A 3-D Computational Model to Investigate the Role of Small Colony Variants (SCVs) in the Antibiotic Susceptibility of a Polymicrobial Biofilm

Nikhita Joy

A Dissertation Submitted to

Indian Institute of Technology Hyderabad

In Partial Fulfillment of the Requirements for

The Degree of Master of Technology

भारतीय प्रौद्योगिकी संस्थान हैदराबाद

Indian Institute of Technology Hyderabad

Department of Chemical Engineering

June, 2016

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CH14MTECH11011

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Dr. Phanindra Jampana
Examiner

Dr. Lopamudra Giri
Examiner

Dr. Parag D. Pawar
Adviser

Dr. Basant Kumar Patel
Chairman

Acknowledgements

Foremost, I take this opportunity to express my profound gratitude and sincere regards to my advisor, Dr. Parag D. Pawar, for his exemplary guidance, motivation, supervision and constant encouragement in the successful completion of this thesis work.

I would like to thank my committee members, Dr. Basant Kumar Patel, Dr. Phanindra Jampana and Dr. Lopamudra Giri for their insightful remarks and valuable advices during the discussions of progress of project work.

I am grateful to my parents who always encouraged and supported me.

My special thanks to Mayur Gaikwad, Virendra Nerune, Mrunalini Gaydhane and Lakshmi Machineni for their moral support, cooperation and guidance in one way or the other during my research work.

Abstract

Biofilm formation has been demonstrated for several pathogens and is evidently a significant microbial survival strategy. They have immense clinical relevance for their role in various infectious diseases and a range of device-related infections because of their increased tolerance to antibiotics. Intra- and inter-species interactions play an important role in the population dynamics and distribution of species within the biofilm community, thereby altering the course of infections and response to antimicrobial therapy. For instance, *P. aeruginosa* in the cystic fibrosis airway produces an exoproduct, 4-Hydroxy-2-heptylquinoline N-oxide (HQNO), that causes wild-type cells of *S. aureus* to phenotypically switch to small colony variants (SCVs). SCVs are problematic in chronic infections; even though they comprise a minor proportion of the population, they persist by virtue of their inherent resilience and host adaptability.

A comprehensive 3-D computational cellular automaton model of biofilm dynamics was developed to investigate the role of SCVs in the antibiotic tolerance of polymicrobial biofilms. This model integrates the following processes: nutrient transport and utilization, biomass growth, division, death and detachment, quorum sensing, phenotypic switching, EPS production, and antimicrobial therapy. Biofilm growth dynamics were explored for two cases: (i) in the absence and (ii) presence of SCVs. A single growth-limiting nutrient was taken into consideration whose concentration was taken to be 4 g/m^3. To inspect the recalcitrance in response to antibiotic-dependent killing, the biofilm was subjected to a range of antibiotic concentrations (7, 9, 11, 13, 15 g/m^3). The antimicrobial treatment was initiated 12 hours prior to the maxima of *P. aerginosa* (slower-growing species) population dynamics curve and was continued for 12 hours. The model predicted the following features: treating a biofilm with antibiotic concentration less than a specific threshold extends its lifetime, external stress can influence interspecific competition, production of SCVs is potentiated in response to antibiotic treatment, and stratified patterns of survival.

These studies will possibly give some insight and provide data for the refinement of existing treatment strategies and/or to develop novel effective therapies for biofilm infections.

Nomenclature

μmax	Maximum specific growth rate
m	Maintenance coefficient
Y_{BN}	Yield of biomass from nutrient
Y_{EN}	Yield of EPS from nutrient
Y_{HQNO}	Yield of HQNO from nutrient
$C_{N,bulk}$	Bulk nutrient concentration
$C_{Ab,bulk}$	Bulk antibiotic concentration
D_i	Diffusivity of component ‘i’ in biofilm
$D_{i,aq}$	Diffusion rate of ‘i’ in aqueous phase
$D_{i,e}/D_{i,aq}$	Relative effective diffusivity
K_i	Half-saturation coefficient
v	Local fluid velocity
α	Conversion rate for up-regulation (QS)
β	Spontaneous down-regulation rate (QS)
γ	Transition constant (QS)
α'	Conversion rate for SCV-switching (PS)
β'	Spontaneous WT-switching rate (PS)
γ'	Transition constant (PS)
$r_{A,u}$	Autoinducer production rate by up-regulated cells
$r_{A,d}$	Autoinducer production rate by down-regulated cells
$r_{HQNO,u}$	HQNO production rate by up-regulated cells
$r_{HQNO,d}$	HQNO production rate by down-regulated cells
$K_{B,max}$	Maximum specific reaction rate of anitibiotic with respect to biomass
$K_{E,max}$	Maximum specific reaction rate of anitibiotic with respect to EPS
$m_{OneCell}$	Mass of one cell
$C_{OneCell}$	Concentration of one cell
C_{BIC}	Bacterial inhibitory concentration
C_{max}	Concentration corresponding to maximum consumption rate
Δt	Time step used for CA
Δx	Element size

