprising result suggests that these bacterial cells interact with light differently compared to conventional fillers like ceramic particles. We hypothesize this phenomenon may be a product of multiple scattering and an increase in light path length caused by light interactions with the suspended cells ⁶⁻⁸. While adjustments to printing parameters, such as photoblocker concentration and light exposure, may be able to account for these changes in cure depth, it is crucial to consider how these adjustments affect the embedded organisms and the properties of the resulting hydrogel.

Further research is needed to explore the relationship between photopolymerization and resin microbial density.

2.6.3. Limitations of Individual Colony Resolution

Several limitations may impact the accuracy of colony quantification in synthetic biofilm systems when using fluorescence microscopy. Microscope resolution and light attenuation can significantly affect image quality, particularly in dense biofilm regions. High-density biofilms are known to cast shadows and scatter light in accordance with Beer's Law, complicating the ability to observe fluorescent biovolume within high density structures and limiting the ability to resolve individual colonies ^{1,2}. Despite these challenges, we find that measuring the total biovolume remains a reliable indicator of biomass accumulation within these systems. This metric is less susceptible to the difficulties of resolving individual colonies, providing a more consistent measure across different experimental conditions.

2.6.4. Viability of 3D Printed *P. fluorescens* Bacteria

To assess biocompatibility of this resin system with *P. fluorescens*, we 3D printed hydrogels with a wild-type population and performed a live/dead stain. The wild-type was chosen to avoid fluorescent signal crosstalk between the mCherry RFP and the propidium iodide fluorescence signals. In the killed control, 99.24% of the observed cell population encapsulated was stained with propidium iodide two hours after printing. For the experimental treatments, average dead cell fractions of 30.67% were observed after two hours, 37.67% after 22 hours, and 29.33% after 48 hours. Several factors could contribute to this reduction in cell viability. Delayed negative effects of the printer laser on the cell population are possible. For instance, exposure to visible light

irradiation in a seawater environment has been shown to produce a viable but non-culturable state in *E. coli* in the presence of a methylene blue ⁹. Physiological differences among individuals that are missed when describing the cell population with average values (such as intrinsic growth rate or generation time) may contribute to sensitivity towards laser exposure and/or the unpolymerized resin, though exposure to these conditions is only minutes long.

Under the chosen incubation conditions, the fluorescent stains penetrated approximately 500 μm into the hydrogels. This is likely due to diffusion constraints of the stains into the hydrogel matrix. Thus, the observable cell population was restricted to the outer $\sim 500\text{ }\mu\text{m}$ edge of each hydrogel. While several fields of view could be obtained to calculate an average dead cell fraction, the interior regions of the hydrogels could not be imaged using fluorescence microscopy with this methodology. Although we expect uniform cell distribution throughout the hydrogel, occasional settling at the substrate interface might lead to variations in the dead cell fraction between the internal regions and the periphery of the gel.

Hydrogels were prepared with identical printer settings and resin preparation as described in the Methods. A wild-type culture of N2E2 was grown in TSB to an OD_{600} of 0.7 also as described in the Methods, but omitting the kanamycin, as the wild-type carries no plasmid expressing mCherry or kanamycin resistance.

Live and dead stains were applied 2, 22, and 48 h after standard printing and rinse procedures, and each was then immediately imaged. Three fields of view were imaged for each hydrogel at each time point with a 20X magnification dry objective. The dead cell fraction was determined by the proportion of cells in each field of view that stain with both live (Cyto) and dead (propidium iodide) stains. A spot filter was applied in Imaris for each fluorescent stain channel and

automatic thresholding filters were applied then inspected for accuracy. A killed control was included for the purpose of evaluating the efficacy of the live/dead stain. For the killed control, a hydrogel was immersed in 70% ethanol for 30 minutes before staining, then imaged 2 hours post-printing with the same methodology.

2.6.5. Supplemental Text References

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2.7. Supplemental Tables

Supplemental table 2.1. Minerals stock solution recipe for defined groundwater media recipe (UGA)

Component	Amount	Units
NTA Disodium Salt	1.5	g/L
MgSO ₄ *7 H ₂ O	3	g/L
MnSO ₄ * H ₂ O	500	mg/L
NaCl	1000	mg/L
FeSO ₄ *7 H ₂ O	100	mg/L
CaCl ₂ *2 H ₂ O	100	mg/L
CoCl ₂ *6 H ₂ O	100	mg/L
ZnCl	130	mg/L
CuSO ₄ *5 H ₂ O	10	mg/L
AlK(SO ₄) ₂ *12 H ₂ O	10	mg/L
Boric Acid	10	mg/L
Na ₂ MoO ₄ *2 H ₂ O	25	mg/L
NiCl ₂ *6 H ₂ O	24	mg/L
NaWO ₄ *2 H ₂ O	25	mg/L
Na ₂ SeO ₄	20	mg/L

Supplemental table 2.2. Salts stock solution recipe for UGA

Component	Amount	Units
Ammonium Chloride	5	g/L
Potassium Chloride	2.5	g/L
MgSO ₄ *7 H ₂ O	12.32	g/L
CaCl ₂ *2 H ₂ O	367	mg/L

NaCl	250 mg/L
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Supplemental table 2.3. Vitamins stock solution recipe for UGA

Component	Amount (mg/L)
d-Biotin	2
Folic Acid	2
Pyridoxine HCl	10
Riboflavin	5
Thiamin	5
Nicotinic Acid	5
Pantothenic Acid	5
Vitamin B12	0.1
p-Aminobenzoic Acid	5
D,L-6,8-Thioctic Acid	5

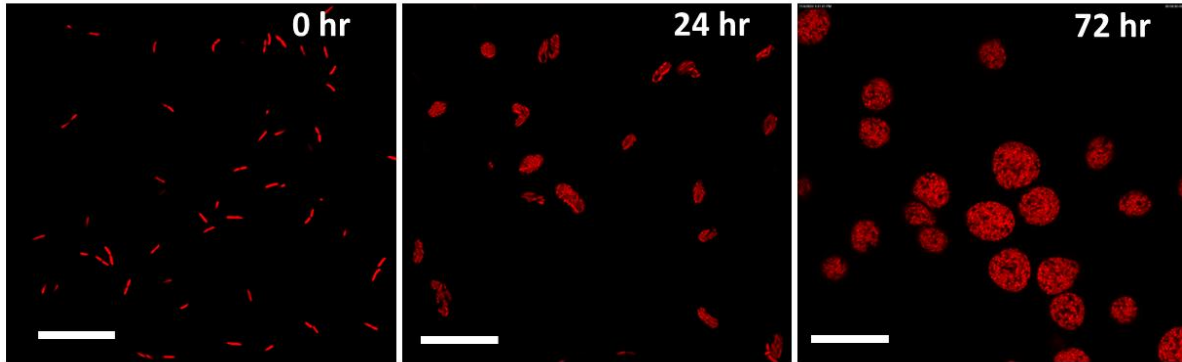
Supplemental table 2.4. Defined groundwater media recipe (UGA)

Component	Amount
dd H ₂ O	467 mL
Salts Stock Solution	20 mL
Vitamins Stock Solution	5 mL
Minerals Stock Solution	5 mL
NaH ₂ PO ₄ (1M stock)	2.5 mL
NaHCO ₃	1.25 g

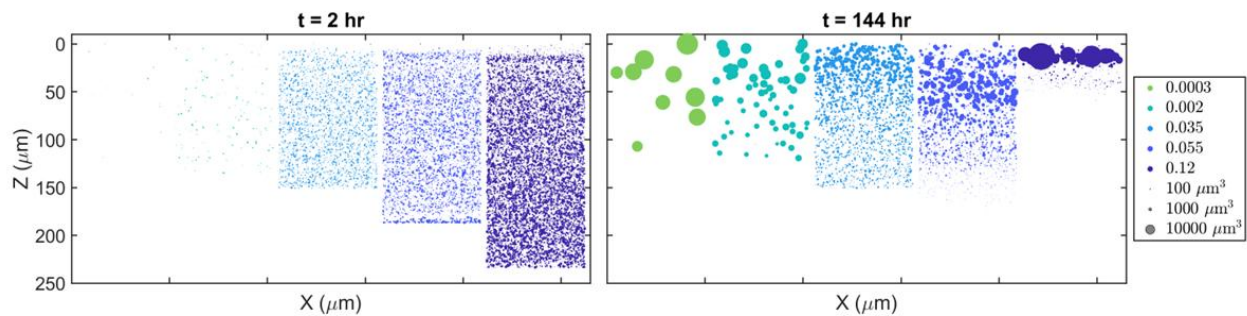
Supplemental table 2.5. UGA recipe post-filter sterilization additions for 45 mL aliquots.

Component	Volume	Stock concentration
C ₂ H ₃ NaO ₂	900 µL	1 M
NaNO ₃	4.5 µL	1 M

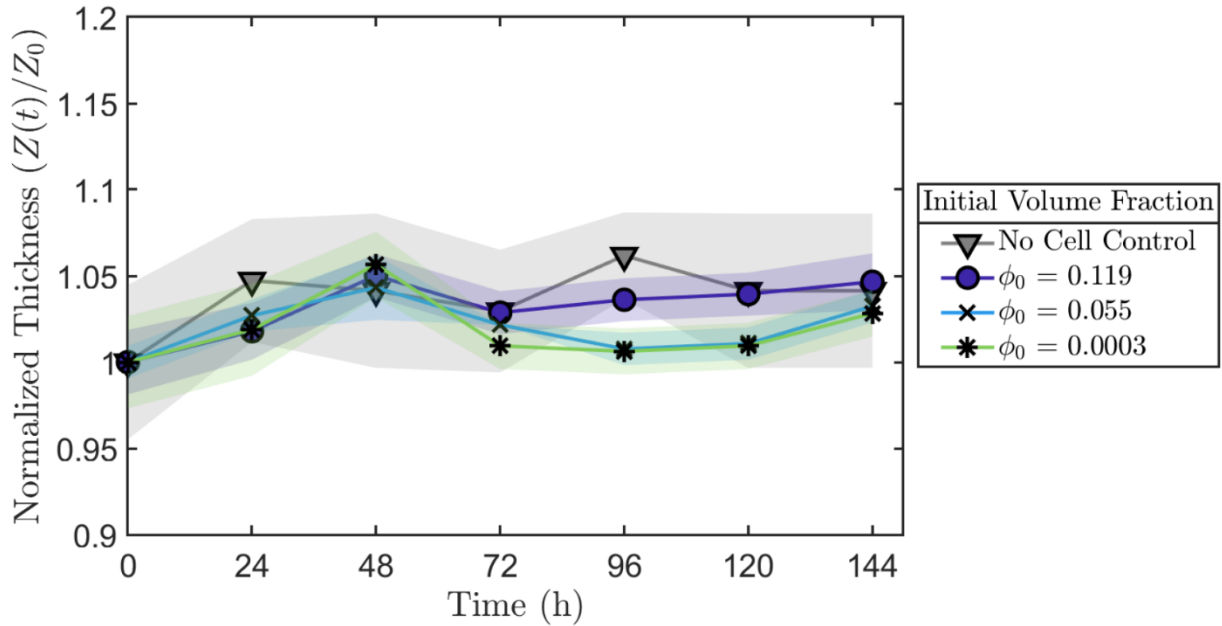
2.8. Supplemental Figures



Supplemental figure 2.1. Growth of discrete colonies following bacterial inoculation in printed hydrogels. Representative high resolution confocal microscopy images (Leica Stellaris 8, 63 $\times$ oil immersion objective) show growth of encapsulated bacterium *Pseudomonas fluorescens* over 72 hours. Colonies start as individual cells or small groups of cells and grow into densely packed discrete colonies. Initial volume fraction shown is approximately 0.035. Scale bars represent 20 μm .



Supplemental figure 2.2. Colony distributions at early and long times. Image analysis data showing colony location and size as xz max projection. **(A)** Max projection at 2 hours following printing. Cell distribution is nearly homogenous. We observe slight increases in cell density at the bottom of high density prints and attribute this to settling of cells and cell aggregates during the printing process. **(B)** Colony distribution following 6 days of culture. Heterogenous distributions of colonies emerge in mature biofilms. Large colonies depicted in higher density biofilms can include multiple discrete colonies counted together as one larger colony (Supplemental Text 3).



Supplemental figure 2.3. Time-dependent analysis of normalized gel thickness. Hydrogels prepared at three initial volume fractions: $\phi_0 = 0.119$ ($\circ$), 0.055 ($\times$), and 0.0003 ($*$), along with a no-cell control (∇) were imaged with confocal microscopy for six days. Normalized thickness, $z(t)/z_0$, is plotted here as a function of time reflecting the relative changes in volume over time. Gel thickness was determined by measuring the vertical distance between the well plate glass bottom and fluorescent microspheres that allowed to settle to the hydrogel surface. Shaded areas around each line represent standard deviation. All conditions, including the control, exhibit ~5% swelling over the experimental time course, indicating that swelling is minimal and cell-laden gels swell comparably to the control.

2.9. References

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CHAPTER THREE

COMPARISON OF SELF-ASSEMBLED AND SYNTHETIC
BIOFILMS UNDER DIFFERENT FLOW CONDITIONS

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3.1. Abstract

Microorganisms and the predominant biofilms that result from microbial activity and growth play vital roles in ecosystem functions, industrial processes, and environmental and human health. Biofilms are often structured in three dimensions, and the inherent small-scale heterogeneities of cell spatial distribution, nutrient availability, and extracellular matrix characteristics are consequential for community-level function and ecology. However, biofilms are typically defined and studied as self-assembled systems in which assembly rules are not completely understood. Due to the scale(s) involved, achieving consistent control of the physical forces governing biofilm formation are challenging as are understanding the relationships between physical constraints and the resultant changes in structure. Thus, to advance biofilm science, methods of manipulating biofilm structure are needed. We developed a laser lithography-based 3D bioprinting method that enables submillimeter control over biofilm structure and composition, and fabricated synthetic biofilms with encapsulated *Pseudomonas fluorescens* in a PEG-based hydrogel. We integrated bioprinting and reactor logistics with flow cell microscopy and spatially and temporally quantified growth to determine synthetic biofilm responses to changing nutrient and transport conditions. In addition, self-assembled biofilm growth was compared to synthetic biofilm growth. We systematically varied fluid velocity over three orders of magnitude and observed that increasing velocity increased apparent carrying capacity of the hydrogel, measured by total biovolume image analysis, when initial cell density was held constant. Behavioral responses of self-assembled biofilms to changing fluid velocity and nutrient conditions generally did not predict synthetic biofilm responses. Growth was not homogeneously distributed in the synthetic biofilms at the two highest fluid velocities and appeared to be impacted by local flow

and surface-boundary conditions. We found that the behavior of self-assembled biofilms under changing mass transport and nutrient conditions was not predictive of synthetic biofilm behavior, and continued research into the behavior and physiology of cells encapsulated in 3D printed material matrices will contribute valuable insight into synthetic biofilm design and the growing field of engineered living materials.

3.2. Introduction

Biofilms are microbial communities where cells thrive attached to each other and/or to surfaces, and can grow embedded in self-produced extracellular matrices ¹ that confers three-dimensional structure in response to the selective environment. While biofilms are not strictly multicellular², biofilms differ from populations of individual cells in physiology, evolution, and emergent properties derived from attachment and structure ³. In fact, biofilm itself can be viewed as an emergent property as opposed to a general phenotype of microorganisms in which biofilm is a higher-order system that cannot be represented or explained by the lower order systems (*i.e.*, cells)⁴. By synthesizing extracellular polymeric substances in tandem with growth, biofilms can exist in flow regimes but also incur mass transport limitations which contribute to the formation of internal nutrient gradients and ecological heterogeneity ³. Compared to planktonic, populations, microbial growth and survival in biofilms has been shown to select for elevated genetic and physiological diversity ^{5,6}, and sometimes for resistance to selective sweeps of a few dominant genotypes ⁷ although in some microorganisms numerous sub-types can persist ⁸.

The natural world, from subseafloor sediment to mammalian gut microbiomes and everything in between, hosts abundant and diverse biofilms ⁹. Biofilms impact every ecosystem

(environmental and human) on the planet both positively and negatively. Over the billions of years of Earth's history, microorganisms have radically transformed Earth's atmosphere and geology and have come to directly and/or indirectly impact resource processing across all environments¹⁰. However, microorganisms are also massively destructive to human industry and health, especially in a biofilm growth context where cells are recalcitrant to many methods of removal and resist antibiotic treatment to a greater degree than planktonic cells¹¹. The dichotomy of biofilm relevance is widespread in terms of beneficial or detrimental effects, for example: protection at the mammalian gut lining versus deleterious infections at different body locations, or mineral interactions for metal recovery versus pipeline deterioration/degradation. These examples exemplify the importance of biofilm in terms of context but also relevance both in positive and negative connotations.

Despite the exact conditions, contexts, or connotations of biofilms, improved understanding of biofilm fundamentals through engineering principles (design, build, test, learn) will advance progress for both biofilm challenges and opportunities. A critical component of understanding biofilms in the context of their early formation, growth and accumulation, and potential beneficial or detrimental behaviors is their structure-function relationships at small scales. Biofilm structure and function can be dictated by local environmental conditions such as exposure to shear forces³⁰ and reaction and diffusion of nutrients and electron donors/acceptors³¹; conditions which are often spatially heterogeneous at sub-millimeter scales in and around biofilms. Controlling biofilm structure, and this motivation has driven the integration of small-scale additive manufacturing with biofilm science

Microbial 3D bioprinting is a nascent field of research in which additive manufacturing techniques are used to control the structure of synthetic biofilms at scales ranging from meters¹² to micrometers^{13,14}. Cells are embedded in a variety of synthetic or natural extracellular matrices and fashioned into specific hydrogel structures by controlling matrix deposition with a 3D printer¹⁵. A dominant focus of microbial bioprinting literature is fabrication of engineered living materials which are rationally designed to perform specific functions such as rare element sequestration¹⁶, photosynthesis in flexible materials¹⁷, and fabricating behaviorally tunable materials¹⁸. The microbial 3D bioprinting literature is diverse, owing partly to the accessibility and low cost of 3D printing for both home-built and modified commercial printers, and partly to the metabolic diversity and flexibility of microorganisms. Microbial 3D bioprinting has been used to develop *in vitro* materials for antimicrobial testing¹⁹, to build synthetic environments that replicate physical characteristics of the environment such as soil particle size distribution²⁰, and for fundamental research in how initial structure affects the outcomes of strain competition²¹. The ability to control biofilm structure, particularly at small scales, serves both applied and fundamental research.

We have developed a 3D hydrogel printing platform based on stereolithography and previously investigated temporal and spatial patterns of bacterial population growth as a function of initial population density under batch conditions²². In this study, PEG-DA (Poly(ethylene glycol) diacrylate) hydrogels were printed with encapsulated *Pseudomonas fluorescens* in a BioSurface Technologies Transmission Flow Cell to explore how changing mass transport conditions affect population growth and distribution in the printed matrix. External transport and nutrient conditions can affect internal self-assembled biofilm characteristics (*i.e.*, spatial heterogeneity of nutrients²⁶), but it is unknown how cells immobilized in a synthetic biofilm with

a controlled structure and matrix respond to similarly changing conditions. The experiments enabled by integrating flow cell microscopy with 3D bioprinting begin to define unknowns of synthetic biofilm behavior.

Fluid velocity of incoming minimal growth media was systematically altered over three orders of magnitude in addition to a no-flow condition. We observed microbial growth dynamics in synthetic 3D printed biofilms over space and time with confocal microscopy and compared printed biofilm growth patterns to self-assembled *P. fluorescens* biofilms under identical flow conditions. The growth and behavior of self-assembled biofilms was not predictive of growth and behavior of synthetic 3D printed biofilms under batch conditions, under laminar flow conditions across three orders of magnitude, or when the predominant carbon source was suddenly removed from the media. It is not known whether growth and behavior differences between synthetic and self-assembled biofilms are generalizable across microbial species, but results from this study indicate that cells encapsulated and growing in a synthetic matrix may interact with changing external transport and nutrient conditions differently than self-assembled biofilms of the same population.

3.3. Materials & Methods

3.3.1. Preparation of Recombinant Cells and Growth Media

Pseudomonas fluorescens N2E2 was transformed with a pGinger expression plasmid via conjugation from *E. coli* ²³. The plasmid confers constitutive expression of mCherry and kanamycin resistance to maintain selection pressure on plasmid retention. 1X tryptic soy broth (TSB) was prepared by dissolving 15 g TSB powder (MP Biomedicals LLC) in 500 ml Milli-Q®

water. Following sterilization, kanamycin was added to a final concentration of $50 \mu\text{g ml}^{-1}$ (Sigma-Aldrich). Agar plates were prepared by supplementing TSB with agar ($1.5\% \text{ w v}^{-1}$) and filter sterilized kanamycin sulfate to a final concentration of 50 mg ml^{-1} .

All flow cell experiments were conducted with a defined minimal media recipe²² under ambient atmosphere and room temperature ($\sim 21^\circ\text{C}$). Media was prepared by mixing the salt stock solution, mineral stock solution, sodium bicarbonate, and Milli-Q® water in a 1L beaker. The vitamin stock solution was added to media a final dilution of 1X. Sodium acetate, monobasic sodium phosphate, and sodium nitrate were added to a final concentration of 20mM, 5mM and 0.1mM, respectively, and stock solutions were prepared at 1M by dissolving into Milli-Q® water and filter sterilizing. Stock solutions were stored at 4°C .

3.3.2. Inoculum Storage and Growth

P. fluorescens cells were stored in 2ml cryogenic vials (Corning) at -80°C in 1 ml 20% glycerol. Colonies on agar plates were incubated in the dark at room temperature for 36-48 hours, then single colonies were inoculated into 10 ml sterile TSB. Overnight TSB cultures were incubated at 30°C , 130 RPM, for approximately 24 hours, and then diluted 1:50 into 20mL minimal media in sterile flasks. Cultures were grown in defined minimal media until an OD_{600} of 0.45-0.5 was reached (corresponding to approximately $1.6\text{-}1.8 \times 10^8 \text{ CFU ml}^{-1}$). The cultures were transferred to Falcon tubes and centrifuged at 10,000G for 10 minutes at room temperature. The supernatant was poured off, and the cell pellet was resuspended in 2mL media to concentrate cells prior to mixing with the photoactive resin. Resuspended cells were stored in Falcon tubes at room temperature for no more than twenty minutes before 3D printing.

3.3.3. Preparation of Photoactive Resin/Cell Mixture

The photoinitiator, lithium phenyl-2,4,6-trimethyl-benzoylphosphinate (LAP) (Sigma Aldrich), was dissolved in room-temperature PBS to a final concentration of 0.1% (w/v). Poly(ethylene glycol) diacrylate (PEG-DA) oligomers (Sigma Aldrich PEG-DA Mn 700) were mixed with the LAP solution to an 11% v/v concentration in the resin mixture. The photoabsorber tartrazine (Alfa Aesar) was added into the resin at a final concentration of 0.05% (w/v) to achieve consistent print thickness. Photoabsorber concentration and light exposures were tuned based on desired cure depth ²⁴. The resin solution was filter sterilized and stored in the dark at room temperature before printing. Each resin solution was used for two weeks, then any remaining resin was discarded. To make the bioink, 900 μ L resin was mixed with 100 μ L concentrated *P. fluorescens* culture to achieve a 10% (v/v) final PEG-DA concentration. Cells were suspended in the unpolymerized resin for no more than three minutes. The solution was gently vortexed and 1mL was spotted onto a sterile glass coverslip in the 3D printing chamber.

Prior to hydrogel printing, 900 μ L resin was mixed with 100 μ L concentrated *P. fluorescens* culture to achieve a 10% (v/v) final PEG-DA concentration. Cells were suspended in the unpolymerized resin for no more than five minutes. The solution was gently vortexed and 1mL was spotted onto a sterile glass coverslip in the 3D printing chamber.

3.3.4. Hydrogel Printing with Encapsulated Bacteria

Print geometries were designed with computer-aided design software (Autodesk, Fusion 360), exported as standard triangle language files, and uploaded to the OpenFL slicing program (Formlabs). The resin and cell mixture spotted onto the coverslip was mounted securely in the SLA (Form 1+, Formlabs) printer using a custom-printed PLA insert.

3.3.5. Flow Cell Assembly and Incubations

A BioSurface Technologies Transmission FC-81 AL flow cell was assembled with loosened screws and aluminum foil covering the inlet and outlet, then sterilized. The flow cell was disassembled after sterilization in a biosafety cabinet, and the coverslip was removed. After printing, excess resin was aspirated with a pipette and the coverslip was transferred to a PBS rinse solution in a Falcon tube for ten minutes. The coverslip was removed and immersed in 70% ethanol for fifteen seconds, then transferred to a fresh PBS solution for ten minutes. Finally, the flow cell was reassembled, and tubing (Masterflex Precision Pump Tubing, C-flex ULTRA, L/S, 16) was attached to the influent and effluent. The influent tubing was attached to either a 50mL BD syringe (0.1mm and 1 mm/min treatments) and syringe pump (WPI Aladdin AL-1000), a peristaltic pump (Thermo Scientific Dry-Bags™ 1300-3600) and 2L bottle with minimal media (10 mm/min treatment), or tubing was plugged (0 mm/min treatment). The peristaltic pump was necessary for the 10 mm/min treatment because a larger reservoir volume was required that could not be accommodated by the syringe pump.

The peristaltic pump was calibrated to dispense 300 μ L per minute and the syringe pump was calibrated to dispense either 3 or 30 μ L per minute. While imaging, flow cells were detached from the pumps and plugged for approximately one hour, after which flow cells were re-attached. During imaging, the syringe ends and peristaltic pump tubing were covered with flame-sterilized aluminum foil.

For self-assembled biofilm experiments, a sterilized flow cell was inoculated with *P. fluorescens* and given a batch phase of thirty minutes prior to beginning flow treatments. Flow treatments were applied for another thirty minutes, then the initial timepoint was imaged.

The nutrient environment surrounding synthetic and self-assembled biofilms was altered to compare responses to sudden carbon depletion and evaluate N2E2 growth in the hydrogel matrix when the aqueous media contained other microorganisms. Carbon depletion experiments were performed at 1mm/min flow velocity. Minimal media without sodium acetate was introduced to the flow cell chamber at 10mm/min for five minutes to flush the chamber of the previous media, then velocity was reduced to 1mm/min for the duration of the incubation. For the non-sterile groundwater incubation, nearby well water was taken from a tap and then chilled at 4°C within an hour of retrieval. An aliquot was poured into a 50mL falcon tube and spiked with sodium acetate to 20mM, then immediately transferred to a 50mL syringe.

3.3.6. Growth Curves and Plate Reader Incubations

To evaluate the effect of the printing process on cell counts, and to confirm that *P. fluorescens* does not grow in the minimal media without sodium acetate, growth curves were conducted in 96 well plates and test tubes. Growth curves were all performed in triplicate in sterile minimal groundwater media under antibiotic selection. 5mL of media was inoculated with a 1:50 dilution of an overnight culture in test tubes, and in 96 well plates (Costar® 96 well, black with clear bottom), 200uL of media was also inoculated with a 1:50 dilution. Both overnight cultures were grown in minimal media. Optical density was measured at a wavelength of 600nm, and fluorescence was measured at 560/610 on a Biotek Synergy Neo2 plate reader. The 96-well plate was statically incubated in the dark at room temperature, and prior to measurements, the plate was orbitally shaken in the plate reader for five minutes.

3.3.7. Confocal Laser Scanning Microscopy

All flow cell experiments were imaged with an upright Leica Stellaris 8 Confocal Laser Scanning Microscope (CSLM) equipped with a white light laser. mCherry was imaged with 587/610 ex/em and a 10X objective (0.3 numerical aperture) or 40X objective (0.8 numerical aperture). Flow cells were mounted on the stage with tubing plugged and imaged every 24 hours for three to six days, depending on the experiment. Resolution for all images was set to 512x512 pixels with an acquisition speed of 600 and a Z-step size of 1.8 μm .

For 2D images, a 10X dry objective lens (HC PL APO 10x/0.40 CS2) was used to image across the length of the hydrogel ranging from the influent to effluent edge. 3D image stacks of the entire hydrogel thickness utilized a 40X water immersion objective lens (HC APO L 40x/0.80 W U-V-I), corresponding to an xy field of view of 244 X 244 μm . Six image stacks were taken of the influent, middle, and effluent regions of the hydrogels, providing a total of 18 image stacks per timepoint, per flow treatment. Because flow cells were removed from the instrument after imaging was complete for each timepoint, different locations within the same region were imaged at each timepoint for 40X magnification images. For self-assembled biofilm experiments, the influent was designated as the 1/6th of coverslip area closest to the influent-side tubing, the middle designated as the middle 1/6th area of the flow cell, and the effluent as the 1/6th area closest to the effluent.

Intensity analysis of 2D images

Files containing influent, middle, and effluent images of the printed hydrogels were analyzed in BiofilmQ for thresholding and segmentation. A manual threshold was applied to every image and adjusted to remove background noise. A top-hat filter of 31 or 35 was used for self-assembled biofilms and printed hydrogels respectively, according to software recommendation.

Each image was segmented using the cube segmentation process, dividing fluorescent signal into pseudo-cells, with each cubic pseudo-cell treated as an individual point of analysis²⁵. A cube side length of 2 was chosen for each image, corresponding to a length of 0.13 μm .

Integrated fluorescence was calculated on a by-cube basis for each 2D biofilm image. Data were imported into the BiofilmQ visualization panel with the figure type set to 2D XY-scatterplot. CentroidCoordinate_X was plotted on the X-axis, CentroidCoordinate_Y was plotted on the Y-axis, both with a range of 1:512 (i.e., the pixel resolution in the xy plane). Intensity_Integrated was selected for the color-code parameter.

Plots of fluorescence intensity resolved by X position were made using the 1.5D histogram figure option. CentroidCoordinate_X was selected for the X-axis parameter with a range of 1:512, and Intensity_Integrated for the Y-axis parameter with a variable range. Bin size was selected as the square root approximation of the number of intensity observations.

3.3.8. Biomass Quantification of 3D Images

Eighteen 3D image stacks were acquired for every time point, flow velocity treatment, and growth treatment. Analysis of 3D image stacks was performed in Oxford Instruments Imaris (version 11.0). Image files were directly imported into Imaris, and a surface object was created to threshold fluorescent signal from background. Surface detail was set to 0.5 μm using the automatic absolute intensity thresholding setting. For the self-assembled biofilm treatments, a center of homogeneous mass (Z-direction) filter was applied to exclude planktonic cells from the biovolume analysis. A Wolfram Mathematica script was used to calculate average and standard deviation colony biovolume, colony count, and biovolume sum versus time.

3.3.9. Acetate and Oxygen Measurements

For all flow cell experiments, 1.5-1.8mL of effluent was collected over ice, filter sterilized through a 0.2 μm polycarbonate filter, and frozen at 20 °C for acetate measurements via HPLC. Three technical replicates per treatment were measured. A standard curve for sodium acetate was prepared by serially diluting a 1M stock solution to 20mM in 10mL volumetric flasks. The 20mM solution was serially diluted further to 10, 5, 1, 0.5, 0.1, 0.05, and 0.01mM. The lowest concentration, 0.01mM, was beyond the limit of detection, so the standard curve ranged from 20 to 0.05mM.

Dissolved oxygen measurements were performed with a FireSting optical O₂ sensor. A sensor spot was glued onto the flow cell glass coverslip with a silica gel-based glue. The sensor spot was placed on the coverslip such that it was visible in the flow chamber and situated between the hydrogel and effluent-side tubing. The 0% O₂ calibration standard was prepared by dissolving two OXCAL tablets in 100mL MilliQ water, then immediately transferring this volume to fill 70mL serum bottle with a glued-on O₂ sensor spot. The serum bottle was crimp sealed. The ambient O₂ calibration standard was prepared by gluing a sensor spot onto an open serum bottle exposed to ambient air. The optical fiber was held steadily to sensor spots for calibrations and measurements for at least one minute to allow readings to stabilize.

3.4. Results

Using a custom FormLabs 3D printer, PEG-DA hydrogels with encapsulated *Pseudomonas fluorescens* were printed directly in a BioSurface Technologies Transmission Flow Cell (FC-81 AL). The velocity of incoming minimal growth medium was systematically altered over three

orders of magnitude, including a batch culture condition (**Figure 3.1.a**). Population growth was imaged with CSLM at the influent facing edges, the middle regions of the hydrogel, and the effluent facing edges. Individual *P. fluorescens* cells or small clusters were initially homogeneously distributed, with differences in the extent and location of population growth in the hydrogels emerging over time (**Figure 3.1.**). In all flow treatments, individual cells developed into distinct microcolonies by 72 hours. Qualitative 2D images of the hydrogels show that microcolonies at the influent-facing edge grew larger when exposed to the two fastest flow treatments, 1 and 10 mm/min (**Figure 3.1.c**).

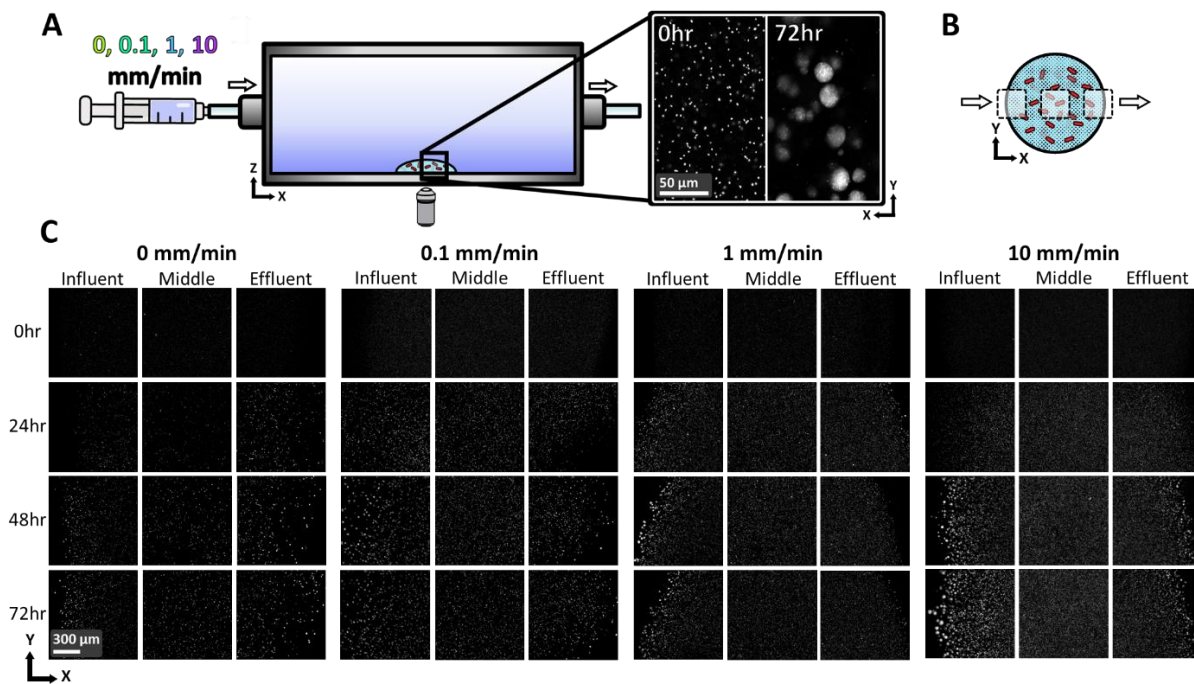


Figure 3.1. A) Diagram of Biosurface Technologies FC-81 AL flow cell. Hydrogels are printed in the center of the flow chamber on top of the glass coverslip bounding the chamber. Cells encapsulated in the hydrogel are initially distributed as single cells or small clusters of a few individuals, and over time, a subset of the inoculum grows into larger microcolonies. The flow chamber is to-scale in relation to hydrogel size. Bacterial population dynamics are observed through the entire hydrogel depth with confocal fluorescence microscopy. B) Top-down view of the printed hydrogel with encapsulated *P. fluorescens*. The square boxes the microscopy field of

view for influent, middle, and effluent positions displayed in part C. C) Fluorescence microscopy images of encapsulated *P. fluorescens* at the influent edge, middle, and effluent edge positions for all four timepoints and flow conditions. Images are taken in approximately, though not exactly, at the same position between timepoints with a 10X magnification dry objective.

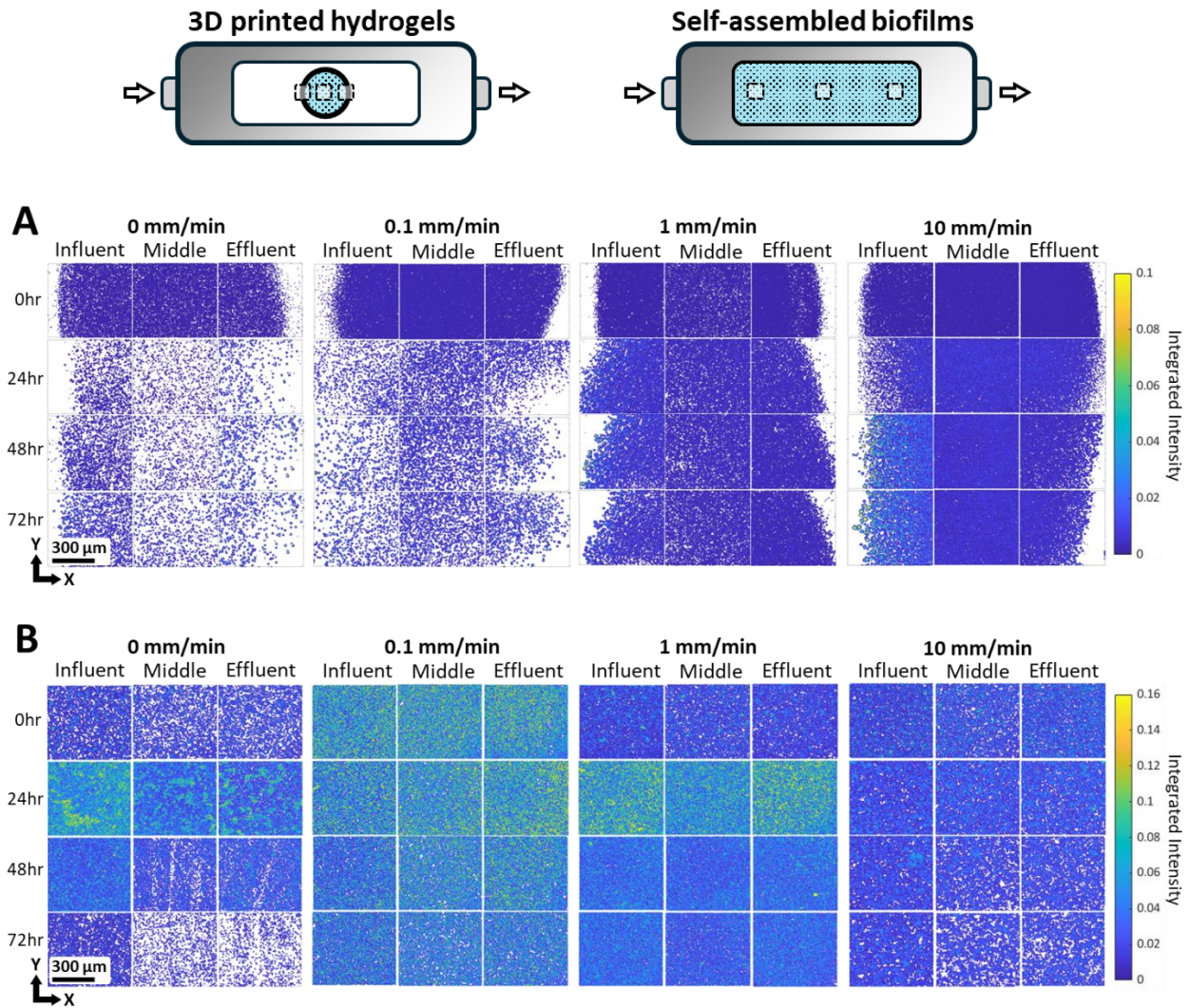


Figure 3.2. A) Fluorescence intensity of hydrogel-encapsulated cells at the influent, middle, and effluent positions for every timepoint and flow velocity combination. Raw fluorescent signal images from Figure 3.1.c were imported into BiofilmQ and each image was manually thresholded to separate signal from background. After thresholding, images were cube segmented in BiofilmQ, and integrated fluorescence intensity was calculated for each segmented object. Intensity is plotted for each xy position, generating a scatterplot of local intensity hotspots at the influent edge for 1 and 10 mm/minute treatments, and at the influent and effluent edges for

0 and 0.1 mm/minute treatments. Each image spans approximately 1 millimeter on each axis. All scatterplot color scaling is plotted on the same intensity scale. B) Representative images of fluorescence intensity of self-assembled *P. fluorescens* biofilms at the influent, middle, and effluent positions for every timepoint and flow velocity combination. Raw fluorescent signal images (supplemental figure 3.1.) were imported into BiofilmQ. Each image of self-assembled biofilms was manually thresholded to separate signal from background, and images were cube segmented in BiofilmQ with identical segmentation parameters. Integrated fluorescence intensity was calculated for each segmented object. Intensity is plotted for each xy position, generating a scatterplot of integrated intensity for each 2D biofilm image. Each image again spans approximately 1 millimeter. Corresponding .tif images are included in supplemental information.

3.4.1. Fluorescence Intensity Changes with Respect to Cell Colony Position in the Hydrogel Matrix

We plotted the fluorescence intensity of hydrogel-encapsulated microcolonies in figure **3.1.B** as a measurement of microcolony growth at each region relative to the direction of incoming flow (**Figure 3.2.a**), and as a function of fluid velocity, xy position, and time. Integrated fluorescence intensity plots at each spatial position reveal sparse population distribution at 0 and 0.1 mm/min flow treatments, compared to 1 and 10 mm/min treatments. Local fluorescence intensity hotspots develop at the influent-facing edge for all flow treatments by 48 and 72 hours. At the effluent-facing edge, by contrast, intensity hotspots develop only under the 0 and 0.1 mm/min flow treatments. The same analysis was applied to images of self-assembled biofilms (**Figure 3.2.b; Supplemental Figure 3.1**). While fluorescence intensity changes as a function of time for all but the 10mm/min flow treatment, there are no intensity differences with respect to incoming flow for self-assembled biofilms. Except for the transient, loosely associated biofilms observed at the 24-hour timepoint of the 0 mm/min treatment, self-assembled biofilms did not exceed a depth of approximately 30 μ m. 3D printed biofilms were fabricated at a depth of 150-180 μ m, depending on exact position in the matrix, though the biovolume was more dispersed than the compact self-assembled biofilms

To quantify patterns of fluorescence intensity as a measurement of microcolony growth, intensity data (**Figure 3.2.a,b**) were binned with respect to x position (perpendicular to the direction of flow). The intensity mean and standard deviation are shown in **Figure 3.3.** plotted as a function of time and bin position. First, some symmetry is observed between the influent and effluent regions for the 0mm/min treatment: the highest mean intensity at the influent and effluent positions are observed at 72 hours and are 0.0537 and 0.0542, respectively. The influent/effluent symmetry in intensity at 0mm/min is diminished at 0.1mm/min, though still observable, with a maximum intensity of 0.096 at the influent, and 0.0496 at the effluent. The concomitant increase in mean intensity at each edge region is absent for the two faster flow treatments, 1 and 10mm/min, where an increase in fluorescence intensity is observed only at the influent region. Finally, in the middle of each hydrogel, intensity does not change relative to time or x position and remains uniformly low below 0.02. The images of self-assembled *P. fluorescens* biofilms (**Figure 3.2.b**) were binned with the same methods as the printed hydrogel images, and no change in intensity relative to x position was observed at any time, position, or flow velocity (**Figure 3.4.**).

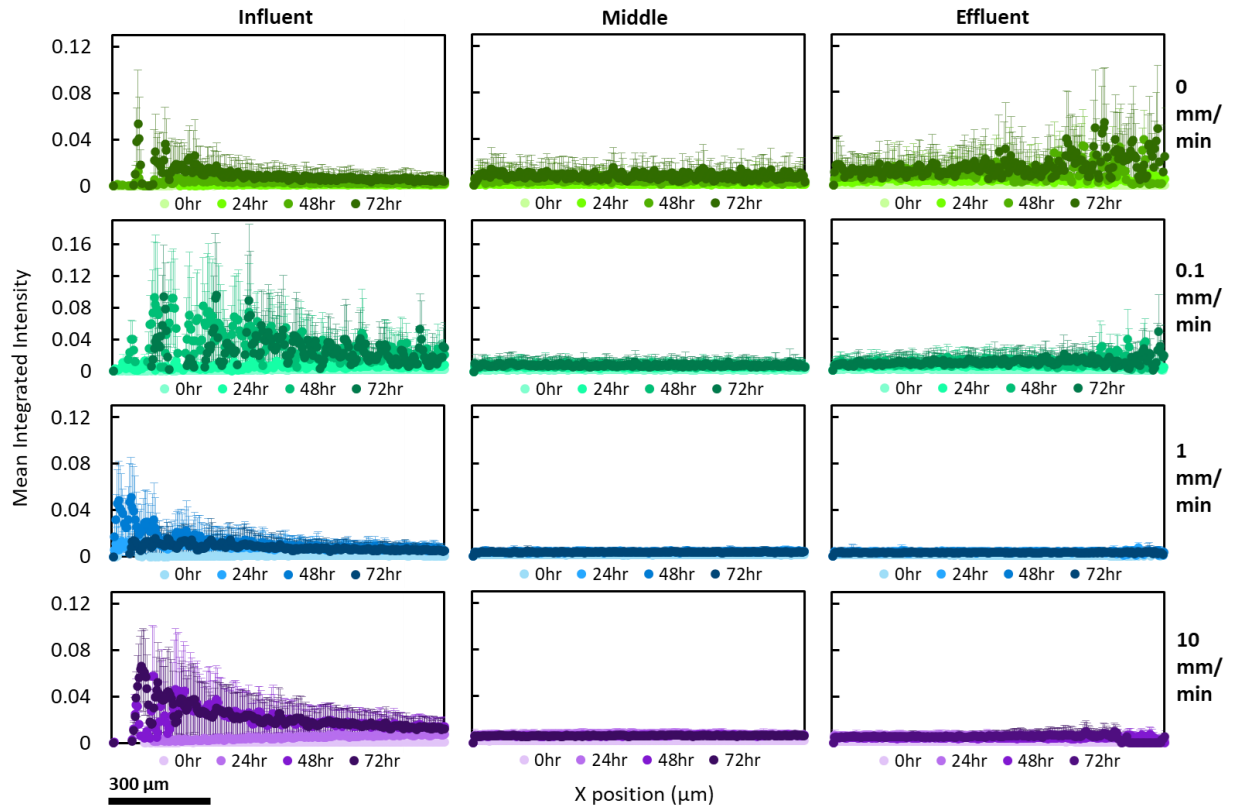


Figure 3.3. *P. fluorescens* microcolonies in 3D printed hydrogels develop fluorescence intensity hotspots that differ depending on position and fluid velocity. Integrated fluorescence intensity measurements from Figure 3.2. are binned according to X position, perpendicular to the direction of flow. Qualitative observations of fluorescence intensity hotspots are reflected in quantitative data: intensity peaks and decays at the influent edge of each hydrogel, with maxima at different times depending on flow velocity. Maximum integrated intensity occurred at 72 hours for 0, 0.1, and 10 mm/minute treatments, and occurred at 48 hours for the 1 mm/minute treatment. Hydrogels exposed to the 0 and 0.1 mm/minute treatment developed integrated intensity peaks at the effluent edge, while hydrogels exposed to the 1 and 10 mm/minute treatments did not. Note the different Y-axis range for the 0.1mm/minute treatment: higher intensity maximums were observed under this flow treatment.

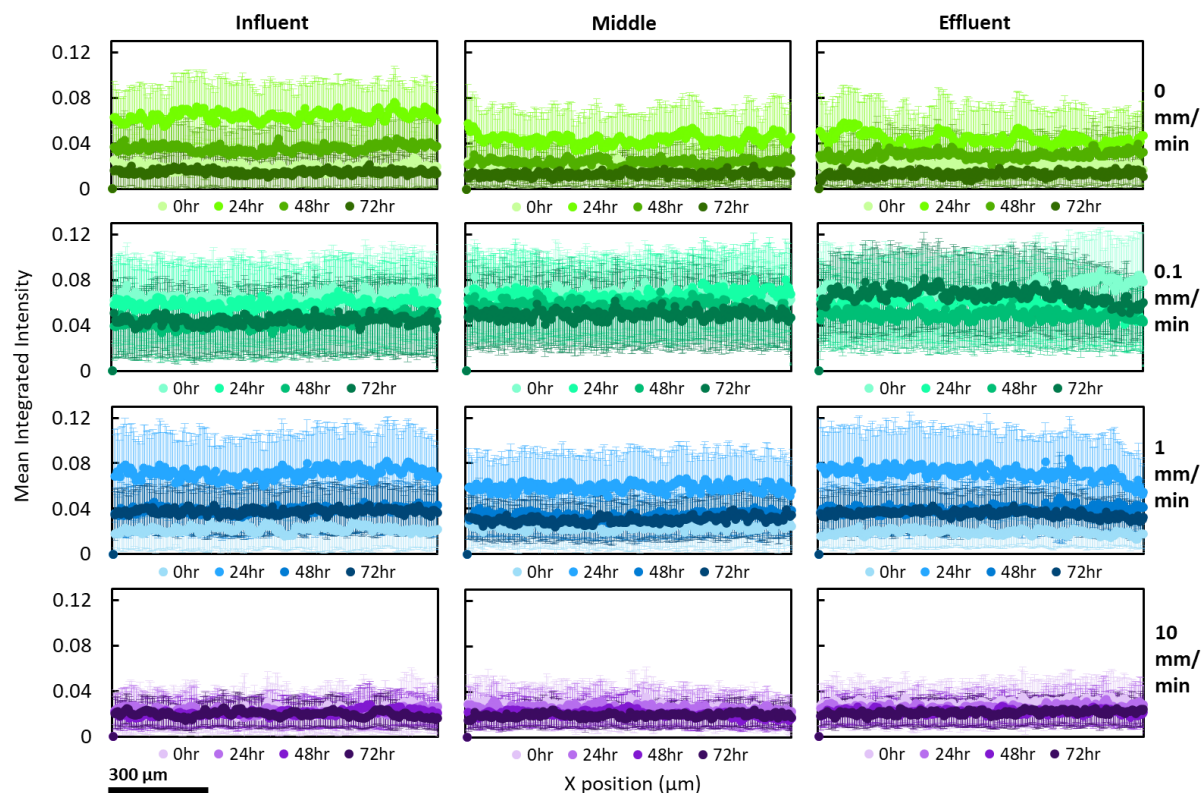


Figure 3.4. Self-assembled *P. fluorescens* biofilms do not develop heterogeneous fluorescence intensity perpendicular to the direction of flow. Integrated fluorescence intensity measurements self-assembled biofilms (Figure 3.2.b) are binned according to X position. Though mean integrated intensity values show time dependence for some flow treatments, the position of biofilms relative to incoming flow does not affect fluorescence intensity.