Contents

Chapter 1

Introduction

1.1 Overview

Antonie van Leeuwenhoek, who discovered microbial attachment to his own tooth surface, is credited with the discovery of biofilm. But the theory describing the process was not developed until 1978 [1]. A biofilm is any aggregate of microorganisms in which cells adhere to each other and/or to a surface. They may form on solid surfaces (living or non-living) in contact with moisture or at air-fluid interfaces and can be prevalent in natural, industrial and hospital settings [2][3]. These cells are quite often embedded in a self-produced matrix of extracellular polymeric substances, EPS, which are high-molecular weight compounds (generally composed of proteins, extracellular DNA, polysaccharides, etc) secreted by microorganisms into their environment. They acts as "intercellular cement" [4], establishing the functional & structural integrity of biofilms. They constitute 50-90% of a biofilm's total organic matter [5-7].

1.1.1 Biofilm vs. Planktonic Organisms

Microbial cells in a biofilm are physiologically distinct from their planktonic counterparts by reduced growth rate, up- and/or down- regulation of certain operating genes, etc.[6] Estimates vary, but it has been suggested by some that as much as 40% of the genes of a bacterium may undergo up- or down- regulation in the transition from planktonic to biofilm state [2,8,9]. So, even though genetically they are the same, expression of some of the genes has undergone a dramatic shift and it is almost as if the two states (planktonic and biofilm) were entirely different organisms.

Growth within biofilms offers protection from a wide variety of environmental challenges like acidic pH, metal toxicity, antibiotics etc [2] and shields against host immune responses

(even though phagocytes penetrate biofilms via fluid-filled channels, they fail to access bacteria encased within the EPS matrix) [10].

1.1.2 Clinical Relevance of Biofilms

Infectious processes in which biofilms play a role include catheter infections, urinary tract infections, middle-ear infections, formation of dental plaque, endocarditis, bacterial vaginosis, coating contact lenses, infections in cystic fibrosis, infections of permanent indwelling devices such as joint prostheses and heart valves etc.

Despite the sophistication, microbial infections can develop on all medical devices and tissue engineering constructs. 60-70% of hospital acquired infections are associated with the implantation of a biomedical device [11].

1.1.3 Antibiotic Tolerance of Biofilms

It is often said that new antibiotics are needed to combat drug-resistant pathogens. But we, in fact, never had antibiotics capable of eliminating an infection completely; therefore this is only a part of the requirement [12].

The antimicrobial drugs that are traditionally developed are designed and tested to eradicate planktonic bacteria under the assumption that they would kill the same bacteria wherever they are found. However, we now know that, planktonic bacteria are more susceptible to those agents when compared to their biofilm brethren. Also, it has been found that biofilms are involved in a number of microbial infections in the body, by one estimate 80% of all infections [13]. Thus, we can begin to understand why antibiotics don't work well enough against infections, especially those caused by biofilms.

1.2 Present Day Scenario

1.2.1 Current Understanding of Controlling Factors

Microbial biofilms are thought to employ spatial organization and physiological cooperation to potentiate their metabolic efficiency and their resistance/tolerance to changes in their local environment [14]. The current understanding is that biofilms, in response to varying environmental conditions, can adopt different structures, which can range from homogeneous monolayers to heterogeneous structures including mushrooms and filaments [15]. A range of factors have been suggested to influence biofilm structure, including cell

death [16], hydrodynamic shear [17-19], mass transport [20,21], adhesion[22], cell motility [23], and detachment [24].

Biofilms can be formed by a single bacterial strain and most biofilm studies examine such cultures. However, majority of the naturally occurring biofilms are actually formed by multiple bacterial species [25] and understanding of the molecular mechanisms involved in the intercellular interactions within such biofilms is limited. Numerous studies have evaluated the susceptibility of multi-species vs. mono-species biofilms against antibiotic, and in most cases, polymicrobial biofilm was notably more resistant [26-30].

1.2.2 Computational Modeling

A computational approach that has revolutionized biofilm modeling is cellular automata (CA) [31,32]. CA are discrete models, in which space, time, and properties of the system can only have a finite number of possible states [33]. To date, the relative contributions of the parameters mentioned in section 1.2.1 have not been elucidated. Furthermore, previous work investigating the effect of cell death on biofilm development had considered 2-D systems [33]. Another study investigated the protection from antibiotic killing in biofilms based on a mechanism of localized nutrient limitation and slow growth; however the computational model used for it was one-dimensional [34]. Hence, the data obtained from these studies may not be physiologically relevant.

1.3 Motivation

Computer models of biofilm dynamics are valuable tools in exploring the formation of biofilms. Models can be used to test hypotheses or make predictions about how specific processes influence the biofilm structure or function. Often there are multiple mechanisms that are difficult to distinguish experimentally and in such cases theoretical investigations are particularly appealing.

To date, biofilm models used to explore the degrees of recalcitrance to killing by antimicrobials that take account of inter-species interactions are limited. The species present and the interactions between them critically influence the development and shape of the community. Understanding these mechanisms will aid in the development of techniques for combating bacterial biofilms in clinical areas.

One possible mechanism which explains the antibiotic tolerance of biofilms and depends on the inter-species interaction is the emergence of small colony variants (SCVs). Although SCVs have been known to exist for over a century, little attention was given to them originally as they were believed to be non-virulent and hence clinically insignificant. However, as their role in persistent infections began to unravel, it became crucial to thoroughly explore the mechanisms of SCV persistence and tolerance.

Thus, there was a need to develop a comprehensive 3-D computational CA model for polymicrobial biofilm growth which would include cell growth, cell division, cell death, attachment and detachment of cell, EPS production, cell-signaling process, and antimicrobial therapy. A detailed understanding of spatio-temporal variation in biofilm structures may lead to the development of novel therapeutics, and other potential biotechnological applications via the formation of synthetic biofilms. The identification of potential targets for inhibition of biofilm development may ultimately provide the means to diminish the impact of polymicrobial diseases on human health.

1.4 Objective

Specific Aim 1:

(a) To incorporate the ability to resume simulations by reading data from files.
This enables one to resume simulations from a specific time point with the existing circumstances or begin an antibiotic treatment. Thus the effect of adding antibiotic at different concentrations could be seen for the same biofilm morphology.
(b) To add a cell ID number to every cell that is generated so that the location of individual cells can be tracked within the biofilm.
(c) To make the code more efficient (by reducing the space and time complexity of the algorithm) and eliminate any redundancy in the code.

Specific Aim 2:

(a) To make the code specific to a cystic fibrosis by incorporating species interactions of *S. aureus* and *P. aeruginosa* involved in the disease.
(b) To investigate how the cell-cell interactions (like competition, cooperation), presence of SCVs, environmental heterogeneity etc. play a role in the antibiotic tolerance of biofilms.

Chapter 2

Literature Review

2.1 Introduction to Biofilm Development

Biofilms are omnipresent. Almost all species of microorganisms, not merely bacteria, have mechanisms by which they can adhere to surfaces and to each other. Biofilms form on nearly every non-shedding surface in contact with moisture or at air-fluid interfaces. Depending on the environmental conditions, they can be as thin as a few cell layers or many inches thick. There are five stages of biofilm formation: (i) Conditioning film; initial attachment, (ii) Adsorption and irreversible attachment, (iii) Growth and colonization (iv) EPS production; biofilm formation, (v) Dispersion. It has been shown that when biofilm is being created, the pathogens inside it can communicate with each other to coordinate gene expression according to the density of their local population. This bacterial communication system to coordinate behaviors at the population level is known as quorum sensing.

2.1.1 Formation of Biofilms

Development of a biofilm begins with the conditioning films altering the surface properties of the substratum and allowing microbes to adhere to the surface. For instance, when sterile medical implants are exposed to bodily fluids, proteins, polysaccharides, ions and various other components adhere to the surface and form a conditioning film which “attracts” microorganisms that would otherwise be unable to attach to the original surface [35]. The colonizers initially use weak, reversible adhesion via van der Waals forces to adhere to the surface. If they are not removed straight away, they can fasten themselves more permanently using cell adhesion structures such as pili. In the next step, production of EPS anchors the bacteria to the surface allowing colonies to grow. A fully developed biofilm contains an EPS matrix and vertical structures separated by interstitial spaces where the

fluid channels provide hydration while removing wastes from different regions of the biofilm. The final stage of biofilm formation is known as dispersion, and is the stage in which the biofilm is established and may only change in shape and size.

2.1.2 Quorum Sensing (QS)

Bacterial species that use quorum sensing, constitutively produce and release certain signaling molecules called autoinducers. They also have a receptor that can specifically detect these signaling molecules. But the odds of a bacterium detecting its own secreted autoinducer are pretty slim. Moreover, when only a few bacterial cells are in close proximity, diffusion reduces the concentration of autoinducer in the surrounding medium to negligible amounts. However, as the population grows, the autoinducer concentration passes a certain threshold, and the receptor becomes fully activated thereby inducing the up-regulation of specific genes, causing all of the cells to begin transcription at approximately the same time.