3.4.2. Fluid Velocity Positively Correlates with *P. fluorescens* Carrying Capacity in Printed Hydrogels

At each fluid velocity and timepoint, high-magnification 3D image stacks were acquired to quantify growth in the hydrogel matrix: six at the influent-facing region, six approximately in the middle, and six at the effluent-facing region. We quantified *P. fluorescens* population dynamics in 3D by segmenting fluorescent signal in each field of view and used the volume occupied by signal to calculate colony biovolume, colony count, and biovolume sum. These methods allow population growth analysis at the level of individual colonies (colony biovolume), and at the level

of each field of view (colony count, biovolume sum). Qualitatively, colonies appeared to grow largest for the 10mm/min flow treatment (**Figure 3.5.a**).

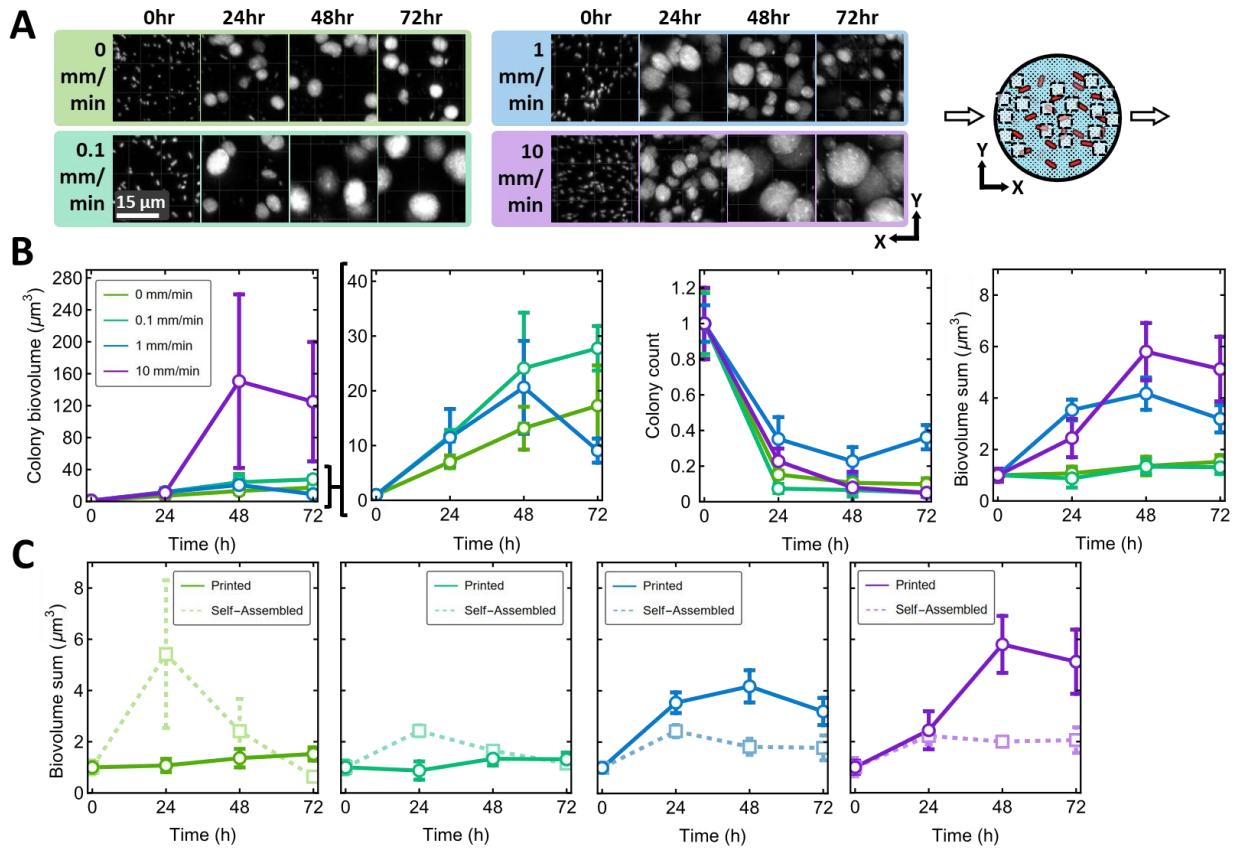


Figure 3.5. A) Representative 3D image stacks of microcolonies encapsulated in hydrogels for each timepoint and flow velocity. Image stacks spanned the entire depth of the hydrogels. Fluorescent signal of all 3D image stacks is thresholded and binarized, and mean colony biovolume (i.e., colony size), mean colony count, and mean biovolume sum are calculated per field of view. B) Time-evolved growth of *Pseudomonas fluorescens* 3D printed hydrogels under variable flow conditions. All measurements consist of the mean and standard deviation of eighteen fields of view. Initial inoculation density was equal for each condition, and data are normalized to biovolume sum, colony count, and colony biovolume at 0 hours. Mean colony size is similar for all conditions except for the 10 mm/minute treatment, which resulted in both a higher mean and substantially greater variability. Colony count decreased regardless of flow treatment, predominantly within the first 24 hours, to between 10-40% of the initial total. Biovolume sum across each field of view was affected by flow velocity: while the 0 and 0.1

mm/minute treatments are indistinguishable, 1 mm/minute flow velocity produced a three-fold higher average sum at 72 hours, and 10 mm/minute produced a five-fold higher sum at 72 hours. The fastest flow treatment prolonged the duration of exponential growth. C) Mean biovolume sum plotted for printed hydrogels and self-assembled biofilms grown at each respective flow velocity. At 0 mm/minute self-assembled biofilms develop large, but loosely associated and transient biofilms that ultimately disappear. Large, transient biofilms were not observed at 0.1, 1, and 10 mm/minute flow treatments, and these flow treatments produced no differences in biovolume sum over a 72-hour period, in contrast to biovolume sum differences observed for *P. fluorescens* populations encapsulated in 3D printed hydrogels. Per field of view, the inoculation density of the biofilm fraction in the self-assembled biofilm treatments was equal to the inoculation density of *P. fluorescens* in the 3D printed hydrogels, though the overall biomass in the flow chamber is larger for the self-assembled biofilm treatments.

At the individual colony level, the fastest flow treatment produced both larger colony sizes and colonies with greater size variability. The size and variability increase with respect to flow velocity was observed at 48 and 72 hours, while at 0 and 24 hours, colony size remained homogeneous between all four flow conditions (**Figure 3.5.b**). While colony size increased, colony count decreased for all flow conditions between 0 and 24 hours, eventually stabilizing between 10-40% of the initial count. *P. fluorescens* viability, in terms of CFU/mL, is not affected by exposure to the unpolymerized resin or laser over the short timescales (~5 minutes) required for single-layer hydrogel printing (**Supplemental Figure 3.4.d**). These results suggest that the printing process did not increase the baseline viability level and that bio-printing enabled the ability to visualize inherent physiological heterogeneity within the bacterial culture. The colony counts observed here are generally lower than observed in our previous study that used aerobic batch conditions²³. In comparison, even though aerobic medium prepared at ambient atmosphere was used, the flow cells described here lack a liquid-air interface that exists in well plate batch culture system. Aqueous oxygen concentrations were not measured in our previous study, but in flow cells, the *P. fluorescens* population in the hydrogels deplete the available oxygen by 48 hours for the 0, 0.1, and 1 mm/min treatments (**Figure 3.6.a**). The 10 mm/minute condition was not tested

due to the rapid replacement rate of the flow cell chamber: at a fluid velocity of 10 mm/minute, the flow chamber is completely replaced with oxygenated media every five minutes.

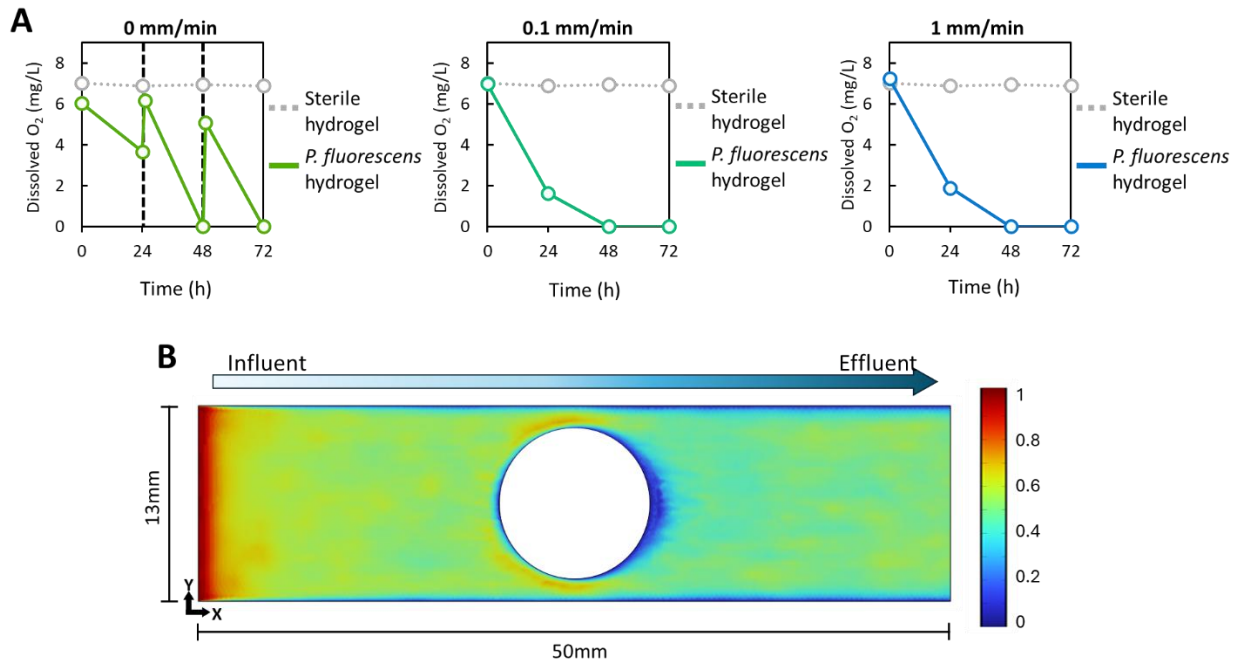


Figure 3.6. A) Aqueous dissolved oxygen measurements in the flow chamber for printed hydrogels at 0, 0.1, and 1 mm/minute. For the 0mm/minute treatment, media was replaced at 24 and 48 hours, indicated by the dashed lines, and oxygen measurements were performed before and after replacement. Dissolved oxygen was measured in the flow chamber using a FireSting optical oxygen meter. Hydrogels with encapsulated *P. fluorescens* were at an approximately equal initial inoculation density to previous experiments. B) *xy* planar view of modeled velocity distribution in microfluidic devices with a 3D printed hydrogel, generated with COMSOL. The plane is set to the Z-center of the hydrogel. Velocity was set to 1m/s for this simulation, but because this is a laminar flow model with a no-slip boundary condition at the exact hydrogel/aqueous media interface, the flow predictions are independent of velocity within a laminar flow range and scale to actual flow velocity. Heat map color scaling indicates normalized velocity across the flow chamber. Flow proceeds from left to right.

To understand broader population dynamics, we analyzed biovolume at the field of view level with respect to time and flow velocity. Differences in growth as a function of flow emerged in the first 24 hours: the 1 and 10 mm/min treatments produced over a two-fold average increase

in biovolume compared to the 0 and 0.1 mm/min treatments. At longer times, the 1 mm/min flow increased average biovolume sum approximately three-fold compared to the batch treatment at 72 hours, and 10 mm/min flow produced an average biovolume sum approximately five times higher than the batch treatment. Growth at 0.1 mm/min did not produce meaningful average growth differences compared to the 0 mm/min treatment. When average growth metrics are resolved by position (influent, middle, and effluent), the main contributor to greater population growth in the fastest two flow conditions is the influent edge region. This holds true for individual colony biovolume at 10mm/min as well (**Supplemental Figure 3.2.a**). Incoming flow could produce a rain shadow effect behind the effluent edge of the hydrogel and restrict nutrient access compared to the influent edge. To evaluate this possibility, we performed a laminar flow velocity model with a no-slip boundary condition between the hydrogel and aqueous environment in the flow chamber (**Figure 3.6.b**). Flow modelling, with velocity plotted on a normalized scale, predicts several millimeters of near-zero velocity at the effluent hydrogel edge compared to the leading influent-facing edge, which may partially explain positional growth differences.

3.4.3. Printed and Self-Assembled Biofilms Respond Differently to Changing Flow Velocity and Nutrient Conditions

We compared biovolume sum in 3D printed hydrogels to self-assembled *P. fluorescens* biofilms, where free-living cells were inoculated into the flow cell and allowed to settle on the cover glass, disperse, and re-attach at will (*i.e.*, natural biofilm life cycle). The initial biovolume of self-assembled biofilms is equal to the initial biovolume of hydrogels on a per-field-of-view basis, though the self-assembled biofilm treatments contain a higher population overall because the cells are not constrained to only occupy the hydrogel matrix. While changes in flow velocity

substantially affected biovolume sum in the printed hydrogels, the 1 and 10 mm/min treatments had only a modest effect on self-assembled biofilm growth by the time steady state was reached at 72 hours (**Figure 3.5.c**). The 0 mm/min batch growth treatment produced transient and loosely associated biofilms at 24 hours that dispersed at longer times. At the population level, increased flow velocity is positively correlated with carrying capacity of synthetic *P. fluorescens* biofilms, but not self-assembled *P. fluorescens* biofilms.

Biofilms in nature are exposed to fluctuating nutrient and mass transport conditions, and a biofilm's potential structure and function are particularly shaped by the availability of electron donors and acceptors for respiration in line with the biochemical capacity of microbial community members. Flow reactors allow tight control of biofilm nutrient environment by changing the characteristics of incoming media. The main carbon source accessible to *P. fluorescens* in the experiments here is 20mM sodium acetate (**Supplemental Table 3.1.**). While cells in the hydrogel matrix depleted the available aqueous oxygen by 48 hours, aqueous acetate concentration never decreased below 16mM, and there were no distinctions in acetate consumption between the self-assembled and hydrogel biofilms (**Supplemental Figure 3.3.**). Though there may be specific locations in the hydrogel matrix where carbon availability is diminished or even limiting, cells in the hydrogel matrix were likely not deprived of carbon source based upon bulk aqueous-phase levels. This was true for self-assembled and synthetic biofilms, at all flow velocities (**Supplemental Figure 3.3.**).

Given the broad application context of bioprinted hydrogels, it is materially relevant to evaluate the response of hydrogel-encapsulated cells to fluctuating environmental conditions and nutrient deprivation. When *P. fluorescens* hydrogels were incubated in non-sterile groundwater

(i.e., with a native microbial community) spiked with 20mM sodium acetate, *P. fluorescens* grows in the hydrogel matrix albeit to a lesser extent than growth with sterile minimal media (**Figure 3.7.a,b**). The reduced growth likely reflects both competition for resources with an extrinsic community and a changed nutrient environment compared to sterile minimal media.

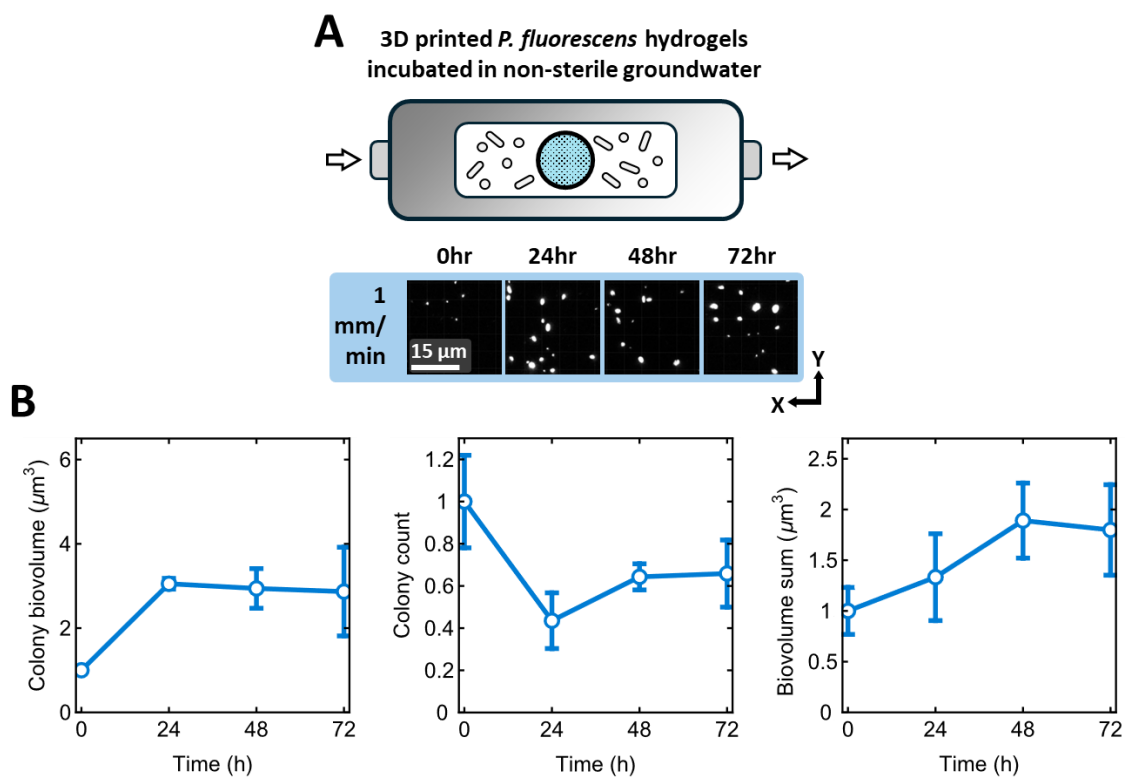
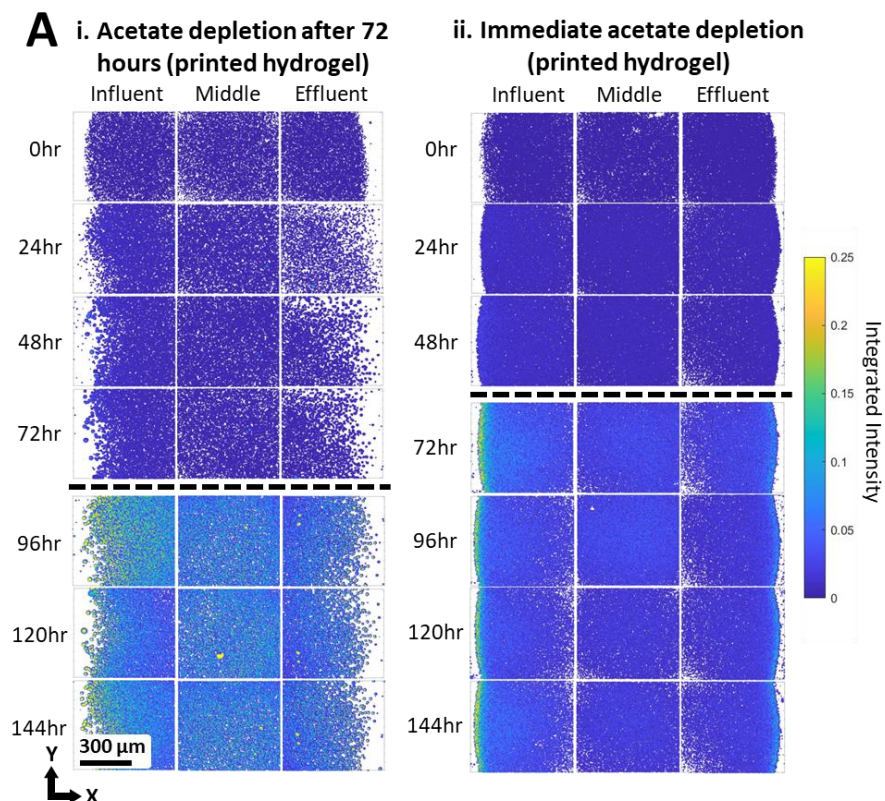


Figure 3.7. A) Graphic of 3D printed hydrogels encapsulated with *P. fluorescens* incubated in a non-sterile groundwater microbial community spiked with 20mM sodium acetate. *P. fluorescens* colonies grow in the hydrogel matrix over 72 hours, though not to the same extent as previous experiments where the population is incubated in sterile minimal media. B) Average colony biovolume, colony count, and average biovolume sum of hydrogel-encapsulated *P. fluorescens* at 1mm/minute flow. Instead of defined minimal media, the hydrogels were incubated in groundwater with its native microbial community spiked with 20mM sodium acetate. Despite the resource competition from the groundwater community in the aqueous phase, *P. fluorescens* was still able to grow in the hydrogel matrix, albeit to a lesser degree than with sterile minimal media.

Synthetic *P. fluorescens* biofilms were grown for 72 hours at a flow velocity of 1mm/min, but after this growth period, the minimal media was flushed from the flow chamber and replaced with acetate-free minimal media. After incubation in carbon-depleted conditions, cells remained in the hydrogel matrix, and mCherry signal intensity increased and remained elevated up to 144 hours for all positions measured (**Figure 3.8.ai**). The black dashed line between the 72- and 96-hour intensity plots demarcates the intensity increase coinciding with pre- and post-acetate depletion in the flow chamber environment. In parallel, a *P. fluorescens* hydrogel was printed and given acetate-free minimal media immediately and for the entire incubation period (**Figure 3.8.iii**). Even under conditions where cells in the hydrogel matrix were immediately exposed to carbon-depleted media, the population in the hydrogel unexpectedly doubled in biovolume in the first 48 hours of incubation.



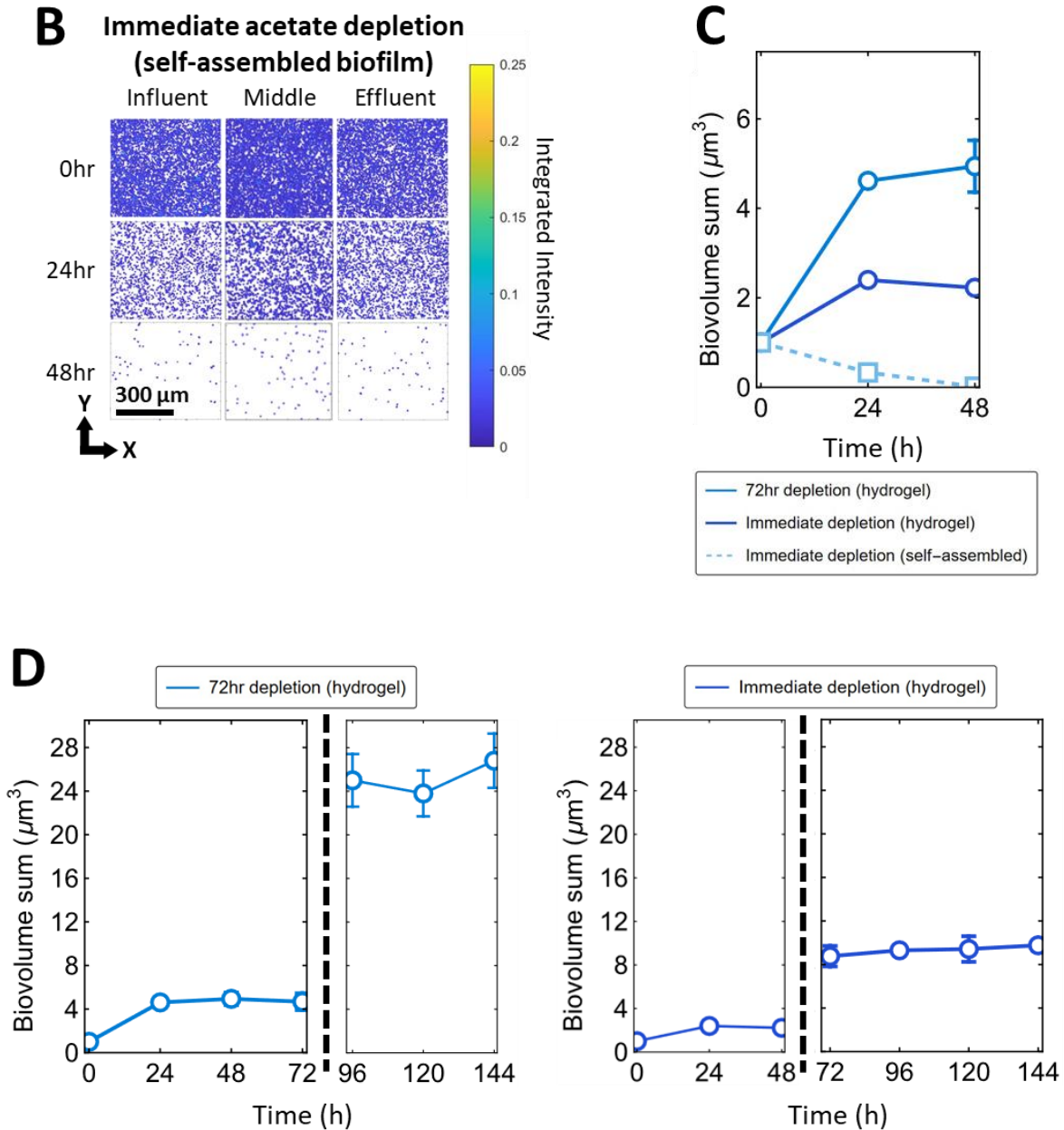


Figure 3.8. A) Integrated fluorescence intensity scatterplots of 3D printed hydrogels grown under two different acetate-depletion conditions: acetate depletion immediately after the 72hr measurement (i) and acetate depletion immediately upon printing and flow cell incubation (ii). The dashed lines indicate pre- and post- fluorescence intensity increase between 72 and 96 hours, and between 48 and 72 hours, respectively. B) Integrated fluorescence intensity scatterplots of self-assembled biofilms given acetate-depleted media immediately upon flow cell inoculation and incubation. *P. fluorescens* cells settle on the coverslip surface of the flow cell, but the initial near-confluent biofilm is reduced over time, and by 48 hours only single cells and very small

clusters remain. C) Biovolume sum calculations of 3D high-magnification images from treatments in parts A(i-ii) and B for the first 48 hours of incubation. Data for each hydrogel and the self-assembled biofilm are normalized to each respective 0hr timepoint. D) Biovolume sum calculations from binary thresholding of 3D high-magnification images from treatments in parts A(i-ii) up to 144 hours of growth. The dashed lines again represent pre- and post-fluorescence intensity increases.