As a result, regulation by QS would allow the cells to save their resources under low density conditions by expressing appropriate behavior only when it is effectual. This coordinated behavior of bacterial cells at the population level is useful in a variety of situations like biofilm formation [36,37], virulence [36,37], symbiosis [36], competence [36], antibiotic production [36], motility [36], etc. Studies have shown that this cell-cell communication via autoinducers can be both intra- and inter-species as well [36,37].

2.2 Polymicrobial Biofilm

Biofilms can be formed by a single bacterial strain and most biofilm studies examine such cultures. However, majority of natural biofilms are actually produced by multiple bacterial species. Numerous studies have evaluated the susceptibility of multi-species vs. mono-species biofilms against antibiotic, and in most cases, the former was notably more resistant [30-34]. The species that constitute a polymicrobial biofilm and the interactions between them (synergistic or antagonistic) critically influence the development and shape of the community[38]. Understanding these mechanisms will aid in the development of techniques for combating bacterial biofilms in clinical areas.

The interactions between different species within a biofilm could be cooperative or competitive. One possible cooperative interaction is where one member provides conditions that promote survival of other members [38]. For instance, when anaerobic bacteria are

grown along with aerobic bacteria in a mixed biofilm, the aerobic bacteria at the top, consume oxygen thereby providing anaerobic conditions within the deeper layers of the biofilm in which anaerobic bacteria can multiply [39]. Thus, in this case, even though anaerobic bacteria are sensitive to oxygen, they can survive and persist under aerobic conditions [40,41]. One possible competitive interaction is where one species actively inhibits the growth of others, by producing inhibitory compounds or consuming essential nutrients [38]. For example, a marine bacterium *Pseudoalteromonas tunicate* hinders the growth of other marine bacteria isolated from the same environment by producing an antibacterial protein (AlpP) [42]. These interactions are important factors in determining the architecture of the polymicrobial biofilm community.

2.3 Proposed Mechanisms for Antibiotic Tolerance in Biofilms

The four primary theories explaining the reduced susceptibility of biofilms are: (i) Physical or chemical diffusion barriers to antibiotic penetration into the biofilm; the charge of polymers [43] and antibiotic-degrading enzymes [44] in the matrix may lead to binding and/or deactivation. (ii) Altered microenvironment and slow growth; most antimicrobials are effective in killing actively growing cells and nutrient concentration gradients within biofilms lead to gradients in microbial growth rate and activity. (iii) Activation of general stress response; for example: drug efflux pumps (they pump out unwanted toxic substances via an energy-dependent mechanism). Antibiotics can act as inducers and regulators of the expression of some efflux pumps [45] which may confer resistance to a wide range of antimicrobials. (iv) Emergence of biofilm-specific phenotype; a small subpopulation of microorganisms in a biofilm adopt a highly protected phenotypic state. These are dormant cells and are highly tolerant to killing by antibiotics. Example: persisters, small colony variants (SCVs). The dormancy of persisters can be observed by a typical biphasic antibiotic killing pattern with rapid killing of bulk population in the initial stages, followed by a plateau where only the persister subpopulation remains alive or are slowly killed. This biphasic pattern is observed both with increasing antibiotic concentration and with increasing treatment time [46].

2.4 Small Colony Variants (SCVs)

Small colony variants (SCVs) are a slow-growing phenotype that are part of the normal life cycle of bacteria, and often arise in response to environmental stresses, such as antibiotic treatment, starvation, host cationic peptides [47]. They are characteristically slow growing

and therefore constitute a small fraction of the population from which they arise but continue to persist because of their inherent resilience and host adaptability [48].

Apart from slow growth rate (in the case of *S. aureus*, SCVs divide at about one-ninth the rate of wild-type cells [49]), typical characteristics of SCVs include atypical colony morphology, lack of pigmentation, reduced hemolytic activity, reduced coagulase activity, reduced carbohydrate utilization, low virulence potential [49-51], reduced colony size (nearly one-tenth the size of the colonies associated with wild-type bacteria) [49], increased resistance to aminoglycosides (susceptibility of SCVs to aminoglycosides was one-tenth to one-thirtieth that of the parent strain) [49], diminished electron transport (observed in various species of *Staphylococcus*, *Enterococcus*, and *Pseudomonas*) [48], higher tendency for adhesion (express more surface adhesins compared to wild-type) to biotic or abiotic surfaces with enhanced intracellular persistence [52,53], increased biofilm-forming capacity in comparison with the wild-type parent [54]. They are capable of reversion to a wild-type or wild-type-like variant; thereby constituting a highly dynamic subpopulation serving as a reservoir for recurrent infections as illustrated in Fig. 2.1 [48]. Thus, phenotype switching enables bacteria to hide inside the host cell without inducing a strong host immune response.