The cause of the drastic increase in fluorescence did not appear to be linked to cell growth based upon assessment of total biovolume. In addition, a sub-sample of cells prepped for bio-printing were incubated in acetate-free medium and growth (OD increase) was not observed although fluorescence increased (7x; **Supplemental Figure 3.4.b**). Moreover, strain N2E2 does appear to grow with the low levels of tartrazine (0.05%, w/v) used as a photo-absorber during the print process (**Supplemental Figure 3.4.e**). A drastic increase in signal intensity occurred in the hydrogel immediately incubated in carbon-depleted media, but this time between 48 and 72 hours of incubation. It is likely that the demarcated intensity increase coincides with carbon depletion, but we expected that carbon depletion would occur immediately, given that acetate was never introduced to the flow chamber. In contrast, self-assembled *P. fluorescens* biofilms inoculated in the flow cell and immediately deprived of carbon waned until only dispersed individual cells remained on the coverslip surface (**Figure 3.8.b**). When cells are encapsulated in the hydrogel matrix, dispersion from an unfavorable environment is not possible and instead are essentially trapped and subjected to environmental fluctuations. Average biovolume sum results are aggregated and normalized to initial biomass (**Figure 3.8.c**) for the two hydrogels carbon-depletion conditions from **Figure 3.7.a**, and the self-assembled biofilm in **Figure 3.7.b**.

Over the entire 144-hour growth period for the acetate-depleted hydrogels (**Figure 3.8.d**), biovolume sum for the 72-hour carbon-depletion treatment reached steady-state by 24 hours, but post-intensity increase, the segmented biovolume increased substantially. A similar pattern was

observed for the immediate carbon depletion treatment, though to a lesser extent. Based upon the results without acetate and other possible trace nutrients, the increases in fluorescence are not reflective of growth but rather cell maintenance. The results without exogenous carbon source suggested that cells bio-printed in the synthetic biofilms could be maintained for the tested time periods (hours to days).

3.5. Discussion

The deep connection between structure and function observed at the macroecological scale, such as the stratified environments of an old-growth forest, is equally defining of biofilms at the microecological scale. Biofilm structure at sub-millimeter scales can have macroscopic consequences. For example, aerobic granular sludge particles are intricately structured microbial communities, typically one or a few millimeters in diameter, that potentiate industrial-scale wastewater treatment ²⁶. Biofilm structures are formed in diverse selective environments and influenced by mass transport conditions (i.e., diffusion and advection) within and outside of a biofilm. In controlled flow cell experiments with *Pseudomonas fluorescens*, the interaction between gravity and shear stress influences patterns of surface colonization by changing bacterial motility and biofilm structure ²⁷. In complex environments like the terrestrial subsurface, groundwater flow is a selective pressure influencing cell partitioning into the planktonic or biofilm phase, though information about how specific flow regimes influence partitioning in the subsurface is limited ²⁸. Experimentally controlling biofilm structure and the surrounding environmental characteristics is crucial for improving our predictive power over biofilm function, formation, and growth. However, controlling the structure and composition of biofilms at sub-millimeter scales

has been a historical challenge for experimental microbiology. Microbial 3D bioprinting offers potential to provide such small-scale control.

Advancements in microbial 3D bioprinting have enabled exploration of a vast synthetic biofilm design landscape. However, an enduring knowledge gap remains regarding the fundamentals of microbial behavior within 3D printed hydrogel matrices, and whether the constraints associated with holding cells captive in synthetic environments impact our ability to predict microbial behavior, physiology, and population dynamics. The conditions of encapsulated growth in a 3D printed hydrogel may be substantially different from the conditions in which a population was naturally selected to grow. To design effective synthetic biofilms for both fundamental and applied research, it is crucial to understand and predict how encapsulated microorganisms will behave in a printed hydrogel matrix and material application context.

Biofilms grow pervasively on surfaces that interface with fluids and/or gases where mass transport is an important selective pressure on biofilm structure, physiology, and function. The *Pseudomonas* genus is arguably one of the most studied bacterial genera and commonly used as a biofilm ‘model’ system, and some strains of the biofilm-forming *Pseudomonas fluorescens* are impacted by changing flow conditions within a laminar regime. For instance, Arúajo et. al. developed a flow cell to grow biofilms on stainless steel coupons and found that the two fastest flow velocities formed thinner, more densely packed biofilms with higher EPS content compared to the slowest laminar flow velocity²⁹. Using a different *P. fluorescens* strain, Jara et. al. observed that hydrodynamic stress increased cell density and EPS production compared to static biofilm growth conditions³⁰. Encapsulating cells in a synthetic matrix adds an additional layer of environmental context. Namely, the interaction between fluid flow and encapsulation affects *P.*

fluorescens population dynamics in patterns not predictable from observing self-assembled biofilms under the same nutrient and extrinsic mass transport conditions. Population dynamics of 3D printed biofilms differed from self-assembled biofilms by every metric observed, except for acetate consumption measured in the aqueous media. These results suggest that despite similar acetate flux, utilization, and depletion, the distribution and growth of biomass differed between the self-assembled and synthetic biofilms.

Synthetic and self-assembled *P. fluorescens* biofilms respond differently to identical flow treatments. Increasing velocity substantially increased carrying capacity of the hydrogel-encapsulated population, and most of this increase is attributed to growth at the influent-facing region of the hydrogel. Conversely, increasing velocity had only minor effects on self-assembled biovolume up to 72 hours of growth, and no effect on carrying capacity measured by biovolume analysis. Repeatable differences were also observed in the relationship between fluid velocity and fluorescence heterogeneity between the synthetic and self-assembled biofilms. Notably, neither synthetic nor self-assembled biofilms depleted aqueous carbon in the flow channel. However, synthetic and self-assembled biofilms responded distinctly to sudden carbon depletion. Overall, spatial and temporal patterns of self-assembled biofilm development were not predictive of patterns in 3D printed hydrogels. The integration of flow cell microscopy and 3D bioprinting demonstrated here allows precise control over the mass transport and nutrient environment and enables population-level investigation of encapsulated cell behavior in living materials.

The fastest flow condition produced a nearly six-fold increase in average biovolume compared to batch for printed hydrogels, while no biovolume difference was observed for self-assembled biofilms. While the fastest flow condition may have altered the internal structure of

self-assembled biofilms and affected the number of individual cells in the biofilms, the carrying capacity of self-assembled biofilms still would likely not approach the increase observed in hydrogels at 10 mm/min velocity (**Figure 3.5.b**). It is possible that shear force applied by this flow treatment caused cells in the self-assembled biofilms, especially at the biofilm-aqueous interface, to slough off and disperse. When cells are trapped in the PEG-DA hydrogel matrix, cells are deprived of the opportunity to respond to shear force by detaching and reattaching to a surface or staying partitioned in the planktonic phase. The long-term physiological and evolutionary effects of encapsulating cells and altering the ‘typical’ biofilm life cycle are unknown, but the hydrogel matrix appears to act as a selection bottleneck where only 10-40% of the initial population (**Figure 3.5.b**) replicates in the hydrogel matrix. This observation coincides with suspected physiological heterogeneity within microbial populations that is challenging to parse out. The ability to visualize over time individual cells within a population will greatly advance the ability to decipher the underlying population genetics of the genotype-phenotype relationship in microorganisms. In addition, the bio-printing method will enable a method to test responses of a given population to a given suite of conditions in a systematic fashion.

Spatial patterns of printed biofilm population growth observed in two and three dimensions seem consistent with laminar flow mechanics. Regarding the positive correlation between fluid velocity and carrying capacity in synthetic biofilms, and the physical context in which population size increased, the PEG-DA hydrogel pore sizes are on the nanometer-scale, and the bulk material suppresses advection ³¹. In other words, diffusion into the hydrogel matrix is the dominant mode of transport and increasing flow velocity does not actively force nutrients through the hydrogel matrix via advection. Rather, increased shear force from increasing fluid velocity may diminish

the diffusive boundary layer between the synthetic biofilm and aqueous environment. Diminishing the diffusive boundary layer would permit easier nutrient diffusion into the hydrogel at relatively fast flow velocities, observed most clearly at the influent-facing edge at 1 and 10mm/min (**Supplemental Figure 3.2.a**). The 10mm/min flow treatment also prolonged the duration of the *P. fluorescens* exponential growth phase from 24 to 48 hours, which was not observed for any other flow or nutrient treatment, but only for cells distributed at the influent edge (**Supplemental Figure 3.2.a**).

Modelling normalized flow velocity suggests that a rain shadow effect exists on the downstream surface of the hydrogel (**Figure 3.6.a**), which is consistent with observations of lower effluent-edge biovolume at 1 and 10 mm/min (**Supplemental Figure 3.2.a**). However, differences in effluent-edge fluorescence intensity (**Figure 3.3.**) between the 0 and 0.1 mm/min treatments and the 1 and 10mm/min treatments cannot be fully explained by modeling the relative normalized flow velocity, as velocity predictions in the flow chamber are independent of the imposed flow conditions under the model assumptions of laminar flow and a no-slip boundary. The increased effluent-edge fluorescence intensity and influent/effluent intensity symmetry at 0 and 0.1 mm/min could be attributed to a combination of sparse cell distribution allowing the remaining cells easier nutrient access compared to the 1 and 10mm/min conditions (**Figure 3.2.a**), and no upstream/downstream flow differences at all in the case of the 0 mm/min flow condition.

The 1mm/min flow condition was chosen for more detailed exploration of the nutrient environment in the flow chamber because this condition produced an observable difference in growth above the 0mm/min baseline and was logistically simpler than the 10mm/min condition. First, the *P. fluorescens* population encapsulated in 3D printed hydrogels grew even when

competing for resources with an external community in unfiltered groundwater spiked with 20mM sodium acetate. For consistent real-world applications of synthetic biofilms, particularly engineered living materials, it is desirable for encapsulated population(s) to be resilient to influences of an external microbial community. While overall population growth was diminished compared to the 1mm/min flow condition with sterile minimal media, growth was still temporally predictable and biovolume growth occurred predominantly during the first 24 hours then plateaued (**Figure 3.7.b**). The consistency of growth duration with previous results in sterile minimal media suggests that the groundwater community did not disrupt the ability of *P. fluorescens* to acquire nutrients and begin replication in the hydrogel matrix, though disruptions may be discovered over greater time periods.

Nutrient environments fluctuate in nature and in natural application contexts of synthetic biofilms, and biofilms have diverse individual and communal responses to stress from sudden nutrient deprivation.^{32, 33} It is unclear whether encapsulation in the synthetic hydrogel matrix would alter population behavior in the face of rapid shifts in nutrient availability. We manipulated the nutrient environment surrounding the synthetic and self-assembled biofilms by introducing minimal media with no acetate at the beginning of incubation, or after 72 hours of normal incubation. We hypothesized that cells in the hydrogel matrix would persist and retain fluorescent signal during carbon deprivation, but that there would be no population growth when they were given carbon-depleted media immediately after printing. Next, we hypothesized that when carbon-depleted media is introduced after 72 hours of normal incubation, no population-level changes observable by fluorescence microscopy would occur given that the population is expected to reach steady-state by 72 hours. Finally, contrasting with the synthetic hydrogel-encapsulated biofilms,

we hypothesized that self-assembled biofilms would steadily disperse under carbon-depleted conditions. By 48 hours, self-assembled biofilms dwindled from a nearly confluent biofilm to dispersed individual cells and small clusters (**Figure 3.8.b**). For the immediate and 72-hour carbon depletion treatments in synthetic biofilms, *P. fluorescens* persisted in the hydrogel matrix, and introducing carbon-depleted media did not change the number of colonies observed or the expected proportion of colonies observed (**Supplemental Figure 3.6**).

However, fluorescence intensity increased between 72 and 96 hours when carbon-depleted media was introduced after 72 of normal incubation (**Figure 3.8.ai**), and between 48 and 72 hours when carbon-depleted media was introduced immediately (**Figure 3.8.ii**). We also observed some population growth between 0 and 24 hours in the immediately incubated in carbon-depleted media (**Figure 3.8.c, 3.8.d**). The fluorescence intensity increase observed in **Figure 3.8.a-ii** is remarkably similar to the increase observed in **Figure 3.8a-i** and likely demarcates the time that cells actually experience carbon deprivation in both cases. Given that there was a delay in elevated fluorescence intensity for the hydrogel immediately incubated in carbon-depleted media, and a twofold increase in biovolume was observed, cells in the hydrogel matrix may have utilized an alternative carbon source(s). There is not sufficient alternative carbon in the media for *P. fluorescens* to grow without acetate (**Supplemental Figure 3.4.a-c**), but one possibility enabling growth derives from evidence in **Supplemental Figure 3.6**. By 24 hours of growth, the observed *P. fluorescens* colony count decreases to 40% of the initial total for the immediate carbon depletion treatment (similar to carbon replete conditions²³). It is feasible that at least a portion of cells may lyse due to printing and encapsulation and thus release low levels of nutrients into the surrounding matrix. Residual tartrazine from the printing process, though likely minimal, may also contribute to population

growth, as this *P. fluorescens* strain can use tartrazine as a growth substrate in the absence of acetate (**Supplemental Figure 3.4.e**).

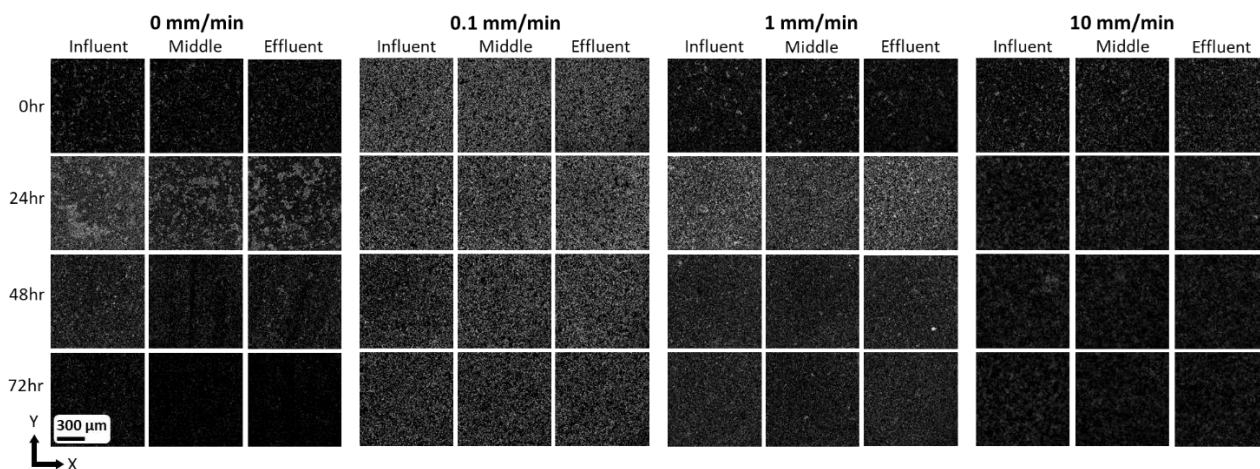
Increased fluorescence intensity after carbon depletion likely does not correlate to growth, despite fluorescence increasing. **Supplemental figure 3.5.a** shows an example field-of-view for the 72hr-depletion dataset where signal attenuation is visible at the top of the hydrogel but not the middle and bottom regions. After introducing carbon-depleted media into the flow cell, signal intensity overcomes attenuation. If the biovolume increase after carbon depletion is due to signal intensity increase, and not cell growth, then any biovolume increase for the cell population at the bottom of the hydrogel should be mitigated or nonexistent. **Supplemental figure 3.5.b** shows a repeat analysis of the same dataset from **Figure 3.8.**, but only the bottom-quarter of each field-of-view is analyzed, instead of the entire depth. Fluorescence did not increase in the bottom quarter of the hydrogel immediately incubated in carbon-depleted media. Fluorescence still increased in the bottom quarter of the 72hr-depletion hydrogel, though reduced from 23-fold when the entire depth is analyzed (**Figure 3.8.**) to 6-fold. The utilization of trace nutrients may explain the observed fluorescence without acetate, but additional explanations should be explored, including unique physiological responses of cells in bio-printed matrices.

The design of engineered living materials with 3D bioprinting should be informed by understanding how encapsulated microorganisms will behave in the different materials that will impact nutrient and cell movement as well as the different application contexts. We have observed that, under changing flow and nutrient conditions, the population dynamics of self-assembled *P. fluorescens* are not predictive of *P. fluorescens* population dynamics in a 3D bioprinted material matrix. A method was developed to directly bio-print a synthetic biofilm on a surface that was

placed into a flow reactor and sterilized in place. This new method allows the building and testing of synthetic biofilms under different flow and growth conditions that can be assessed via quantitative confocal microscopy.

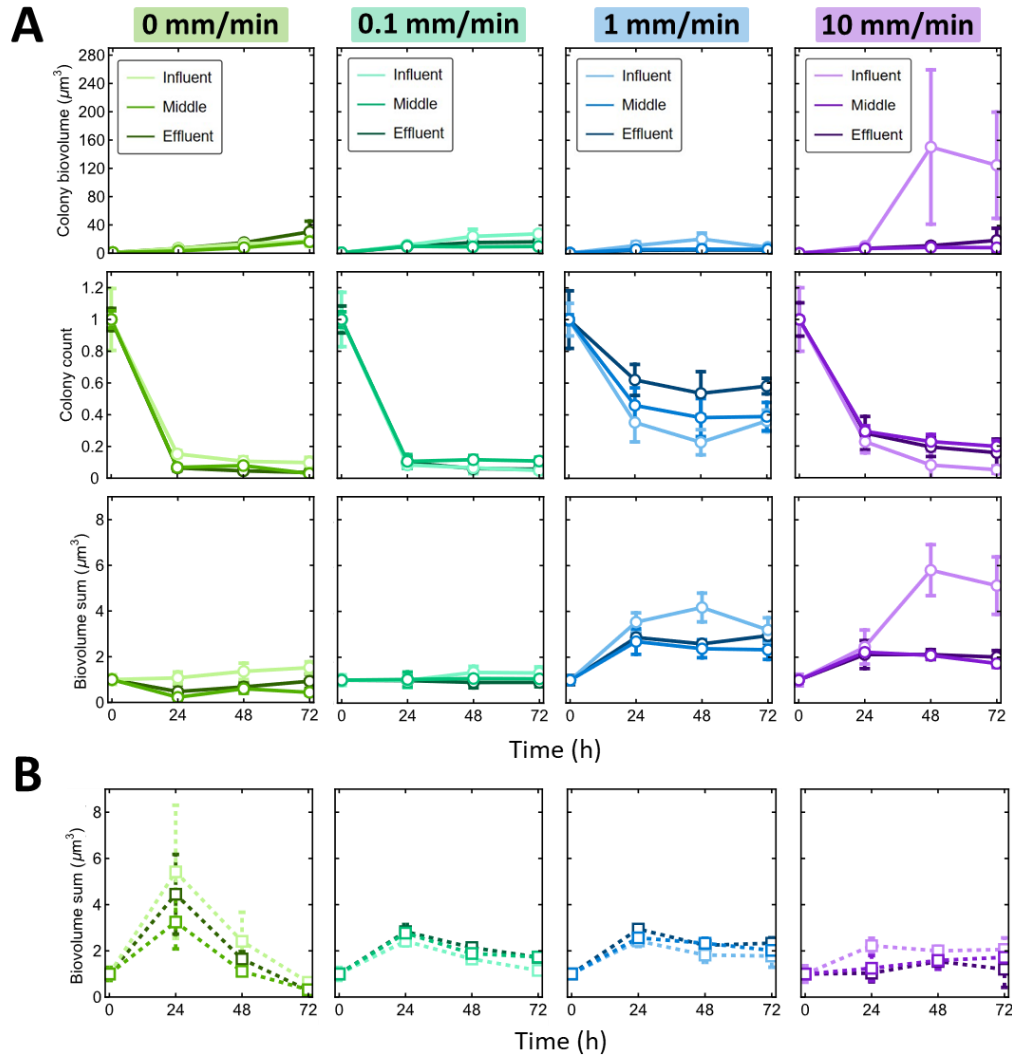
Increasing the flow of growth medium resulted in increased carry-capacity of the synthetic biofilms, particularly at the influent-facing surface of the hydrogel. Interestingly, the hydrogel matrix acted as a selection bottleneck for a proportion of the initial population ranging from approximately 10-40%, though the underpinning mechanisms influencing which individuals grow in the hydrogel matrix are currently unknown. In the future, integrating observations of synthetic biofilm population dynamics with transcriptomics would provide a wealth of correlative information about cell physiology in the hydrogel matrix, especially how gene expression may differ across a given population as well as compared to cells across self-assembled biofilms.

3.6. Supplemental Figures

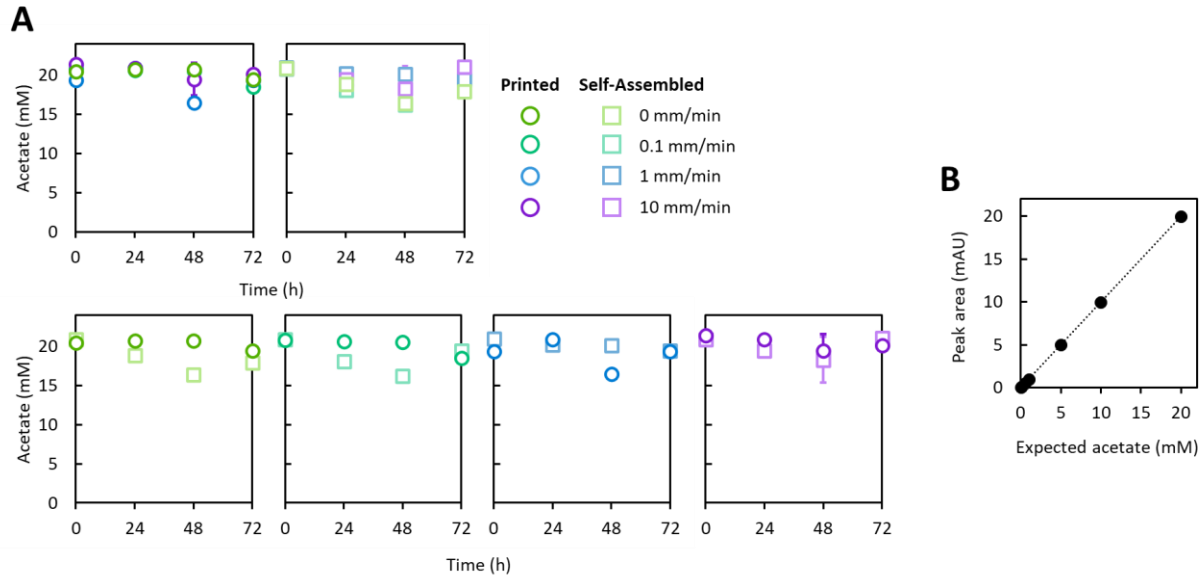


Supplemental Figure 3.1. Representative 2D images of self-assembled biofilms at each time and flow velocity combination used for fluorescent signal analysis in figures 2 and 4. The self-assembled biofilm is confluent over the coverslip surface area. “Influent, middle, and effluent”

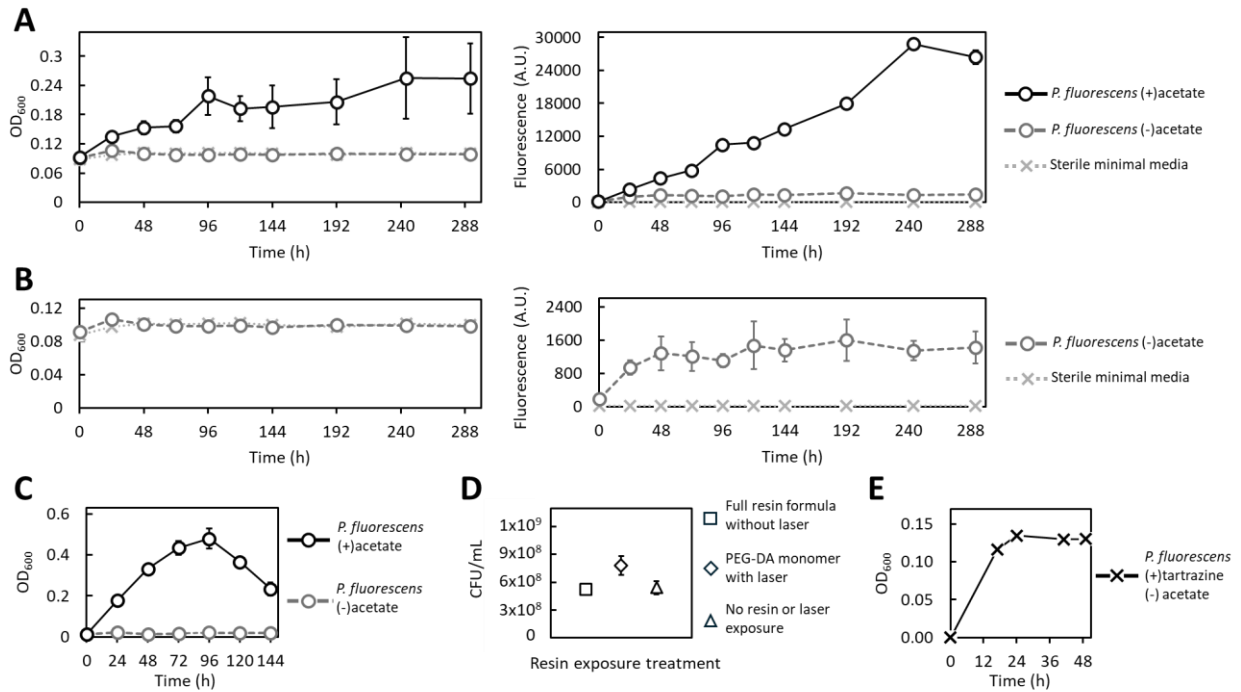
refer to regions across the entire flow chamber relative to incoming flow (see **Figure 3.3.b**). Biofilms were grown under each flow treatment for one hour after inoculation prior to imaging.



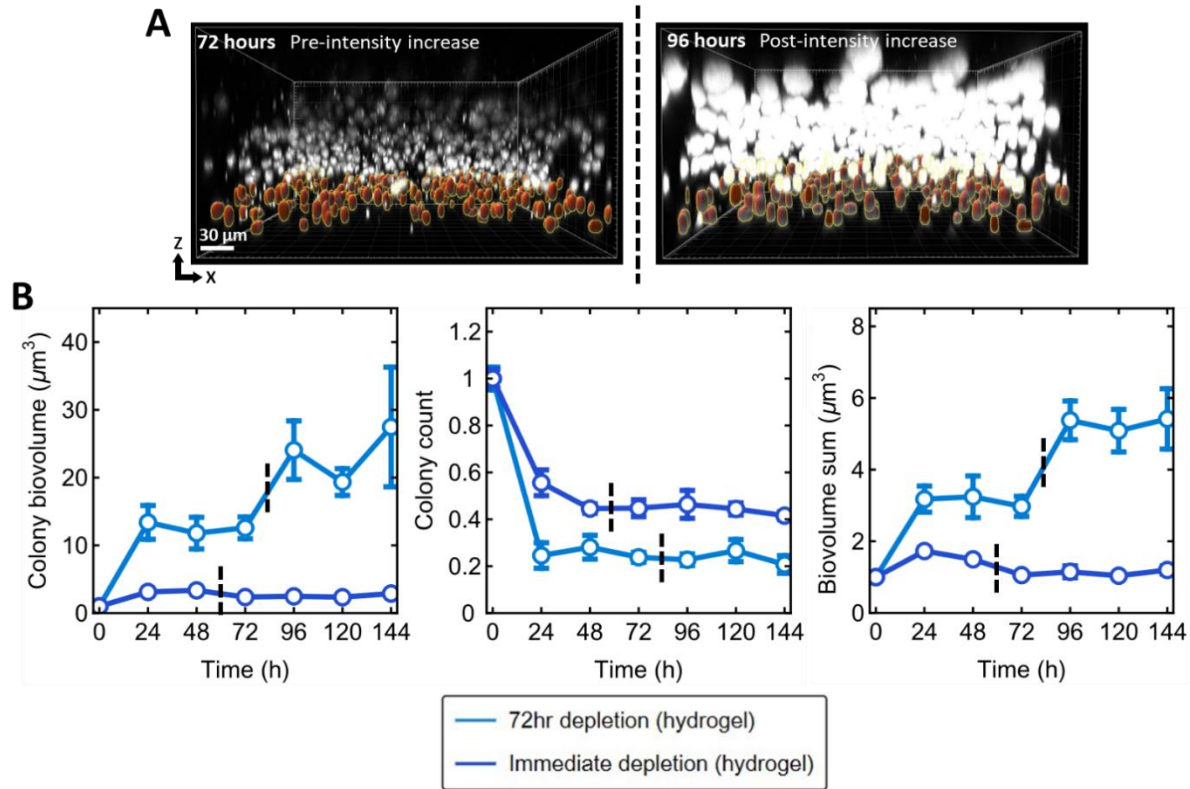
Supplemental Figure 3.2. A) Time-evolved growth of *Pseudomonas fluorescens* 3D printed hydrogels under variable flow conditions separated by hydrogel position (influent, middle, and effluent). Measurements at each position are the mean and standard deviation of six fields of view for each positional category. The greatest contribution to increases in mean colony biovolume and mean biovolume sum between the 10 mm/minute treatment and the other three flow conditions are from the *P. fluorescens* population distributed at the influent edge. B) Differences in mean biovolume sum as a function of spatial position relative to the media influent are not observed for self-assembled biofilms at any of the four flow velocities.



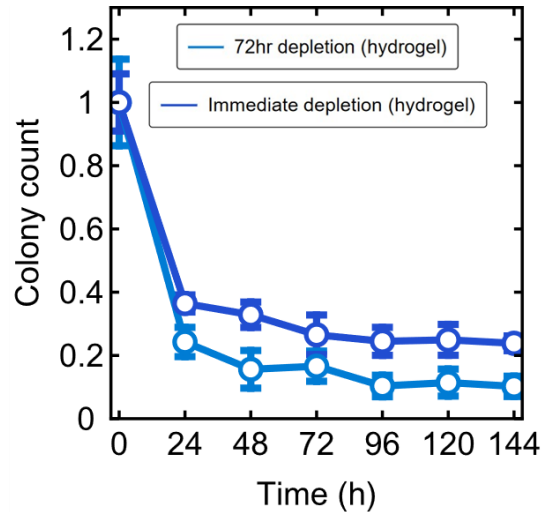
Supplemental Figure 3.3. A) Acetate concentration, measured with HPLC, in the aqueous effluent for both printed and self-assembled biofilms at each time point and flow velocity. Acetate is not depleted below approximately 16mM under any flow condition for the printed and self-assembled biofilms. B) Calibration curve plotting expected acetate concentration against each peak area HPLC measurement.



Supplemental Figure 3.4. A) Growth (OD_{600}) and mCherry signal (560/610 ex/em) of *P. fluorescens* in a 96-well plate with and without acetate, and a sterile minimal media negative control. For the (+)acetate condition, when optical density reaches steady-state, mCherry signal continues to accumulate over time. B) Figures from part A re-scaled and omitting the (+)acetate growth condition. While OD_{600} of the (-)acetate growth condition is indistinguishable from the sterile minimal media control, except for a slight increase at 24 which may be due to a small amount of acetate carry-over from the rinsed inoculum, mCherry signal is distinguishable from the control, though relatively small compared to the (+)acetate condition. C) CFU/mL counts of *P. fluorescens* on tryptic soy agar after exposure to two different resin treatments and a no-resin control. Cells were exposed to the full resin formula without the printer laser, or the PEG-DA monomer with the printer laser, for three minutes prior to 10^6 serial dilution and plating on agar. A slight increase in CFU mL^{-1} count was observed for the PEG-DA monomer + laser treatment. However, given that the PEG-DA monomers inhibit cell growth during longer exposure period (>12 hours), the observed increase in CFU mL^{-1} count is not likely due to monomer exposure improving cell growth over a shorter exposure period. D) Growth curve of planktonic *P. fluorescens* in 10mL minimal groundwater media under an aerobic headspace. The dashed gray line represents growth without sodium acetate in the media, while the solid black line represents growth with the standard 20mM sodium acetate in the media. Each point is the mean and standard deviation of three replicates. The *P. fluorescens* population does not grow in acetate-depleted media in a planktonic growth context and does not form liquid-air interface biofilms. E) Growth curve of planktonic *P. fluorescens* in 10mL minimal groundwater media under an aerobic headspace, but with 0.05 wt% tartrazine as the carbon substrate, and without sodium acetate. Each point is the mean and standard deviation of three replicates.



Supplemental Figure 3.5. A) Example images of the 3D printed hydrogel pre- and post-intensity increase. The two images are from the same dataset as figure 7 in the main text, but only the bottom quarter of the biofilms are utilized for biovolume analysis in this figure. Signal attenuation is clearly visible in the top portion of the 72-hour incubation image compared to the 96-hour image, where signal intensity massively increases after carbon-depleted media is introduced into the flow chamber. The white volume represents raw fluorescent signal, while the red volume is thresholded signal used for part B calculations. B) Average colony biovolume, colony count, and biovolume sum from the bottom quarter of two hydrogels: the hydrogel subjected to carbon depletion after 72 hours of standard incubation, and the hydrogel subjected to carbon depletion immediately. In contrast to figure 7 in the main text, where the entire depth of the biofilm is used for biovolume analysis, an increase in biovolume is not observed in the immediate depletion hydrogel when only the bottom quarter of the biofilm is analyzed. Biovolume analysis for the 72-hour depletion hydrogel still shows an increase in biovolume sum from 72 to 96 hours when only the bottom quarter is used for analysis, but to a much lower degree than when the entire biofilm depth is analyzed in figure 7.



Supplemental Figure 3.6. Average colony counts of hydrogel-encapsulated *P. fluorescens* for the 72-hour and immediate carbon depletion treatments. Colony count averages are not affected by acetate depletion in the minimal media. The black dashed lines divide the presumed pre- and post-carbon depletion time points. These counts represent the entire depth of the hydrogel in each field of view.

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CHAPTER FOUR

ENCAPSULATION IN A 3D PRINTED HYDROGEL MATRIX
HOMOGENIZES GROWTH PHASE DURATION AND
GROWTH RATE IN *PSEUDOMONAS PUTIDA* ISOLATES

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Co-Author: Heidi J. Smith

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- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

4.1. Abstract

Biofilms are physiologically and structurally complex microbial communities that are ubiquitous on Earth. As a consequence of three-dimensional structure, microorganisms develop emergent properties (*i.e.*, biofilm) not observed in populations of individual free-living cells, including antimicrobial tolerances, diversified evolutionary outcomes, and physiological heterogeneity. Making causal connections between biofilm structure and function relies partly on the ability to manipulate biofilm structure, but methods for finely controlling biofilm structure and physical context have been historically lacking. Microbial 3D bioprinting is an emerging technology where additive manufacturing can control the structural properties of synthetic, human-designed biofilms. Recent advances in microbial 3D bioprinting have centered around designing living materials with goal-directed functions, probing how initial biofilm structure affects microbial physiology, and fabricating *in vitro* mimics of complex, natural biofilms to probe the structure-function relationship (*i.e.*, applied function) in a controlled environment. However, the fundamental differences in physiology and population dynamics between self-assembled biofilms and synthetic 3D printed biofilms are not well understood nor are the impacts of controlled cellular growth within engineered biofilms. The physical constraints of a synthetic material matrix are typically vastly different from the context in which a microbial population was naturally selected to grow, and predicting the effects of synthetic matrix encapsulation on microbial behavior can inform rational design of synthetic biofilms for diverse purposes. Population-level growth rate is a fundamental descriptor of microbial behavior; therefore, growth rate differences were evaluated between closely related *Pseudomonas putida* isolates in traditional planktonic and biofilm growth modes as well as in 3D printed synthetic biofilms. We compared isolate growth rates within each

growth treatment and observed that encapsulation in the hydrogel matrix homogenized population growth rates, and the time elapsed to the beginning of stationary phase. In addition, variation was observed between the strains in self-assembled biofilms, yet synthetic biofilms appeared to dampen and stream-line the physiological responses across the stains. Future work is needed to determine if growth rate homogenization is a generalizable phenomenon among other closely related isolates, and whether physiological states in a microbial population coalesce in different hydrogel matrices under varying conditions.

4.2. Introduction

Natural selection has shaped microbial life to grow predominantly in the context of biofilm communities in many different environments and conditions ¹. Biofilms can develop complex three-dimensional structure where cells are attached to each other and/or to surfaces in which the community develops emergent properties due to the physical structure as well as the resultant behavior of individual cells responding to local heterogeneous environments at the micrometer scale ². Some emergent properties of biofilms include the social evolution of collective matrix resources ³, cell differentiation in the context of structure ⁴, antimicrobial resistance and tolerance due in combination to the extracellular matrix and altered growth ⁵, and population/community level phenotypes such as certain mechanisms of programmed cell lysis in response to nutrient conditions ⁶. Biofilm structure is interdependent with function, and vice versa. Consequently, understanding biofilm structure allows researchers to connect community functions such as metabolic niche development to specific physical conditions within or outside of a biofilm ⁷. For example, biofilm thickness can either permit or exclude anaerobic niches deep in a biofilm under

an aerobic atmosphere due to rapid oxygen consumption of aerobically respiring cells in an outer layer ⁸. Oxygen can be consumed by a dense, metabolically active population within 10s of micrometers, resulting in concentration gradients at very small scales ⁹.

Biofilm structure at sub-millimeter scales can influence larger-scale outcomes of microbial social interactions ¹⁰ and spatiotemporal population dynamics ¹¹. Understanding biofilm structure is an important part of fundamental research in biofilm function but also in the context of rationally designing microbial communities for biotechnology ¹². However, experimentally controlling biofilm structure, especially at small scales, is a historically challenging and elusive goal. Recent advancements in additive manufacturing and microfabrication have permitted small-scale control over biofilm structure, particularly through integrating existing 3D printing technology with biofilm research. Microbial 3D bioprinting is an emerging technology where 3D printers are either re-purposed from commercial applications or homebuilt to design and build synthetic biofilms ¹³. Typically, synthetic biofilms are designed with hydrogel properties using polymers such alginate ¹⁴, PEG ¹⁵, or agarose mixtures ¹⁶ as the scaffold, with microorganisms encapsulated in the matrix. 3D printed hydrogels allow growth substrates to diffuse into the matrix ^{17,18}, while having small pore sizes that trap the encapsulated organisms and retain intended structure while hydrated.

While still a nascent field, microbial 3D bioprinting has been applied to a diverse range of research questions in materials and biofilm science. One branch of the literature is focused on designing *in vitro* synthetic biofilms to mimic interesting and important characteristics of a natural biofilm. Wangpraseurt et. al. developed biomimetic *in vitro* coral habitats with 3D bioprinting that mimicked oxygen diffusion kinetics observed in natural corals at small scales, and encapsulated symbiotic algae in the materials ¹⁹. A small subset of microbial 3D bioprinting research is

concentrated on how initial spatial patterning of different genotypes influences competition, and Kumar et. al.²⁰ reported that spatial separation of colicin-producing and colicin-susceptible *E. coli* genotypes protects the susceptible cells from the colicin toxin. Finally, the bulk of the 3D bioprinting literature surrounds the design and fabrication of engineered living materials. Gantenbein et. al. bioprinted 3D lattices with a fungal bioink and demonstrated utility as a regenerative skin-like material with tunable behavior depending on the nutrient environment²¹. Despite recent advancements in 3D printing synthetic biofilms, it is generally unknown how encapsulation inside a hydrogel matrix alters microbial behavior and population dynamics compared to growth contexts where cells can freely attach, self-assemble, and disperse in response to changing conditions. Understanding population-level growth characteristics of cells inside of a 3D printed hydrogel matrix could inform the design of replicable engineered living materials and can help evaluate the success of a synthetic biofilm in modulating the advantages/disadvantages of corresponding naturally assembled bio-systems.

Population-level growth rate, while not the sole meaningful descriptor of microbial physiology given the complexities that emerge in stationary and starvation phases²², is still fundamental to understanding cells and populations in contexts ranging from a simple growth curve²³ to a natural community. We investigated how encapsulation in a 3D printed PEG-DA hydrogel matrix affects average growth rates of five closely related *Pseudomonas putida* strains isolated from similar topsoil environments and genetically transformed to express fluorescent reporters. We hypothesized that encapsulation in the hydrogel matrix would homogenize the range of growth rates exhibited by the isolates compared to traditional growth contexts, particularly in comparison to self-assembled PDMS flow cell biofilms. To investigate our hypothesis, we

characterized growth rate averages in a planktonic growth curve, a 96-well batch biofilm growth treatment, and in PDMS flow cells molded from PDMS and attached to a glass coverslip. We used a stereolithography printer to fabricate hydrogels with encapsulated *P. putida* and performed flow cell incubations while spatiotemporally measuring population dynamics with fluorescence microscopy. Encapsulation in the hydrogel matrix homogenized average growth rates compared to the other growth modalities, and in addition, normalized the elapsed time to stationary phase. Growth results indicate that the hydrogel matrix acts as a homogenizing environment on many fronts, and while the long-term physiological and evolutionary consequences of hydrogel matrix encapsulation on microbial populations are unknown, it is apparent that even very simple synthetic biofilms can behave in ways not predictable from planktonic or self-assembled biofilms.

4.3. Materials & Methods

4.3.1. *Pseudomonas putida* isolation, transformation, growth, and strain identification

Environmental *Pseudomonas spp.* were enriched from a variety of Montana and California topsoil samples using M9 Minimal Salts broth amended with benzoate and succinate. After 24 hours shaking at 30°C, subsamples were serially diluted and spread onto *Pseudomonas* Isolation Agar containing triclosan (most pseudomonads are resistant) and glycerol (most pseudomonads can use it as sole source of carbon and energy). Well-isolated colonies from the highest dilutions were serially streaked for isolation on PIA before DNA extraction, 16S rRNA PCR, and sequencing.

Five strains were chosen for their robust biofilms and electroporated with plasmid pBBR1k-con-GFPuv (Addgene). Later, the mCherry gene was PCR amplified from pMF440

(Mike Franklin, MSU) and cloned into pBBR1k-con after deleting the GFPuv gene. The five pseudomonads were electroporated with pBBR1k-con-mCherry for a total of ten fluorescent strains.

Partial 16S rRNA genes were amplified (E. coli positions 338 to 907) and sequenced by the Sanger method. Multiple sequence alignments were performed in arb-silva, initial tree construction in Wasabi, followed by final tree generation in treeview.

1X tryptic soy broth (TSB) was prepared by dissolving 15 g TSB powder (MP Biomedicals LLC) in 500 ml Milli-Q® water. Following sterilization, kanamycin was added to a final concentration of 50 $\mu\text{g ml}^{-1}$ (Sigma-Aldrich). Agar plates were prepared by supplementing TSB with agar (1.5% w v⁻¹) and filter sterilized kanamycin sulfate to a final concentration of at 50 mg ml⁻¹. All experiments were conducted in a defined minimal media recipe²⁸ under ambient atmosphere and room temperature (~21°C). Media was prepared by mixing the salt stock solution, mineral stock solution, sodium bicarbonate, and Milli-Q® water in a 1L beaker. The vitamin stock solution was added to media to a final dilution of 1X. Sodium acetate, monobasic sodium phosphate, and sodium nitrate were added to a final concentration of 20mM, 5mM and 0.1mM, respectively. Sodium acetate, sodium nitrate, and monobasic sodium phosphate (Sigma-Aldrich) stock solutions were prepared at 1M by dissolving into Milli-Q® water and filter sterilizing.

4.3.2. *P. putida* Isolate Storage and Growth

Isolates were stored in 2ml cryogenic vials (Corning) at -80°C in 1 ml 20% glycerol, prepared by mixing 500 μl cell culture grown in TSB to an OD₆₀₀ of 0.8 with 500 μl 40% sterile glycerol. A sterile inoculating loop was used to streak cells into TSB agar plates for inoculum growth. Agar plates were incubated in the dark at room temperature for 36-48 hours. To prepare

cells for printing experiments, a single colony of each isolate was inoculated into 5mL TSB in a sterile glass tube. Overnight TSB cultures were incubated at 30°C, 130 RPM, for approximately 24 hours, and then diluted 1:50 into 5mL minimal media in 20mL sterile glass tubes. The isolate cultures in minimal media were used as the inoculum for all subsequent experimentation and were diluted to an OD₆₀₀ of 0.5 if needed for inoculation of experimental treatments.

4.3.3. Preparation of Photoactive Resin/Cell Mixture

The photoinitiator, lithium phenyl-2,4,6-trimethyl-benzoylphosphinate (LAP) (Sigma Aldrich), was dissolved in room-temperature PBS to a final concentration of 0.1% (w/v). Poly(ethylene glycol) diacrylate (PEG-DA) oligomers (Sigma Aldrich PEG-DA Mn 700) were mixed with the LAP solution to an 11% v/v concentration in the resin mixture. The photoabsorber tartrazine (Alfa Aesar) was added into the resin at a final concentration of 0.05% (w/v) to achieve consistent print thickness. Photoabsorber concentration and light exposures were tuned based on desired cure depth ²⁴. The resin solution was filter sterilized and stored in the dark at room temperature before printing. Each resin solution was used for two weeks, then any remaining resin was discarded, and a new batch was prepared.

Prior to hydrogel printing, 900 µL resin was mixed with 100 µL *P. putida* isolate cultures to achieve a 10% (v/v) final PEG-DA concentration. Cells were suspended in the unpolymerized resin for no more than five minutes. The solution was gently vortexed and 500µl was spotted onto a sterile glass coverslip in the 3D printing chamber.

4.3.4. Growth Curves and Plate Reader Incubations

Growth curves (20mL glass tubes) were performed in triplicate in sterile minimal groundwater media under antibiotic selection. 5mL of media was used per replicate and inoculated with a 1:50 dilution of an overnight culture. In 96 well plates (Costar® 96 well, black with clear bottom), 200uL of minimal media per replicate was inoculated with a 1:50 dilution of an overnight culture. Optical density was measured at a wavelength of 600nm, and fluorescence was measured at an excitation/emission of 560/610 on a Biotek Synergy Neo2 plate reader. The 96-well plate was statically incubated in the dark at room temperature, and prior to measuring optical density and fluorescence, the plate was orbitally shaken in the plate reader for five minutes. For the first 24 hours of incubation, optical density and fluorescence were measured every two hours, and for subsequent time points, measured every 24 hours.

4.3.5. PDMS Flow Cell Assembly and Incubations

PDMS flow cells were fabricated by the Montana Microfabrication Facility. The flow channels were cast in PDMS bonded to a 1.5 glass coverslip with channel dimensions of 14 x 3 x 0.35mm. To prepare for biofilm flow cell incubations, approximately 10 inches of tubing (Scientific Commodities Inc. Micro Medical Tubing, #BB31695-PE/2) were first attached to the effluent openings by inserting a tubing end cut to a 30° angle. 15 µl of overnight culture grown in minimal media and diluted to an OD₆₀₀ of 0.1 were inoculated into the influent opening, with excess fluid forming a small bubble on top of the influent opening to prevent the channel from drying out during the 30-minute static inoculation phase. Isolates were each added to separate channels.

After static inoculation, approximately 16 inches of tubing connected to separate 3mL plastic BD syringes filled with minimal media were primed at 5 μ l/minute. The syringes were set in a syringe pump (WPI Aladdin AL-1000). While flow was running at 5 μ l/min, the influent tubing was attached to the influent channel openings through the inoculum bubble. To achieve a flow velocity of 1 mm/min in the PDMS flow channels, a dispense rate of 0.19 μ l/min was programmed on the syringe pump after the 30-minute static inoculation phase. The flow cells were attached flush to a microscope stage with double-sided tape for extra security.

4.3.6. Hydrogel Printing with Encapsulated Bacteria

Print geometries were designed with computer-aided design software (Autodesk, Fusion 360), exported as standard triangle language files, and uploaded to the OpenFL slicing program (Formlabs). The resin and cell mixture spotted onto the coverslip was mounted securely in the SLA (Form 1+, Formlabs) printer using a custom-printed PLA insert.