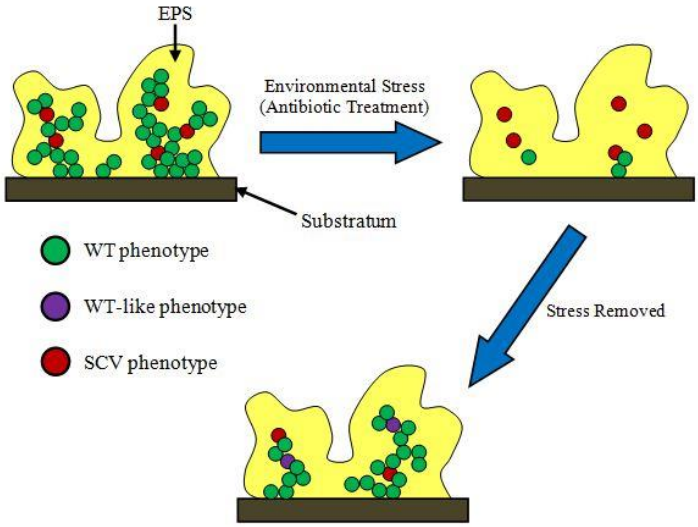


Figure 2.1: A schematic of a recurring biofilm infection. Untreated biofilms of bacteria often consist of WT (major population) and SCV (minor population). The WT population diminishes and SCVs survive under environmental stress, such as antibiotic treatment in turn making SCVs the dominant members of the population. However, when the selective pressure is removed, a proportion of SCVs revert to either the WT phenotype or a WT-like phenotype thereby regaining characteristics that enable faster growth. The WT organisms then proliferate and once again become the dominant members of the population.

SCVs are a challenging aspect in chronic infections such as cystic fibrosis in lung, chronic obstructive pulmonary disease, osteomyelitis, diabetic foot ulcers, chronic wound infections, etc., where antimicrobials are administered during the acute phase of infection but fail to eradicate SCVs, which remain dormant within the host and later cause recurring infection. The occurrence of SCVs has been observed to be the highest in cystic fibrosis and osteomyelitis [49]. SCVs of *S. aureus* were identified in 24% of patients suffering with cystic fibrosis [55].

2.5 Cystic Fibrosis: Interaction Between *S. aureus* and *P. aeruginosa*

Cystic fibrosis (CF) is a genetic disorder that mostly affects the lungs but could also affect pancreas, liver, kidneys, and intestine [56,57]. From an early age, the lungs of individuals with cystic fibrosis are colonized and infected by bacteria which thrive in the altered mucus that collects in the small airways of the lungs and lead to the formation of biofilms. Two of the most common pathogens found in CF lungs are *S. aureus* and *P. aeruginosa*.

P. aeruginosa secretes an exoproduct, 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), that suppresses the growth of many Gram-positive bacteria [58-60]. In the case of *S. aureus*, HQNO activates alternative sigma factor B, which alters the expression of several virulence factors, including those that regulate the ability to adhere, invade, and persist within host cells, and facilitates the emergence of SCV phenotype [61]. Since the biosynthesis of HQNO is up-regulated by the cell density-dependent quorum sensing mechanism [62], HQNO production would be expected to be amplified in biofilms, thus increasing the selection pressure for *S. aureus* SCVs.

Both HQNO and SCVs of *S. aureus* were found in CF lungs, indicating that evolution of these variants does indeed occur within the human host [38]. It has been suggested that the formation of SCVs is a survival strategy adopted by *S. aureus* to withstand competition with *P. aeruginosa* [63]. In any case, this example highlights the assertion that interactions between species within biofilms must be taken into account when designing therapies.

Chapter 3

Methodology

A cellular automaton is a collection of cells on a grid of a specified shape. It evolves through a number of discrete time steps according to a set of rules (generally, some mathematical functions) that determine the new state of each cell (in the next time step) based on the current state of the cell and the states of its neighboring cells. The rules are applied iteratively for as many time steps as desired.

In the case presented here, an individual based cellular automaton was used in order to represent the growth and development of a bacterial biofilm on a surface in an aqueous environment. A useful feature associated with this kind of model is that each agent (bacterial cell) is treated as an individual, independent entity with its own state and behavior. To simulate behavioral variability, parameter values (Table 3.1) for each cell were randomly assigned within a range of ±10% of the mean values taken from literature. This feature is particularly useful for studying complex systems where individual heterogeneity is important, like in the case of biofilms.