4.3.7. Bioprinting Flow Cell Assembly and Incubations

A BioSurface Technologies Transmission FC-81 AL flow cell was used for all bioprinting flow cell experiments. Flow cells were assembled with loosened screws and aluminum foil covering the inlet and outlet, then autoclaved on a fast cycle for 20 minutes at 121°C. Flow cells were disassembled in a biosafety cabinet, and the coverslip bounding the flow chamber was removed and taken to the SLA printer. Coverslips were taken back to the biosafety cabinet and, using a pair of sterile disposable tweezers, gently transferred to a PBS rinse solution in a 50mL Falcon tube where they remained for ten minutes. Coverslips were removed and immersed in 70% ethanol for fifteen seconds, then transferred to a fresh PBS solution for ten minutes. Finally, flow

cells were reassembled, and tubing (Masterflex Precision Pump Tubing, C-flex ULTRA, L/S, 16) was attached to the influent and effluent ends. The influent-end tubing was attached to a 50mL BD syringe and syringe pump (WPI Aladdin AL-1000). The syringe pump was calibrated to dispense at a rate of 30 μ L per minute. While imaging, flow was stopped, and flow cells were detached from the pumps and plugged for approximately one hour, after which flow cells were re-attached. During imaging, the syringe end was covered with flame-sterilized aluminum foil.

4.3.8. Confocal Laser Scanning Microscopy

PDMS flow cell experiments were imaged with an inverted confocal fluorescence microscope (Leica Stellaris 8) using the mCherry preset (587/610 ex/em). Laser power was set at 1% and gain was set at 2% with a *z*-step of 0.3 μ m and zoom factor of 4. The images in **Figure 4.3.** were set to a zoom factor of 5. At least 10 μ m of headspace above each biofilm was captured in the image stack, and the same locations across time were used for each replicate. Biovolume measurements are the mean and standard deviation of ten image stacks.

Bioprinting flow cell experiments were imaged with an upright confocal fluorescence microscope (Leica Stellaris 6) using the mCherry preset (587/610 ex/em). Laser power was set to 1% and gain was minimized. Flow cells were mounted on the stage with tubing plugged. Resolution for all images was set to 512x512 pixels with an acquisition speed of 600 and a *z*-step size of 1.8 μ m. For high-magnification images of colonies in the hydrogel matrix (**Figure 4.5.**), the optical zoom was set to 5 with a *z*-step size of 0.3 μ m.

For 2D images, a 10X dry objective lens (HC PL APO 10x/0.40 CS2) was used to image across the length of the hydrogel ranging from the influent to effluent edge. 3D image stacks of the entire hydrogel thickness utilized a 40X water immersion objective lens (HC APO L 40x/0.80

W U-V-I), corresponding to an *xy* field of view of 244 X 244 μm . Six image stacks were taken of the influent, middle, and effluent regions of the hydrogels, providing a total of 18 image stacks per timepoint for each isolate. Because flow cells were removed from the instrument after imaging each timepoint, different locations within the same region were imaged at each timepoint for the high magnification 3D images.

4.3.9. Intensity Analysis of 2D Images

Files containing influent, middle, and effluent images of the printed hydrogels were opened in ImageJ, and each individual image was saved as a .tif file. The .tif files were imported into BiofilmQ for thresholding and segmentation. A manual threshold was applied to every image and adjusted to remove background noise. A top-hat filter of 31 was selected according to software recommendation. Each image was segmented using the cube segmentation process, dividing fluorescent signal into pseudo-cells, with each cubic pseudo-cell treated as an individual point of analysis²⁵. A cube side length of 2 was chosen for each image, corresponding to a length of 0.13 μm .

Integrated fluorescence was calculated on a by-cube basis for each 2D biofilm image. Data were imported into the BiofilmQ visualization panel with the figure type set to 2D XY-scatterplot (color optional). Parameters were as follows: CentroidCoordinate_X was plotted on the X-axis, CentroidCoordinate_Y was plotted on the Y-axis, both with a range of 1:512 (i.e., the pixel resolution in the *xy* plane). Intensity_Integrated was selected for the color-code parameter. Heatmap figures were exported as MatLab image files, then converted to JPEG images with a MATLAB script. Plots of fluorescence intensity resolved by X position were made using the 1.5D histogram figure option. CentroidCoordinate_X was selected for the X-axis parameter with a range

of 1:512, and Intensity_Integrated for the Y-axis parameter with a variable range. Bin size was selected as the square root approximation of the number of intensity observations.

4.3.10. Biovolume Quantification of 3D Images

Eighteen 3D image stacks were acquired for every time point. Analysis of 3D image stacks was performed in Oxford Instruments Imaris. Image files were directly imported into Imaris, and a surface object was created to threshold fluorescent signal from background. Surface detail was set to 0.5 μm using the automatic absolute intensity thresholding setting for early timepoints and adjusted to 1.79 μm for later timepoints to more accurately segment the upper regions of the printed hydrogels.

For biofilms grown in PDMS flow cells, a center of homogeneous mass (Z-direction) filter was applied to exclude planktonic cells from the biovolume analysis. In addition, some *P. putida* isolate biofilms (P.nmb, P.sjm, P.aec) at later timepoints required adjustment of segmentation parameters: thresholding by local contrast with a threshold of 1.6-1.8 captured biovolume more accurately than the absolute intensity segmentation option. Spreadsheets containing surface object count, microcolony size, and field-of-view biovolume sum measurements were exported from Imaris. A Wolfram Mathematica script was used to calculate average and standard deviation colony biovolume, colony count, and biovolume sum versus time.

4.3.11. Statistical Analysis of Average Growth Rates

Statistical analysis of differences in growth rate averages between isolates within growth treatments was conducted using one-way ANOVA and a Tukey HSD post-hoc test at an alpha-level of 0.05 to evaluate pairwise growth rate differences. All ANOVA analyses were conducted using

statistical summary data (i.e., averages, standard deviations, and sample sizes) instead of growth rates for independent observations because growth rate data for each independent observation was not available for the 3D bioprinting treatments. Calculations of between- and within-group sums of squares, between- and within-group variances, F-statistics, p-values, and honest significant difference thresholds were performed in Excel. ANOVA tables are provided in **Supplemental tables 4.1.-4.5.**

4.4. Results

P. putida isolates were enriched from top-soil environments by incubation of the microbial community in M9 minimal salts amended with benzoate and succinate. To retrieve individual colonies from the community incubation stage, aliquots were serially diluted onto Pseudomonas Isolation Agar and the five strains investigated here (P.aec, P.hcn, P.sjm, P.nmb, P.mb) were selected for robust biofilm formation. PCR amplification and sequencing of their partial 16S rRNA gene revealed their species-level designations as *Pseudomonas putida* spp, which are common Gram-negative soil bacteria distributed ubiquitously. The isolates do not display obvious morphological differences between colonies on agar plates, though there is variability in pigmentation derived from mCherry expression (Figure 4.1. A).

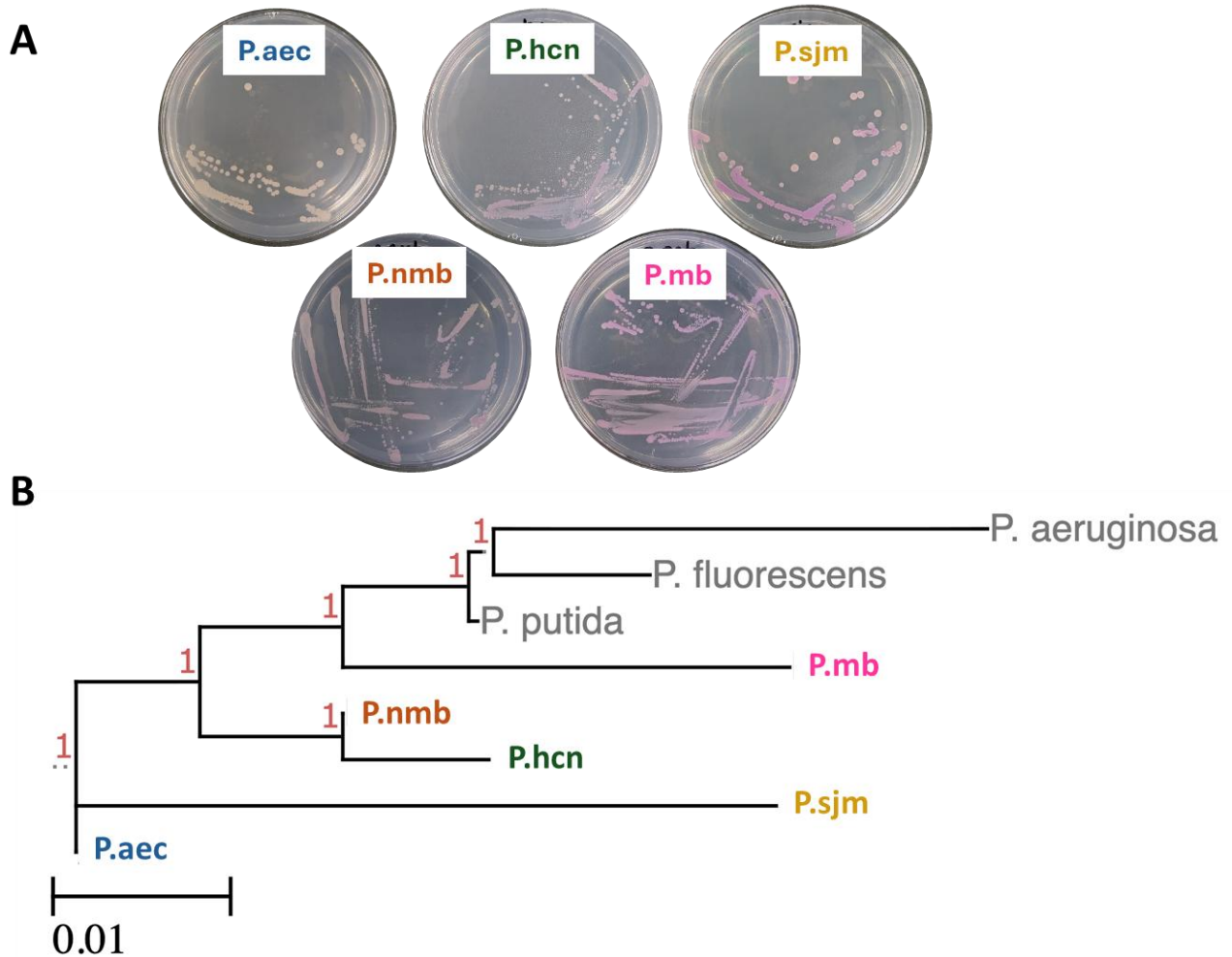


Figure 4.1. A) *P. putida* colonies on R2A agar plates. Though pigmentation differs from strain to strain, macroscopic colony morphology is consistent. B) Phylogenetic tree of the five *P. putida* isolates corresponding to P.mb, P.nmb, P.sjm, P.hcn, and P.aec related to *P. putida*, *P. fluorescens*, and *P. aeruginosa* reference strains constructed with partial 16S rRNA sequence data (see methods section 4.3.1.).

4.4.1. Growth Curves in Glass Tubes and 96-Well Plates Demonstrate Strain-Level Differences in Growth Rate

Basic growth characterizations of each isolate were first conducted with traditional methods. Each isolate was inoculated into minimal media in a 96-well plate, and growth was observed for a 24-hour timelapse, then up to 120 hours. Optical density at 600nm and fluorescence

were measured for each timepoint (**Figure 2a**). While the P.nmb population grew from a starting OD₆₀₀ of approximately 0.09 to an average of 0.17 within the first 24 hours, the other four isolates had a longer lag phase and did not show meaningful growth until the 48-hour measurement. Two isolates (P.hcn and P.sjm) entered stationary phase by the 96-hour measurement, while the others entered stationary phase by 48 hours. Final yields at 120 hours of growth ranged from an average of 0.14 (P.aec) to 0.36 (P.sjm). Fluorescence intensity of P.sjm and P.nmb diverged from the other three isolates beginning at hour 12 and remained elevated, reaching 6006.2 and 2999.8 A.U. at 120 hours, respectively. These results indicated that the five isolates had different planktonic and biofilm growth rates with the following groupings based upon planktonic growth (P.aec/P.hcn; P.sjm/P.nmb; and P.mb) (**Table 4.1., Figure 4.2.**). However, based upon self-assembled biofilms there were two groups (P.aec/P.nmb/P.mb and P.hcn/P.sjm) (**Table 4.1., Figure 4.4.**). Despite population-level growth rate differences, each isolate reached similar final yields within 120 hours, with a relatively small OD₆₀₀ range of 0.438 (P.nmb) to 0.542 (P.sjm) compared to 96-well plates at 120 hours of growth.

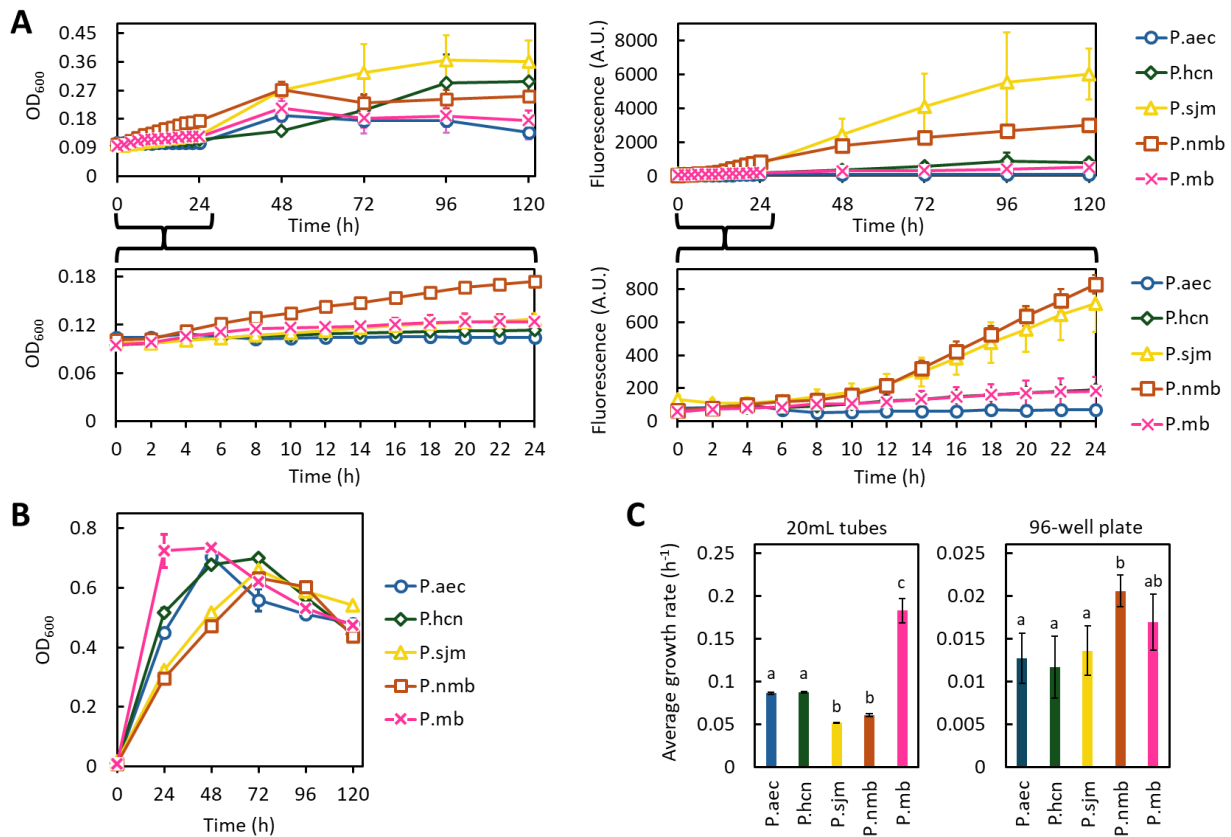


Figure 4.2. A) Optical density and mCherry fluorescence for all five isolates grown in 96-well plates. Fluorescence was measured at an excitation/emission of 560/610. The top figures display the full 120-hour time range, while the bottom show the 24-hour timelapse in more detail. Each point is the average and standard deviation of five independent replicates B) Growth curve for all five isolates in 20mL glass tubes. Each point is the average and standard deviation of three independent replicates. C) Average growth rate constants, based on optical density measurements, for each isolate in 20mL tubes and 96 well plates. The time intervals used to calculate the growth rate constants are listed in Table 4.1.

Population growth rate differences between isolates in the 20mL tube growth curve did not correlate with differences observed in the 96-well plate context. The lack of correlation is unsurprising given the differences in culture volume, gas exchange, and the opportunity for the isolates to form surface-associated biofilms in the 96-well plate. The P.nmb isolate had the highest growth rate average in the 96-well plate context. Among these growth curves conducted with

traditional planktonic and biofilm growth methods, the time interval at which populations transitioned into stationary phase ranged from 24 to 96 hours.

4.4.2. Closely Related *P. putida* Isolates Form Qualitatively Distinct Biofilm Architectures

Isolates formed very similar colonies on complex growth medium. However, when biofilms were grown under flow conditions in PDMS flow cells, differences between the strains were observed (**Figure 4.3.**). Some isolates (P.aec and P.nmb) have noticeable individual differences in cell size and morphology, while others (P.hcn, P.sjm, and P.mb) form more homogeneous biofilms with little qualitative distinction between the appearances of individual cells. Populations generally did not form confluent biofilms on the coverslip surface but did attain vertical structure between 5 and 35 μm thick, depending on the timepoint and isolate (**Figure 4.4.b.**).

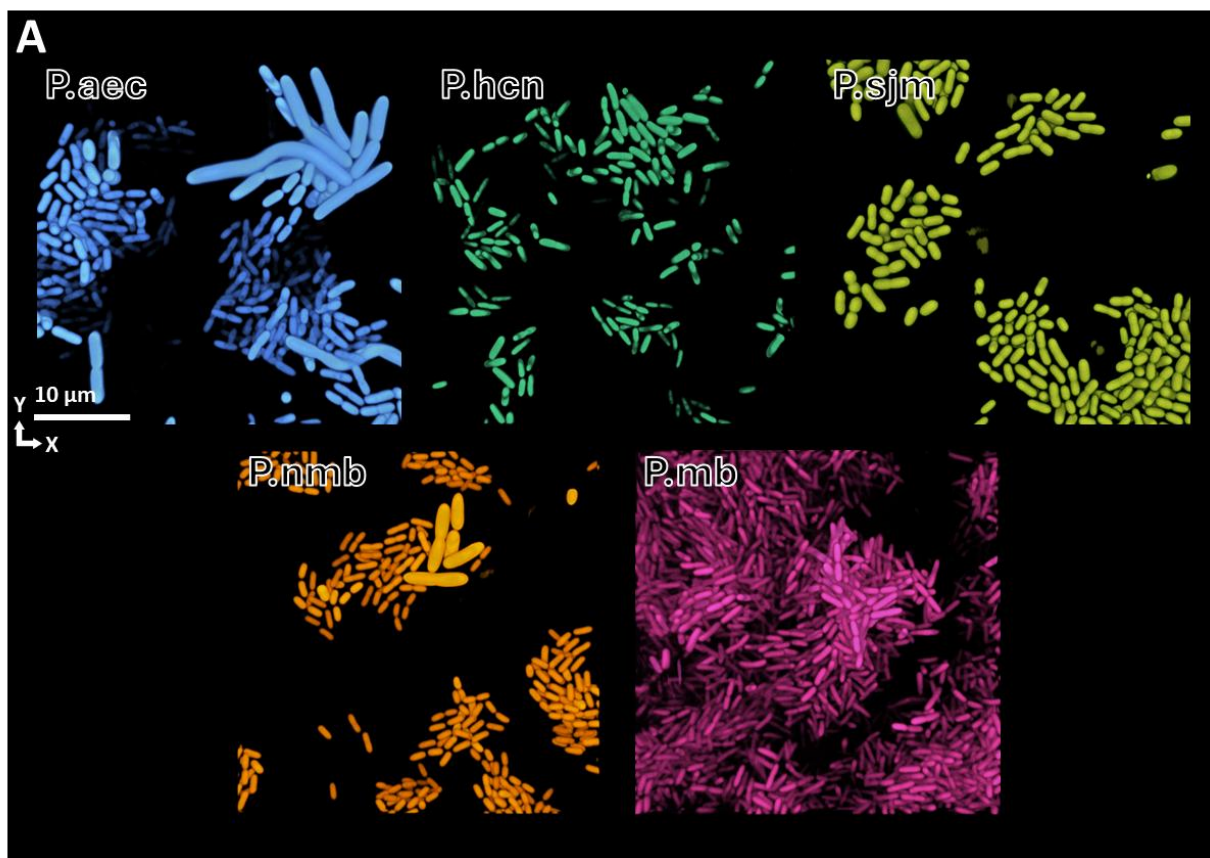
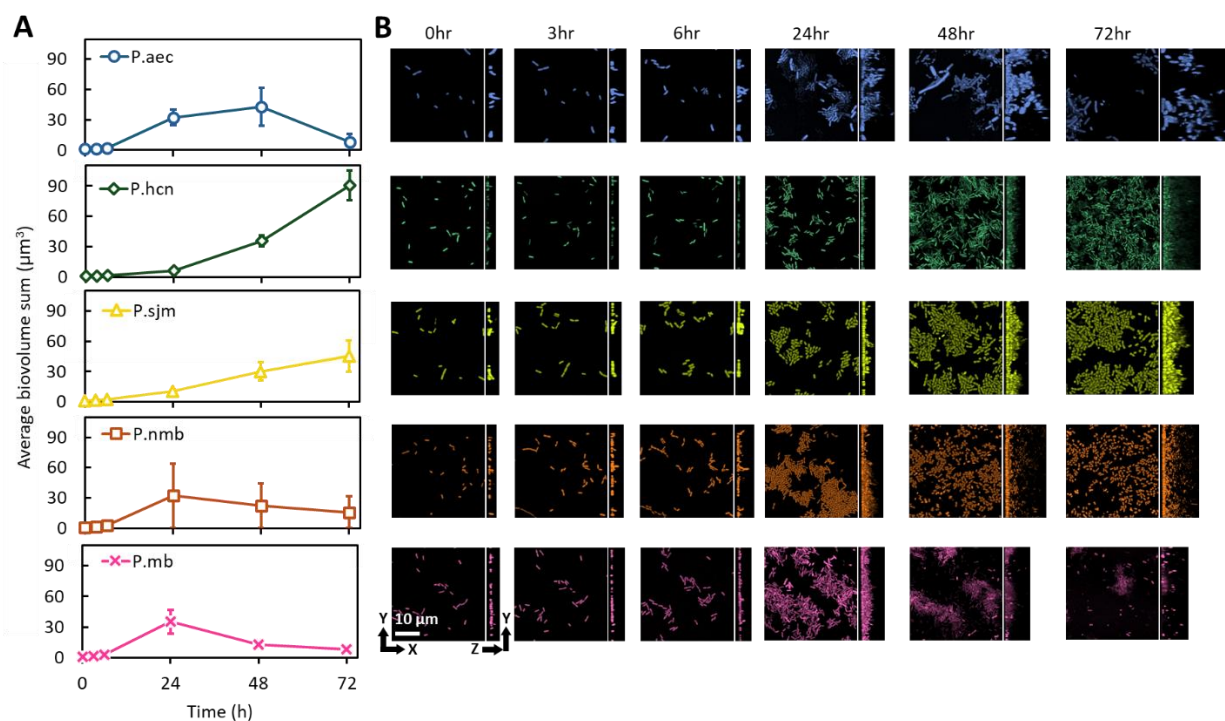


Figure 4.3. Fluorescence microscopy of *P. putida* isolate biofilms after 24 hours of growth in minimal media in PDMS flow cells. Images are false colored.

4.4.3. *P. putida* Isolate Biofilms have Distinct Growth Patterns in PDMS Flow Cells

The five *P. putida* isolates formed distinctive biofilms in PDMS flow cells. We defined isolate growth patterns in a continuous culture context and grew biofilms under the same minimal media conditions in PDMS flow cells with a flow velocity of 1mm/min. Biovolume in μm^3 was measured by thresholding fluorescent signal in 3D images and was used as the parameter for growth rate calculations. Each isolate attached to the coverslip at consistent densities after a static inoculation phase of 30 minutes, mostly existing in dispersed individual cells or small clusters for the first six hours of growth (**Figure 4.4.b**). By 24 hours, P.nmb and P.mb reached the respective

maximum biofilm-phase growth earlier and diminished at 48 and 72 hours (**Figure 4.4.a**). By contrast, *P.hcn* and *P.sjm* biofilms did not reach steady state biovolume by 72 hours (**Figure 4.4.a**). Isolate *P.aec* displayed intermediate characteristics, reaching a maximum biomass at 48 h and appreciably declining by 72 h (**Figure 4.4.a**).



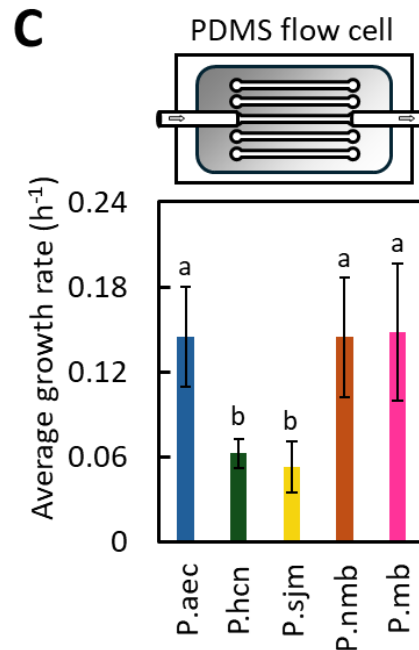


Figure 4.4. A) Average biovolume sum calculated from segmented images of each biofilm isolate in PDMS flow cells. Each data point is the average and standard deviation of ten fields of view. B) Representative *xy* and *zy* projections of each biofilm from 0-72 hours. C) Average growth rate constants, based on segmented fluorescent signal, for each biofilm isolate in PDMS flow cells. The time intervals used to calculate the growth rate constants are listed in Table 4.1.

Calculating and comparing average growth rate constants revealed two distinct rate clusters: P.aec, P.nmb, and P.mb all had average growth rates of 0.14 ± 0.035 - 0.048 h^{-1} in the flow cell biofilm context, while P.hcn and P.sjm achieved growth rates of 0.062 ± 0.01 and $0.053 \pm 0.02 \text{ h}^{-1}$, respectively (**Figure 4.4.c, Table 4.1.**). Qualitatively, most isolates formed dense, multilayer biofilms under $20 \mu\text{m}$ thick. Cells in the upper layers of P.nmb biofilms were loosely associated at 48 and 72 hours while the coverslip-attached layer remained densely packed, and the P.aec isolate formed tall but disperse structures of relatively large cells (**Figure 4.4.b**). Altogether, though average growth rates were on the same order of magnitude as growth rates observed in planktonic

batch culture (**Figure 4.2.c**), patterns of biofilm growth rate differences were not consistent with batch culture growth curves.

4.4.4. Growth variation is dampened when populations are encapsulated in the hydrogel matrix

Each isolate was 3D bioprinted as a PEG-DA synthetic biofilm inside of a BioSurface Technologies FC81-AL flow cell and population dynamics were observed with fluorescence microscopy. By thresholding fluorescent signal in each 3D image stack of the hydrogel, average colony biovolume (*i.e.*, colony size), average colony count, and average biovolume sum (*i.e.*, fluorescent signal in an entire field of view) was calculated over time and across all isolates (**Figure 4.5.a**). Initially, all isolates were dispersed as individual cells and grew inside of the hydrogel matrix with minimal media in the surrounding aqueous phase (as described in Chapter 3). Average colony biovolume and biovolume sum were stagnant for the first six hours for all isolates but increased between 6 and 24 hours, while colony count decreased during the same time interval. Generally, average colony biovolume and biovolume sum positively correlated. The isolate with the lowest average colony size also had the lowest biovolume sum (P.mb), and the isolate with highest average colony size had the highest biovolume sum (P.sjm) with the other isolates in-between, except for P.nmb which had high variability in colony size. Colony count at 72 hours ranged from a proportion of 0.29 to 0.73 of the initial totals (P.nmb and P.mb, respectively). Except for P.sjm which attained an average biovolume sum of $64.3 \mu\text{m}^3$, albeit with high variability, normalized biovolume sum ranged from $18.3 \mu\text{m}^3$ (P.mb) to $33.6 \mu\text{m}^3$ (P.nmb).

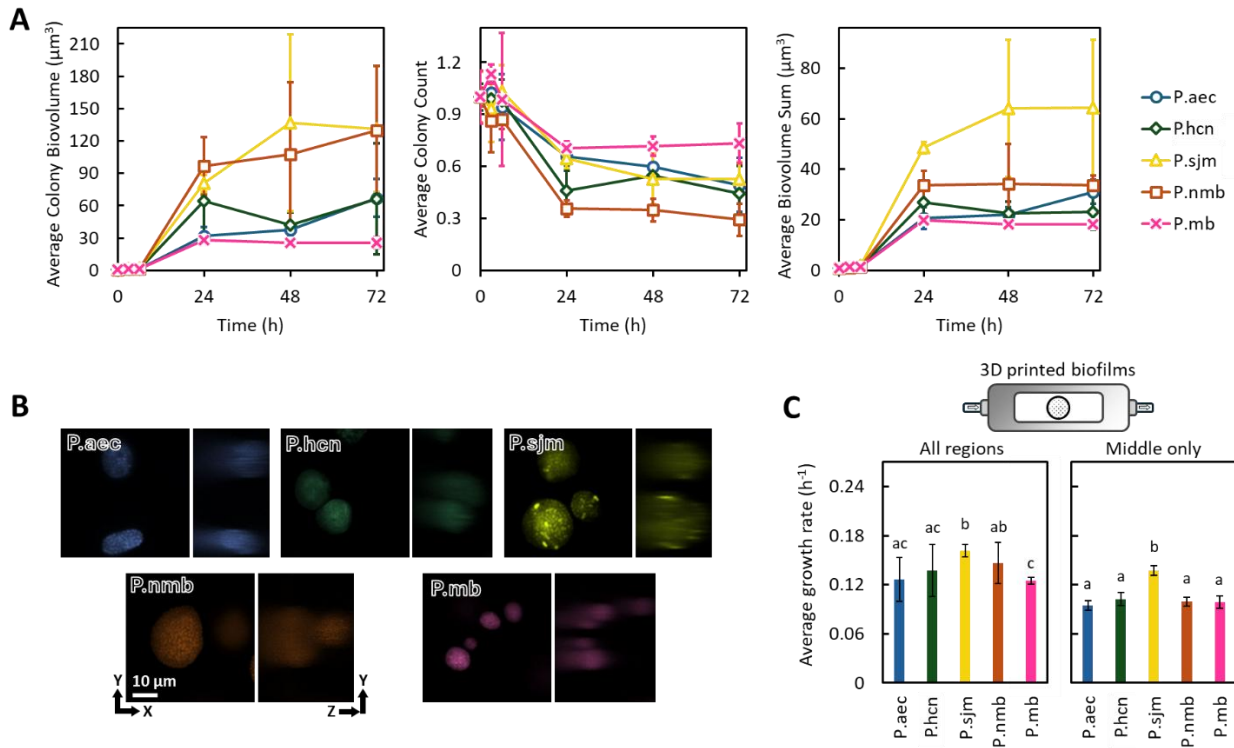


Figure 4.5. A) Average colony biovolume, average colony count, and average biovolume sum for each isolate normalized to each 0hr measurement. Biovolume and colony count measurements are calculated from segmented fluorescent signal throughout the entire synthetic biofilm depth. Each data point is the average and standard deviation of eighteen fields of view: six at the influent-facing edge, six at the middle of the hydrogel, and six at the effluent-facing edge. B) High-magnification images of *P. putida* colonies embedded in the hydrogel matrix. Generally, cells are tightly packed together without much observable difference in morphology, except for P.sjm where a sub-population of cells grow larger and partially exit the tightly packed colony. C) Average growth rate constants, based on average biovolume sum, for each isolate encapsulated in 3D printed hydrogels. The time intervals used to calculate the growth rate constants are listed in Table 4.1.

High-magnification imaging of colonies encapsulated in the hydrogel matrix revealed distinctions among isolates in colony appearance and shape (**Figure 4.5.b**). Most colonies were constrained by the hydrogel matrix to form near-circular shapes, but occasionally the P.aec isolate developed more oblong oval-shaped colonies which may have emerged from large, filamentous individual cells observed in self-assembled biofilms (**Figure 4.3**). P.sjm colonies developed higher

fluorescence intensity at a per-colony level, and distinctive morphological differences in individual cells that were not observed in the respective self-assembled biofilms (**Figure 4.3.**). A small subset of individual *P.sjm* cells were larger, brighter, and occasionally appeared to move through the hydrogel matrix. Calculating growth rate constants using biovolume sum changes from 0-24 hours reveals a homogenization of growth rates compared to previous methods, and homogenization of the elapsed time to stationary phase. Average growth rates of isolates at the field-of-view level ranged from $0.12 \text{ h}^{-1} \pm 0.004$ (*P.mb*) to $0.16 \text{ h}^{-1} \pm 0.007$ (*P.sjm*) (**Figure 4.5.c, Table 4.1.**).

Figure 4.5.a shows isolate population dynamics with all image stacks averaged together, collapsing information about potential growth differences between the influent edge region, the effluent edge region, and the middle of the hydrogel. In **Supplemental figure 4.1.**, average colony biovolume, count, and biovolume sum are separated by hydrogel matrix region. For every isolate except for *P.mb*, population growth at the influent edge region contributed most to total biovolume growth, though growth was observed in each region for all isolates. There were no meaningful biovolume differences at the colony or field-of-view level between the middle and effluent regions for any isolate. Colony count decreased between 0 and 24 hours for all isolates, and for most isolates, count was not dependent on location in the hydrogel matrix. The *P.nmb* population was an exception, and the average normalized colony count at 72 hours ranged from 0.29 (influent) to 0.61 (effluent).

4.4.5. Growth and Fluorescence Intensity are Spatially Heterogeneous for Most Isolates in the Hydrogel Matrix

In addition to high magnification 3D imaging of hydrogel encapsulated cells for each isolate, the influent, middle, and effluent regions were imaged in 2D at lower magnification to understand population dynamics at a larger scale. 2D images of each region were taken at 0 and

72 hours, and segmented while preserving fluorescence intensity to generate scatterplots correlating xy spatial position to integrated fluorescence intensity (**Supplemental Figure 4.2.**). Each population of cells is initially distributed homogeneously at the beginning of incubation, and intensity is relatively low. Intensity increases for each isolate in each region between 0 and 72 hours, though the influent-facing edge develops the clearest intensity hotspots, especially for the *P.sjm* isolate that formed bright biofilms between the bottom of the hydrogel and the glass coverslip due to a subset of cells escaping from the bottom of the matrix.

Intensity data in **Supplemental Figure 4.2.** were binned perpendicular to the direction of incoming flow and average fluorescence intensity was plotted versus x position for each bin (**Figure 4.6.**). All isolates except *P.aec* experienced an increase in fluorescence intensity at the influent region of the hydrogel within the first 150-200 μ m compared to the middle and effluent regions. Intensity increases at the influent edge with accompanying decay as x position moves farther away from the hydrogel-aqueous phase boundary, which is consistent with previous results of *Pseudomonas fluorescens* encapsulated in the hydrogel matrix at a fluid velocity of 1mm/min (Chapter 3).

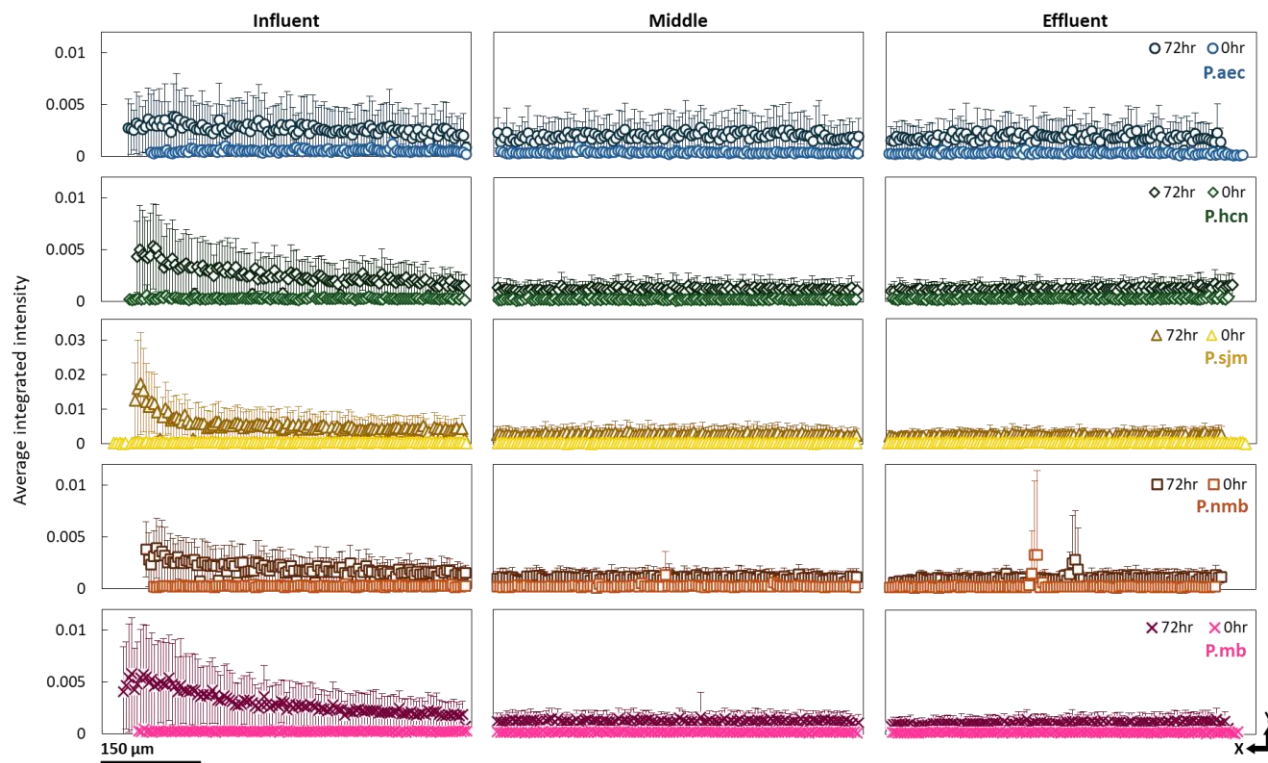


Figure 4.6. Integrated fluorescence intensity measurements from Supplemental Figure 4.2. binned according to x position, perpendicular to the direction of flow. Mean integrated intensity rose between 0 and 72 hours for each isolate at the influent region, though to different maxima depending on the isolate. Note the larger y -axis range for the *P.sjm* isolate, reflecting the ability of this population to grow between the hydrogel matrix and coverslip and develop a bright monolayer at the coverslip surface. The peaks in *P.nmb* intensity data at the effluent region are from a large, bright colony present in the field of view.

Table 4.1. Average growth rate constants and the time interval to stationary phase for each treatment and isolate.

	Average growth rate (h^{-1}), time interval				
	20mL tubes	96-well plate	Flow cell	3D printed biofilms (all regions)	3D printed biofilms (middle only)
P.aec	0.0861 $\pm$ 0.0014, 48h	0.0127 $\pm$ 0.0029, 48h	0.1450 $\pm$ 0.0354, 24h	0.1262 $\pm$ 0.0267, 24h	0.0948 $\pm$ 0.0061, 24h
P.hcn	0.0871 $\pm$ 0.0010, 48h	0.0117 $\pm$ 0.0036, 96h	0.0626 $\pm$ 0.0101, 72h	0.1374 $\pm$ 0.0321, 24h	0.1025 $\pm$ 0.0077, 24h
P.sjm	0.0517 $\pm$ 0.0001, 72h	0.0136 $\pm$ 0.0029, 96h	0.0530 $\pm$ 0.0181, 72h	0.1618 $\pm$ 0.0075, 24h	0.1375 $\pm$ 0.0058, 24h
P.nmb	0.0607 $\pm$ 0.0017, 72h	0.0206 $\pm$ 0.0019, 48h	0.1445 $\pm$ 0.0424, 24h	0.1465 $\pm$ 0.0252, 24h	0.0994 $\pm$ 0.0057, 24h
P.mb	0.1828 $\pm$ 0.0142, 24h	0.0169 $\pm$ 0.0033, 48h	0.1483 $\pm$ 0.0486, 24h	0.1247 $\pm$ 0.0042, 24h	0.0988 $\pm$ 0.0079, 24h
Average growth rate range	0.1311	0.0089	0.1430	0.0371	0.0427

4.5. Discussion

The growth rate of a given microbial population depends on intrinsic characteristics and environmental context, encompassing the chemical, physical, and otherwise selective conditions of each given cell. That said, individual cell growth rates are difficult to ascertain for any given environment, and reported values are typically an average of the given population. The traditional growth environments of glass tubes (planktonic), 96-well plates (planktonic/biofilms), and a straight-channel PDMS flow cell (biofilm only) produced variable between-treatment population growth rates and within-treatment pairwise growth rate differences in five different *Pseudomonas putida* populations isolated from topsoil. Emerging synthetic selective environments for biofilm growth, such as matrices fabricated with microbial 3D bioprinting, are likely to select for different growth behaviors compared to traditional planktonic and biofilm growth methods.

We hypothesized that encapsulating *P. putida* isolates in a 3D printed PEG-DA hydrogel matrix would homogenize average population-level growth rates between isolates compared to the other three growth methods. Diverging growth rates and outcomes were observed in every growth context, but apart from the P.sjm isolate, the hydrogel matrix produced the most similar between-population growth rates and a narrow range of growth rates compared to PDMS flow cells, the most similar treatment (**Table 4.1.**). One-way ANOVA analysis of growth rate differences observed within synthetic biofilms resulted in statistical clustering into three groups (**Figure 4.5.c**) when all hydrogel regions were included in growth rate calculations. However, most growth variability between isolates originated from growth at the influent-edge region that is exposed to shear from fluid velocity (**Supplemental Figure 4.1.**). When only the middle region of the hydrogel was analyzed, statistical growth rate differences clustered in two groups with only the P.sjm growth rate statistically different from the others. Higher growth rate variability at the influent region may be due to some populations responding more favorably than others to increased nutrient diffusion expected when shear forces diminish the hydrogel boundary layer^{26,27}.

The time elapsed to the beginning of stationary phase for each isolate ranged from 24-72 hours in planktonic growth curves (**Figure 4.2.b**), 48-96 hours in 96 well plates (**Figure 4.2.a**), and 24-72 hours in PDMS flow cells (**Figure 4.3.a**). However, when *P. putida* isolates were grown in 3D printed hydrogel matrices, stationary phase was reached by 24 hours for all isolates (**Figure 4.5.a**). Homogenization of between-isolate growth phase duration may be caused by homogenization of initial cell dispersion and the mass transport environment surrounding cells in the hydrogel matrix. Cells in the traditional growth contexts are not constrained by a synthetic matrix and can react to the immediate environment by moving and dispersing, forming liquid-air interface biofilms in

glass tubes (*i.e.*, pellicles), and forming surface-attached biofilms in 96-well plates and flow cells. Three of the five isolates (P.aec, P.nmb, and P.mb) partially dispersed in PDMS flow cells by 72 hours, but the same populations do not have the opportunity to disperse when encapsulated in the synthetic matrix and instead persist in stationary phase for up to 72 hours. By encapsulating cells in a hydrogel matrix, we potentially collapse the phenotypic diversity derived from cellular free movement within a given population and/or the ability to form self-assembled structured biofilms/aggregates. The hydrogel matrix scaffold also provides a consistent reaction-diffusion environment through the entire synthetic biofilm depth at initial growth stages, and at the initial cell densities chosen for experiments herein ²⁸. These attributes may contribute to the apparent normalization of niche space that can enable physiological heterogeneity.

Within growing biofilms (natural or synthetic), cells may be morphologically and/or metabolically constrained by the growth of self and/or nearest neighbors ²⁹. The initial morphology of cells in a nascent biofilm can influence down-stream biofilm architecture between and within populations ³⁰. In general, the morphological diversity observed in self-assembled *P. putida* biofilms was flattened upon hydrogel matrix encapsulation, (**Figure 4.3.**, **Figure 4.5.b**) and encapsulated cells cannot expand laterally towards each other to the same extent as on a glass coverslip. While the turgor pressure of a typical Gram-negative bacterium exceeds the PEG-DA hydrogel yield stress (around 10^5 Pa vs. 10^2 Pa, respectively) allowing cells to deform the matrix through growth, the hydrogel matrix still acts as a constraint on cell and colony morphology. Most of the hydrogel-encapsulated colonies from the *P. putida* isolates adopted a circular or near-circular shape, but a subset of P.aec colonies adopted a more oblong oval shape. We are not certain which

original morphologies gave rise to shape differences, but in self-assembled biofilms, *Paec* populations showed the most variability in cell size and shape.

At the individual cell level, qualitative differences in cell size and fluorescence intensity were observed for the hydrogel-encapsulated *P.sjm* isolate (**Figure 4.5.b**) that were not predictable from observation of self-assembled structure of the isolate. Physiological differences that may exist between the larger and smaller encapsulated *P.sjm* cells are unknown, as is why this isolate in particular developed individual morphological differences upon matrix encapsulation. It is interesting that self-assembled *P.sjm* biofilms with homogeneous cell shapes and sizes displayed qualitative variability in the hydrogel matrix (**Figure 4.5.b**). Culturing bacteria in a confined synthetic matrix has been shown to down-regulate the TCA cycle in *E. coli* and *S. aureus* ³¹ and a confined environment such as a hydrogel matrix can dictate 3D cell ordering within a colony ³². However, the relationships between growth rate, metabolic flux, and C/O ratios experienced by cells in synthetic or natural matrices undoubtedly play a role as well. Taken together, the mechanical constraint provided by the hydrogel matrix may select for expression of cell morphology not observed in a surface-attached, self-assembled context. Thus, synthetic film systems may provide an additional landscape to probe genotype-phenotype relationships of different microorganisms in which environmental gradients can be incorporated with standardized approaches.

In four of five *P. putida* isolates grown in the hydrogel matrix exposed to fluid velocity of 1mm/min, increased biovolume and fluorescence intensity were observed at the influent region. Separating colony biovolume, colony count, and biovolume sum measurements by spatial region revealed that for most isolates, the greatest contributors to growth were cells at the influent region

(**Supplemental Figure 4.1.**), and this result coincides with similar work with *Pseudomonas fluorescens* (Chapter 3). Regardless of location, the onset of stationary phase was consistently observed at 24 hours by biovolume sum analysis, and colony count decreased relative to the initial population for all isolates (**Figure 4.5.a**). The 3D printing process and/or hydrogel matrix encapsulation appear to act as a selection bottleneck, though the characteristics of this bottleneck, and why some cell colonies remain fluorescent and growing while others do not, are currently unknown. One possibility is that dispersed encapsulation in the hydrogel matrix is exposing cell-cell variability naturally present in bacterial populations³³ that is usually imperceptible at a single-cell level with bulk measurements. For each of the isolates, there may be a subset of each population that is not amenable to encapsulation in the hydrogel matrix due to intrinsic characteristics, such as variability in individual growth rates³⁴ or physiological status that renders some individual cells intolerant of encapsulation.

The accessibility and flexibility of 3D printing technology, combined with microbial physiology and ecology, has facilitated synthetic biofilm development for a great range of purposes³⁵. Still, there is little research concerning how encapsulation in a hydrogel matrix may change fundamentals of microbial population dynamics and behavior. The physical context of both synthetic and natural environments constrains potential structure, function, and growth dynamics of microbial biofilms. Dubbin et. al. demonstrated, for example, that hydrogel pore size effects population carrying capacity in a PEG-DA matrix¹⁵, but it is unknown whether this is a generalizable effect across populations. We identified and contextualized how encapsulation changes the population growth rates of closely related *P. putida* isolates, understanding that growth rate is just one of many parameters characterizing natural and synthetic biofilms. From the

perspective of 3D bioprinting for living material design, predicting spatial and temporal microbial growth in a synthetic hydrogel matrix can be important for reaching and maintaining optimal population sizes for the function of a given material. From the perspective of designing *in vitro* synthetic biofilms to mimic a natural counterpart, evaluating basic growth parameters like growth rate may be crucial for evaluating successful or unsuccessful mimicry of a natural biofilm, for example, in an antimicrobial testing context ³⁶.

Growth rate observations of hydrogel-encapsulated isolates suggest that the hydrogel matrix is a homogenizing environment on many different fronts, including that hydrogel encapsulation is a selection bottleneck in and of itself, the matrix likely homogenizes the nutrient environment around individual developing cells, and the matrix constrains morphological potential at the colony level. Future work understanding cell behavior in synthetic biofilms should integrate observations of population dynamics with deeper physiological characterization of encapsulated cells in multiple growth phases, different substrate types, varying conditions, and with multiple species. From the perspective of metabolism, microbial life has adapted to utilize a vast array of chemical species for anabolic and catabolic processes ³⁷, and there is little information about how cells respiring with electron donors and acceptors besides organic carbon and oxygen respond to hydrogel matrix encapsulation. Gathering -omic datasets from hydrogel-encapsulated cells and comparison to self-assembled biofilms could inform our understanding of how biology builds systems at the micro-scale, especially as a function of depth ³⁸. Finally, the long-term consequences of matrix encapsulation on microbial evolution are unknown, and integrating tools from experimental evolution with 3D bioprinting would open a vast array of fundamental biological questions in the context of synthetic biofilms.

4.6. Supplemental Tables

Supplemental table 4.1. One-way ANOVA summary statistics and Tukey pairwise HSD test for the glass tube growth curve treatment.

Glass tubes average growth rate ANOVA table							Group mean difference	HSD	Critical q-value	Statistical difference?
Total average group mean	SSQ between	df	MSB	F	p					
0.093687513	0.03269948	4	0.008175	196.0732	<0.0001	mb-nmb	0.122140364	0.016131	4.327	TRUE
						mb-sjm	0.131137613	0.016131	4.327	TRUE
						mb-hcm	0.095693034	0.016131	4.327	TRUE
						mb-aec	0.096763739	0.016131	4.327	TRUE
	SSQ within		MSE			nmb-sjm	0.008997249	0.016131	4.327	FALSE
	0.000416929	10	4.17E-05			nmb-hcm	0.026447330	0.016131	4.327	TRUE
						nmb-aec	0.025376625	0.016131	4.327	TRUE
	SSQ total					sjm-hcm	0.035444580	0.016131	4.327	TRUE
	0.033116409	14				sjm-aec	0.034373874	0.016131	4.327	TRUE
						hcm-aec	0.001070705	0.016131	4.327	FALSE

Supplemental table 4.2. One-way ANOVA summary statistics and Tukey pairwise HSD test for the 96-well plate treatment.