3.1 Simulation Domain

For the biofilm domain, a cuboid of dimensions L_x x L_y x L_z was considered which was spatially discretized into cubical elements with edge length as 3 μm (refer Fig. 3.1). This elemental volume is suitable to harbor one bacterial cell and its extracellular constituents. Each of the elements is occupied by either a bacterium or an equivalent volume of liquid. The bottom surface (dimension: 120μm x 120μm) represented the substratum upon which the biofilm was allowed to develop. A continuously replenished nutrient reservoir was placed at the top at a distance of 18 μm from the substratum. The interface between the

nutrient reservoir and the biofilm domain is termed as the mass transfer boundary layer (MTBL).

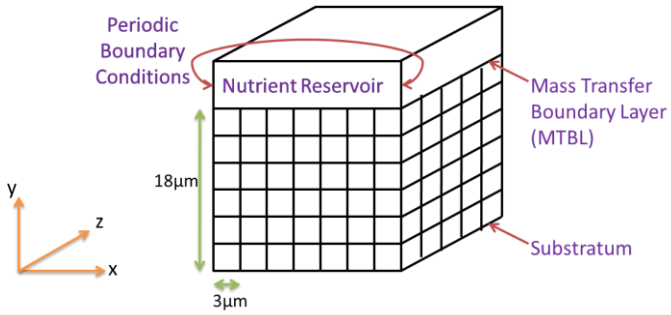


Figure 3.1: A schematic of the modeling domain

Following boundary conditions were applied to the system:

I. Periodic boundary conditions had been assumed in the x- and z- directions. Thus the modelling domain represents a curved surface where biofilm biomass at one edge wraps around to the other edge i.e. the element next to the cell with x- (or z-) coordinate as x_{max} (or z_{max}), in the positive x- (or z-) direction, would have its x- (or z-) coordinate as x = 0 (or z = 0) and the element after would have its x- (or z-) coordinate as x = 1 (or z = 1) and so on. This was done so as to avoid edge effects and to maintain continuity of the biofilm biomass. Thus, a large system was approximated by using a small domain.

II. An ideal planar (120µm x 120µm) source of nutrient was placed just above y_{max} (taken as the combined height of the biofilm as well as MTBL). Dirichlet boundary condition (fixed boundary condition) was imposed for all grid elements at the boundary of the nutrient reservoir i.e. at $y = y_{max}$, concentration of the nutrient was taken as the bulk concentration. Moreover, MTBL is always situated at a fixed distance above the top of the biofilm and so the source of nutrient will move upwards, i.e. y_{max} changes, as the biofilm grows with time.

III. Neumann boundary condition was applied at the substratum (boundary at the bottom of the domain) viz. at y = 0, concentration gradient is zero which then implies that the nutrient concentration at y = -1 (required for calculating concentrations at y = 0 using finite difference method) will be same as the nutrient concentration at y = 1.

3.2 Nutrient Transport and Uptake

The nutrient concentration in the biofilm compartment depends on the balance between rate of nutrient transport due to diffusion and convection, and nutrient uptake rate by the bacterial cells.

This phenomenon was modeled within each element of the simulation domain by using a discretized form of the three-dimensional convection-diffusion equation:

$$\frac{\partial C_N}{\partial t} = -r_N(C_N, C_B) + D_N \sum_{i=1}^{3} \frac{\partial^2 C_{N,i}}{\partial x_i^2} - \nabla.(\vec{v} C_N) \qquad (3.1)$$

Here, r_N is the volumetric rate of nutrient consumption. C_N and C_B represent nutrient and biomass concentration at each discretized element (x,y,z) and at each time point, t, respectively. D_N is the diffusivity of nutrient in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{N,aq}$) with the relative effective diffusivity $D_{N,e}/D_{N,aq}$, v is the local fluid velocity.

Cells consume nutrient from the external environment and channel it into different metabolic pathways. Some nutrient may be directed into growth and product synthesis; another fraction is used to generate energy for maintenance activities. In the absence of product formation, it is assumed that all nutrient entering the cell is used for growth and maintenance functions. A complete account of nutrient uptake should include a maintenance component. The specific rate of nutrient uptake for maintenance activities is known as the maintenance coefficient.

Biomass growth rate is dependent on the availability of nutrients and so it is influenced by the spatial distribution of nutrient concentration within the biofilm. In turn, bacterial growth, division, death, detachment etc. affects nutrient concentration fields. Thus the temporal and spatial distribution of nutrient concentration (C_N) is intimately dependent on the local biomass concentration (C_B). And so the nutrient consumption rate was assumed to be a function of both C_B and C_N, as stated below:

$$r_N(C_N, C_B) = \left(\frac{\mu_{max}}{Y_{BN}} + m\right) C_B \left(\frac{C_N}{C_N + K_N}\right) \qquad (3.2)$$

Here, μ_{max} is the maximum specific growth rate, Y_{BN} is the yield of biomass from nutrient (yield coefficient), m is the maintenance coefficient and K_N is the nutrient constant (half-saturation coefficient). μ_{max} and K_N are intrinsic parameters of the cell-nutrient system.

3.3 Spatial and Temporal Evolution of Cellular Biomass

3.3.1 Growth

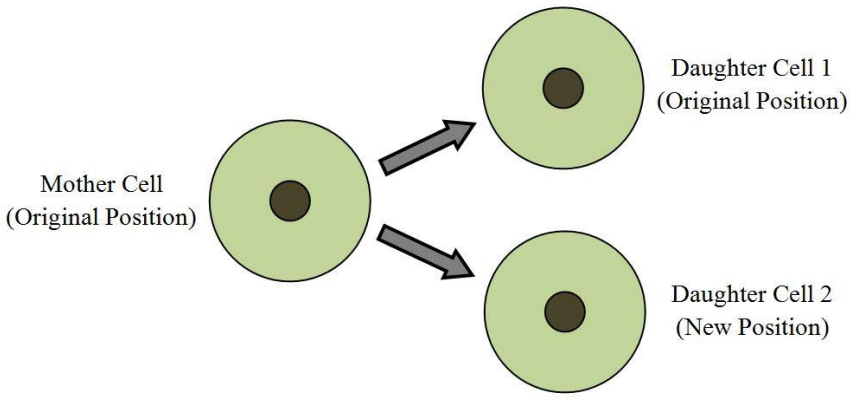
As it was mentioned earlier in section 3.2, inside the cell, the nutrient that has been consumed was utilized for two purposes: cell growth and endogenous metabolism. The endogenous metabolism was assumed to be proportional to the concentration of biomass, i.e. it is given by mC_B. The nutrient that had not been consumed for endogenous metabolism was used for cell growth at an efficiency, Y_{BN}.

$$\frac{\partial C_B}{\partial t} = Y_{BN} * (r_N(C_N, C_B) - mC_B) \tag{3.3}$$

3.3.2 Division

As the bacterial cells consume nutrient, they keep on growing (biomass concentration increases) within their grid location. When the biomass of a bacterium reaches twice its native value, it divides into two daughter cells (refer Fig. 3.2(a)); each having a new set of parameters. One of the two daughter cells remains in the original location of the mother cell, whereas the other one is placed in a neighboring element in the direction that offers least mechanical resistance.

(a)



(b)

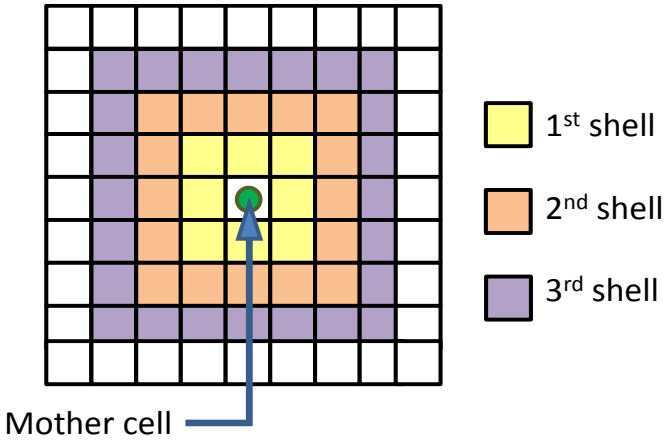


Figure 3.2: (a) A mother cell undergoing division, (b) Two-dimensional view of the neighbouring elements at increasing distances from the dividing cell

During division, each direction is checked for free spaces at increasing distances from the mother cell, and the first direction in which such a free space is found is considered to be the direction of least mechanical resistance. For example, consider a mother cell that is undergoing division. At first, the immediate neighboring elements i.e. the 1st shell (3^3-1^3 = 26 elements) as shown in Fig. 3.2 (b), will be checked for free spaces. If all of them are occupied, then the 2nd shell (5^3-3^3 = 98 elements) is checked and so on. Once an empty grid location is found, the entire line of cells between the mother cell and the closest free space is pushed away from the former by one element in the same line of direction so as to make space near the dividing cell where the second daughter cell is finally placed.

If two or more directions with equal mechanical resistance (i.e. directions having empty positions at equal distances from the mother cell location) are found, one of those is chosen at random.

3.3.3 Death

In the 3-D computational model that has been developed, there are three possible mechanisms by which a cell can die: (i) the cell has been in stationary phase for a pre-determined number of hours ($t_{SP,limit}$), or (ii) the ratio of nutrient consumption to endogenous metabolism (R) falls below a certain threshold, or (iii) due to antibiotic treatment.