96-well plate average growth rate ANOVA table							Group mean difference	HSD	Critical q-value	Statistical difference?
Total average group mean	SSQ between	df	MSB	F	p					
0.015083891	0.000266659	4	6.67E-05	7.529142	0.0007	mb-nmb	0.003683324	0.005267	3.958	TRUE
						mb-sjm	0.003322325	0.005267	3.958	TRUE
						mb-hcm	0.005244145	0.005267	3.958	TRUE
						mb-aec	0.004218121	0.005267	3.958	TRUE
	SSQ within		MSE			nmb-sjm	0.007005649	0.005267	3.958	FALSE
	0.000177084	20	8.85E-06			nmb-hcm	0.008927469	0.005267	3.958	TRUE
						nmb-aec	0.007901445	0.005267	3.958	TRUE
	SSQ total					sjm-hcm	0.001921820	0.005267	3.958	TRUE
	0.000443743	24				sjm-aec	0.000895796	0.005267	3.958	TRUE
						hcm-aec	0.001026024	0.005267	3.958	FALSE

Supplemental table 4.3. One-way ANOVA summary statistics and Tukey pairwise HSD test for the PDMS flow cell treatment.

Capillary flow cells average growth rate ANOVA table							Group mean difference	HSD	Critical q-value	Statistical difference?
Total average group mean	SSQ between	df	MSB	F	p					
0.110663882	0.093676769	4	0.023419	20.02007	<0.0001	mb-nmb	0.003768771	0.040808	3.773	FALSE
						mb-sjm	0.095228092	0.040808	3.773	TRUE
						mb-hcm	0.085681626	0.040808	3.773	TRUE
						mb-aec	0.003299487	0.040808	3.773	FALSE
	SSQ within		MSE			nmb-sjm	0.091459321	0.040808	3.773	TRUE
	0.052640369	45	0.00117			nmb-hcm	0.081912855	0.040808	3.773	TRUE
						nmb-aec	0.000469284	0.040808	3.773	FALSE
	SSQ total					sjm-hcm	0.009546466	0.040808	3.773	FALSE
	0.146317138	49				sjm-aec	0.091928605	0.040808	3.773	TRUE
						hcm-aec	0.082382139	0.040808	3.773	TRUE

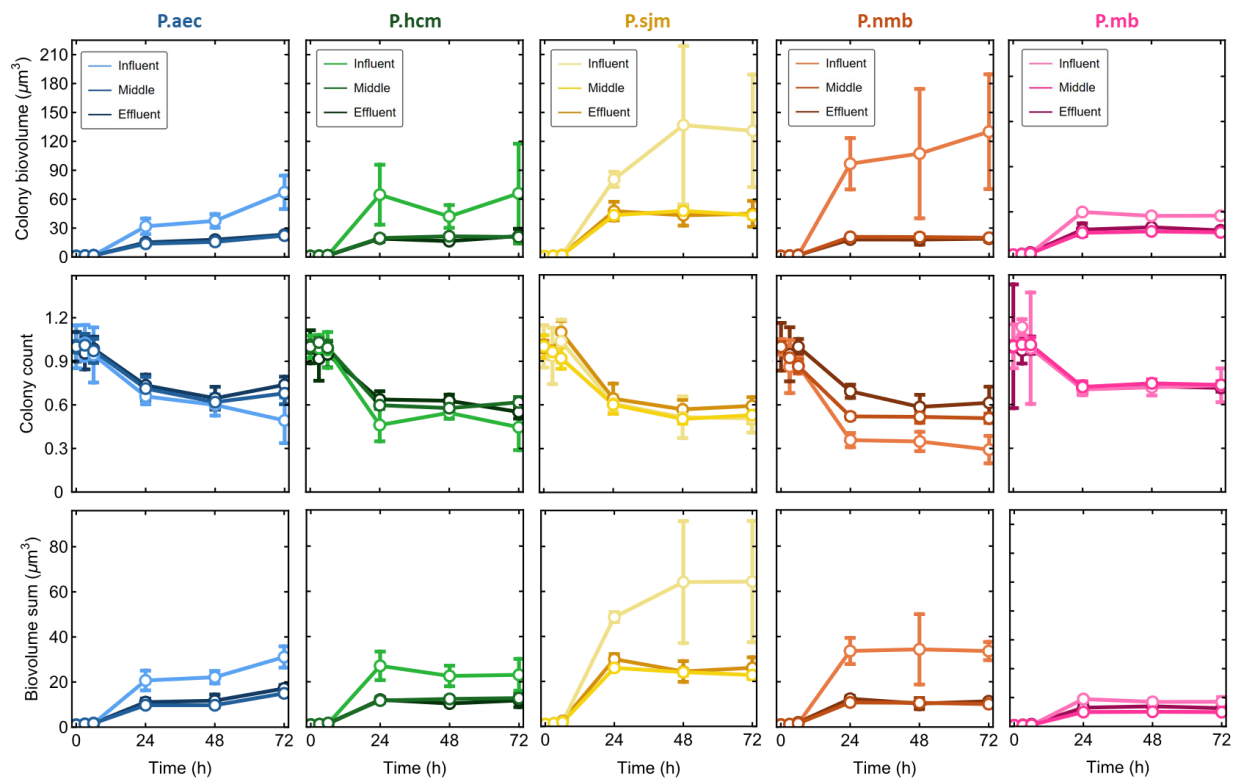
Supplemental table 4.4. One-way ANOVA summary statistics and Tukey pairwise HSD test for the bioprinting treatment with all spatial regions included.

Bioprinting (all regions) average growth rate ANOVA table							Group mean difference	HSD	Critical q- value	Statistical difference?
Total average group mean	SSQ between	df	MSB	F	p	mb-nmb	0.021811522	0.019330	3.706	TRUE
0.139308198	0.017022013	4	0.004256	8.689942	0.0001	mb-sjm	0.037113800	0.019330	3.706	TRUE
						mb-hcm	0.012723545	0.019330	3.706	FALSE
						mb-aec	0.001557087	0.019330	3.706	FALSE
	SSQ within		MSE			nmb-sjm	0.015302278	0.019330	3.706	FALSE
	0.041624878	85	0.00049			nmb-hcm	0.009087977	0.019330	3.706	FALSE
						nmb-aec	0.020254435	0.019330	3.706	TRUE
	SSQ total					sjm-hcm	0.024390255	0.019330	3.706	TRUE
	0.058646891	89				sjm-aec	0.035556713	0.019330	3.706	TRUE
						hcm-aec	0.011166457	0.019330	3.706	FALSE

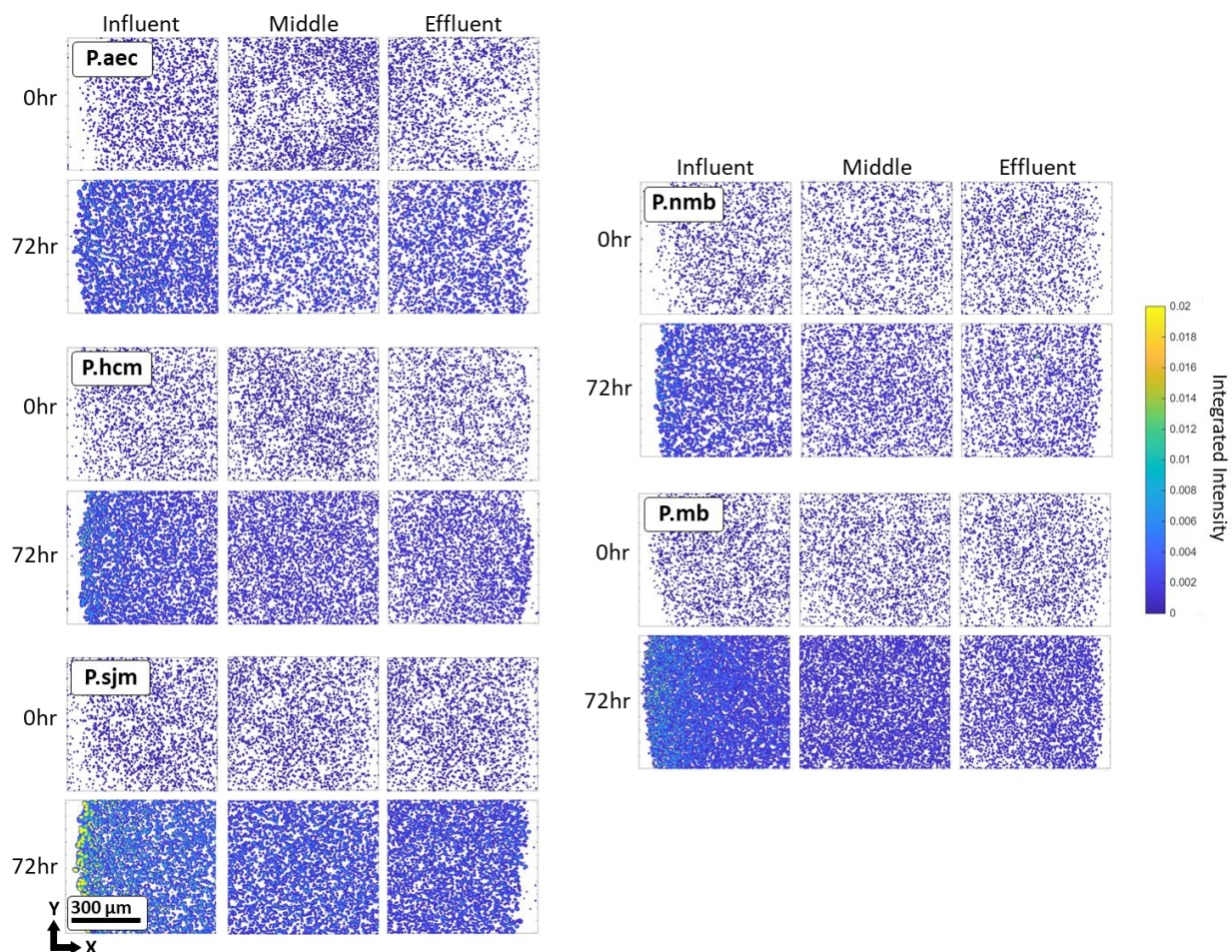
Supplemental table 4.5. One-way ANOVA summary statistics and Tukey pairwise HSD test for the bioprinting treatment with only the middle spatial region included.

Bioprinting (middle only) average growth rate ANOVA table							Group mean difference	HSD	Critical q- value	Statistical difference?
Total average group mean	SSQ between	df	MSB	F	p	mb-nmb	0.000566503	0.010629	3.890	FALSE
0.106575420	0.007341556	4	0.001835	40.9689	<0.0001	mb-sjm	0.038684359	0.010629	3.890	TRUE
						mb-hcm	0.003718691	0.010629	3.890	FALSE
						mb-aec	0.004029164	0.010629	3.890	FALSE
	SSQ within		MSE			nmb-sjm	0.038117856	0.010629	3.890	TRUE
	0.001119989	25	4.48E-05			nmb-hcm	0.003152188	0.010629	3.890	FALSE
						nmb-aec	0.004595667	0.010629	3.890	FALSE
	SSQ total					sjm-hcm	0.034965669	0.010629	3.890	TRUE
	0.008461545	29				sjm-aec	0.042713523	0.010629	3.890	TRUE
						hcm-aec	0.007747854	0.010629	3.890	FALSE

4.7. Supplemental Figures



Supplemental figure 4.1. Average colony biovolume, average colony count, and average biovolume sum calculated from normalized segmented biovolume but with the influent, middle, and effluent regions separated. Each point is the average and standard deviation of six image stacks.



Supplemental figure 4.2. Fluorescence intensity of hydrogel-encapsulated *P. putida* isolates at the influent, middle, and effluent positions. Raw 2D images at each position were imported into BiofilmQ and each image was manually thresholded. After thresholding, images were cube segmented in BiofilmQ, and integrated fluorescence intensity was calculated for each segmented object. Intensity is plotted for each xy position, generating a spatially resolved scatterplot fluorescence intensity. Each image spans approximately 1 millimeter. All scatterplot color scaling is plotted on the same intensity scale.

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CHAPTER FIVE

CONCLUSION

5.1. Synthetic Biofilm Population Dynamics are Distinct from Self-Assembled Biofilms

In this dissertation research, the synthetic 3D bioprinting design landscape was systematically explored by controlling internal (population density, strain variability) and external (fluid velocity, nutrient environment) variables and observing population-level responses of encapsulated cells. Population density was controlled over three orders of magnitude and while initial population density did not affect carrying capacity in the hydrogel matrix, initial population density did affect spatial biovolume distribution. Initial cell density, ϕ_0 , was related to the characteristic length scale of biovolume along the depth axis, λ , and a logarithmic relationship was observed as a heuristic predictor between these two variables, except for the lowest initial cell density. Within the tested cell density range, if a synthetic biofilm with a specific characteristic length scale of biovolume development is desired, it is feasible to vary cell density to achieve this length scale. This insight can be viewed as a tool for synthetic biofilm development, demonstrating the connection between a structural characteristic (initial cell density) and a developmental characteristic of a living material (spatiotemporal patterns of population growth).

With methods for growing *Pseudomonas fluorescens* established in the synthetic hydrogel matrix, the external environment of synthetic biofilms was explored using flow cell microscopy. Fluid velocity of incoming minimal media was systematically altered over three orders of magnitude within a laminar regime, and spatiotemporal population dynamics we quantified and

compared to a batch condition. While carrying capacity was not affected by initial cell density within the range tested, increasing fluid velocity increased carrying capacity in the 3D printed biofilms, predominantly at the influent region. Carrying capacity, measured by biovolume analysis, did not positively correlate with fluid velocity in self-assembled biofilms. When the nutrient environment was altered by depleting the minimal media of organic carbon, cells remained detectable in the hydrogel matrix (and in fact drastically increased in fluorescence intensity) while self-assembled *P. fluorescens* biofilms steadily diminished. Systematic exploration of the nutrient environment revealed diverging growth outcomes between synthetic and self-assembled biofilms.

Finally, responses of closely related *Pseudomonas putida* isolates to synthetic biofilm matrix encapsulation in a flow cell were evaluated and compared to population dynamics observed with traditional planktonic and biofilm growth methods. Population growth patterns of *P. putida* isolates showed that encapsulation in the hydrogel matrix caused the duration of growth phase to converge to 24 hours. Differences in growth phase duration may have emerged with greater time resolution, however, the consistency of growth phase duration was still remarkable given that every other growth context produced between-strain variability in growth phase duration. The range of growth rate averages in the 3D printed hydrogel matrix, when all spatial positions were analyzed and when only the middle region was analyzed, was narrower than the self-assembled biofilm growth rate range. Altogether, inter-isolate population dynamics and growth differences observed with traditional methods did not meaningfully predict population dynamics in the synthetic biofilm matrix.

Several other levers of synthetic biofilm control are available for exploration. For example, interactions between hydrogel matrix pore size (which is tunable by altering the molecular weight

of the PEG-DA monomers ¹). and fluid velocity would likely change observed population dynamics, especially if channels were built into the hydrogel matrix to accommodate advective fluid flow over a larger surface area. Altering hydrogel shape, especially thickness, would likely affect spatiotemporal growth of encapsulated cells and population-level responses to environmental perturbations. For example, a thick synthetic biofilm may develop sharp nutrient gradients reminiscent of natural biofilms compared to a thinner synthetic biofilm. Changing the matrix polymer would likely alter observed population dynamics as well, even if nutrient diffusion remained consistent. *Pseudomonas* populations did not appear to degrade the polymerized PEG-DA matrix over the timescales explored in this research, but pseudomonads are notorious for their metabolic flexibility in terms of organic carbon oxidation ². For example, the *P. fluorescens* strain used in this research grew planktonically with tartrazine as the only available carbon source at the concentration used for hydrogel printing. If natural polymers, such as alginate or agar, were chosen, the *P. fluorescens* and *P. putida* populations may have degraded the matrix and been less spatially constrained as a result. Spatial constraint appears to be a major mechanism by which hydrogel matrix causes growth convergence, and relaxed spatial constraints may have allowed more expression of strain-level differences in colony morphology and growth rate within the hydrogel matrix.

5.2. Population Dynamics Inform Synthetic Biofilm Research across Three Broad Literature Themes

Some observations of synthetic biofilm population dynamics were consistent between the six *Pseudomonas* strains utilized in this research. Among all hydrogels exposed to a 1mm/minute fluid velocity (some *P. fluorescens* synthetic biofilms in chapter 3, all *P. putida* synthetic biofilms

in chapter 4), the most population growth was observed at the influent region, and biovolume was lower in the middle and effluent regions. PEG-DA material properties likely would not change upon encapsulation of different isolates, so it is not surprising that applying fluid velocity would affect the hydrogel boundary layer consistently. Generally, except for the *P.aec* isolate, the cell colonies that developed in the hydrogel matrix adopted near-circular shapes as they pushed outward on the hydrogel matrix through population growth, regardless of whether hydrogels were incubated in batch or continuous culture. Finally, a remarkably consistent result between all synthetic biofilm growth treatments was a reduction in the number of colonies observed with fluorescence microscopy during the first 24 hours of incubation, even while the total biovolume increased on a per-colony basis and a field-of-view basis.

It is not known for certain what happens to the proportion of cells that lose fluorescent signal: cells could lyse or could remain intact but non-growing. The *P. putida* hydrogel incubations were performed under kanamycin selection and colony count still decreased, so the colony count decrease likely does not reflect loss of plasmid-based mCherry expression. Cell loss at early times, interpreted through the lens natural selection, suggests that hydrogel matrix encapsulation acts as a selection bottleneck for a proportion of each population. It is striking that, again, the colony count decrease occurs within the first 24 hours of incubation and generally reaches steady state at longer times, up to six days. Even in the most homogenized scenario of a well-mixed bacterial culture that is not nutrient limited, physiological cell-to-cell variability is always present for natural selection to act on, and there are most likely physiological underpinnings to which cells tolerate hydrogel encapsulation, and which do not.

While growth rate variability in *P. putida* synthetic biofilms was narrowed compared to self-assembled biofilms, the five *P. putida* isolates still reached variable steady-state biovolume in the hydrogel matrix. In addition, biovolume sum variability and colony size were substantially greater for the P.sjm isolate by 72 hours of incubation. Biovolume variability may be related to the ability of P.sjm to escape the hydrogel matrix – cells initially distributed near the hydrogel-coverslip boundary were able to grow out of the matrix and form a biofilm layer between the hydrogel and coverslip. Though growth external to the hydrogel matrix was not included in biovolume analyses, there may be a physiological reason why P.sjm is less constrained by the hydrogel matrix than the other four isolates. In addition, hydrogel matrix encapsulation produced size and fluorescence intensity variability in individual P.sjm cells that was not observed in P.sjm self-assembled biofilms.

Detailed analysis of spatiotemporal population dynamics can inform building synthetic biofilms for *in vitro* replication of natural, self-assembled biofilms. Biofilms can form deadly infections on parts of the human body, like in the respiratory systems of cystic fibrosis patients ³, and on foreign material interfaces such as joint prostheses ⁴ or heart valves ⁵. *In vitro* models of biofilm infections that successfully capture important structural characteristics of an infectious biofilm, and the physical context(s) in which biofilms are formed, are sorely needed, as are mechanisms to assess whether *in vitro* replication of a natural biofilm is successful. While biofilms can hold vast physiological heterogeneity, and at any given time a substantial portion of a biofilm is likely not engaged in active growth, population-level growth metrics (like growth rate and growth phase duration) are still powerful descriptors of microbial behavior in complex environments ^{6,7}. Thus, growth rate of an encapsulated population, or a population seeded on/near

the matrix but interacting with an encapsulated population, may be one heuristic (among many) to evaluate the similarity or dissimilarity of a 3D printed synthetic biofilm to a self-assembled counterpart. As microbial 3D bioprinting research advances, it will be advantageous to understand and predict the growth of encapsulated communities in various hydrogel matrices, not just single populations.

From the perspective of fundamental behavior/sociomicrobiology research, controlling the small-scale spatial structure of biofilms can provide tremendous utility for hypothesis testing of ecological and evolutionary theory. For example, the ability to spatially pattern microbial genotypes with observed or predicted mutualisms could be used to evaluate how spatial mixing impacts community fitness, *i.e.*, the extent to which mutualism improves fitness. While reticulate evolution weakens the tractability of vertical inheritance among microorganisms, particularly asexually reproducing microorganisms, controlling the spatial structure of mixed populations can help contextualize the importance of initial structure and/or disturbance regime on the evolution of selfish or altruistic social phenotypes^{8,9}. Additionally, natural selection within the physical constraints of a synthetic matrix may shape the long-term evolution of encapsulated populations. Phenotypic diversification¹⁰ and parallel adaptation¹¹ can occur over brief timescales when a microbial population is serially passaged in a biofilm growth mode¹², and whether this holds true in synthetic biofilms is unknown.

Finally, fabrication of engineered living materials with 3D bioprinting can be substantially benefited by further definition of the synthetic biofilm design landscape and how structural variables of synthetic biofilms influence one another. For example, it is likely that there are additional predictive relationships like the initial cell density/characteristic length scale

relationship described in chapter 2. Characterizing nutrient heterogeneity in a synthetic biofilm as a function of shape, thickness, and matrix polymer could inform functional capabilities of living materials. Understanding population dynamics in an engineered living material while perturbing structural variables could provide insight into attaining optimal growth conditions for a material function ¹³ , and into how long a living material could perform a function such as logic gate computation ¹⁴ or synthesis of valuable chemicals ¹⁵.

5.3. Unanswered Questions and Future Research Opportunities

The opportunities for future synthetic biofilm design and fundamental research are vast. Individuals in microbial populations are physiologically heterogeneous on many fronts, including doubling time/growth rate ¹⁶ and mutation frequency ^{17,18} even in a homogeneous environment. Physiological variability is generally magnified in a biofilm context compared to individual cells, especially over evolutionary time ¹⁹, and synthetic biofilms could be used as a tool to reveal this variability. For example, the underlying variability between individuals that proliferate in the hydrogel matrix, and individuals that do not, could be interrogated with population-level ‘omics analyses. The influence of hydrogel carrying capacity on the balance between neutral evolutionary forces and selection could also be investigated. The long-range effects of hydrogel matrix encapsulation could be explored with experimental evolution to understand the fundamental impacts of matrix encapsulation on cell morphology and physiology across generations of serial passage.

Analysis at the level of genes and gene expression could also be used to further evaluate the similarity and dissimilarity between synthetic and self-assembled biofilms. From the

perspective of biofilm spatial structure, populations can physiologically diversify²⁰ and display differential gene expression within tens of micrometers²¹. Differential gene expression can be associated with an eventual division of labor within a microbial population²², and the conditions that might produce differential gene expression/labor division in synthetic biofilms are unknown. Given that hydrogel matrix encapsulation produced different population growth patterns to self-assembled biofilms under the same bulk nutrient flux and fluid velocity conditions, and the movement (*i.e.*, dispersal and migration) of hydrogel-encapsulated cells is greatly restricted, it is not clear how physiological diversification would play out under the constraints of a synthetic matrix. It is well known that biofilms can form internal nutrient gradients that enable and restrict the development of ecological niche space²³. A synthetic matrix itself can act as a selective constraint that is vastly different from the context in which a biofilm was naturally selected to grow and therefore result in different localized nutrient conditions. However, there are natural environments where growing cell colonies are physically constrained in a similar manner to a synthetic hydrogel matrix²⁴, such as colonies embedded in the tissue of larger organism. 3D bioprinting could be especially effective at designing *in vitro* synthetic biofilms to study naturally encapsulated microbial communities in a controlled setting.

Variables that were systematically altered in the research described here can be explored further. While strain-level growth variability in the hydrogel matrix was investigated among *P. putida*, multispecies synthetic biofilms were not investigated. Multispecies biofilms have greater functional potential for living material development than single population biofilms, and it is important to characterize the growth of microorganisms in synthetic biofilms that do not utilize oxygen and organic carbon for respiration to tap further into the vast metabolic potential of

microbial life. Additionally, while fluid velocity was altered over several orders of magnitude in chapter 3, all fluid velocities were well within a laminar flow regime. Chaotic turbulent flow, compared to laminar flow, can select for structural and population density differences in biofilms²⁵, cause biofilm sloughing²⁶, and can expose biofilms to a wide range of potential fluid velocities and shear forces²⁷. Applications of synthetic biofilms in natural environments, such as pollutant remediation²⁸, may expose synthetic biofilms to turbulent flow, and it is unclear how matrix-encapsulated populations would respond to turbulence compared to self-assembled biofilms.

All synthetic biofilms explored in this research were fabricated and incubated at the interface of a solid (microscope cover glass) and a liquid (minimal media). While the physical context of synthetic biofilm incubations in chapter 2 contained a liquid air interface, the synthetic biofilms were not directly exposed to air. It is an inherent constraint of 3D hydrogel printing that the synthetic biofilms must remain hydrated to retain their intended structure. Thus, there are many natural biofilms that cannot be adequately modeled by the experimental methods developed herein. Image analysis methods can be pushed further to deepen understanding of population dynamics in synthetic biofilms: emerging methods for single-cell segmentation could facilitate additional analysis of structure and population dynamics at the level of single cells within matrix-encapsulated colonies^{29,30}.

There are vast and fascinating unanswered questions pertaining to the behavior and physiology of synthetic biofilms that will fuel discovery for years to come. The work in this dissertation covers only a narrow breadth of such questions, but it is the sincere hope of the author that the insights into synthetic biofilm population dynamics provided here will aid the research efforts of others.

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