If the ratio, R (= r_N/mC_B), is greater than 1 then the cell exhibits net growth and if it is less than 1 then the bacterium has entered stationary phase. This is recorded with an individual based counter, t_{SP}. If R is below 1 during one hour, t_{SP} increases by one. However, a

bacterium also has the possibility to recover if R increases above 1 before it dies. Consequently, if R is above 1 during one hour, t_{SP} decreases by one. As soon as t_{SP} is greater than or equal to $t_{SP,limit}$, the cell dies.

Bacteria also die if R falls below a certain threshold value. This was done so as to account for bacterial death under circumstances of low (or no) nutrient concentration.

If the biofilm is under antibiotic treatment, then cells die based on certain probability which is a function of rate of consumption of antibiotic and bacterial inhibitory concentration, which will be seen in section 3.7.

A cell that is dead no longer consumes nutrient or divides; it neither plays any role in quorum sensing nor does it undergo phenotypic switching. Hence, it is removed from the simulation domain leaving a free space at its location.

3.3.4 Detachment

Dispersal of cells from the biofilm colony is a crucial stage of the biofilm life cycle. It enables biofilms to spread and colonize new surfaces, thereby contributing to the dissemination of contamination and infection in both clinical and public health settings. However, in the current computational model, this process was not considered in detail.

A cell detaches from the biofilm if it loses contact with the substratum. The contact could be direct or indirect (through a cluster of bacteria in which at least one bacterium is directly bound to the substratum or via EPS). At the end of each time step, just like in the case of bacterial death, detached cells are also removed from the domain & are no longer tracked.

3.4 Quorum Sensing: Autoinducer Production and Transport

Every cell in the domain is modeled as being in either up- or down-regulated state. Initially, during colonization, all the cells are in the down-regulated state. Cells are allowed to switch between the two states, at rates, dependent on the local autoinducer concentration.

The transition rate from down- to up-regulated state was taken as [64]:

$$QS^{+} = \alpha\left(\frac{C_A}{1+\gamma C_A}\right) \tag{3.4}$$

Whereas, the transition rate from up- to down-regulated state was taken as [64]:

$$QS^{-} = \beta\left(\frac{1}{1+\gamma C_A}\right) \tag{3.5}$$

The probabilities of switching from one state to another within a time interval, Δt, was then taken as [64]:

$$P_u = QS^{+}\Delta t \quad , P_d = QS^{-}\Delta t \tag{3.6}$$

Here, α is the conversion rate for up-regulation, β is the spontaneous down- regulation rate, γ is the transition constant, C_A is the local autoinducer concentration, P_u is the probability of a cell switching from down- to up-regulated state, P_d is probability of a cell switching from up- to down-regulated state. During the simulation, a random number is generated and if the probability of switching is greater than the said number, then the cell switches its state.

Up- and down-regulated cells are assumed to produce and release autoinducer molecules at constant rates of $r_{A,u}$ and $r_{A,d}$, respectively.

$$r_A = \begin{cases} r_{A,u} \\ r_{A,d} \end{cases} \tag{3.7}$$

Time evolution of autoinducer concentration within the biofilm is given by:

$$\frac{\partial C_A}{\partial t} = D_A \sum_{i=1}^{3} \frac{\partial^2 C_{A,i}}{\partial x_i^2} + \frac{r_A}{\Delta V} - \nabla.(\vec{v}C_A) \tag{3.8}$$

Here, r_A is the production rate (molecules/s) of autoinducer molecules, ΔV is the elemental volume, C_A is the local autoinducer concentration, D_A is the diffusivity of autoinducer in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{A,aq}$) with the relative effective diffusivity $D_{A,e}/D_{A,aq}$, v is the local fluid velocity.

3.5 EPS Production

Just like the nutrient that had not been consumed for endogenous metabolism was used for cell growth at an efficiency Y_{BN}, a part of it was also used for production of EPS at an efficiency Y_{EN}.

$$\frac{\partial C_E}{\partial t} = Y_{EN} * (r_N(C_N, C_B) - mC_B) \tag{3.9}$$

Here, C_E is the local EPS concentration.

In the current computational model, EPS (if considered), is produced only by up-regulated cells. However, if a cell has entered stationary phase ($R = r_N/mC_B < 1$), then it doesn't produce any EPS even if it is up-regulated.

EPS do not grow, die or consume nutrient, but they occupy space and undergo division. Once the EPS concentration in a given grid element exceeds the threshold value, it divides into two equal parts. One part remains in the original location while the other part moves into a neighboring element in the direction that offers least mechanical resistance, just like in the case of cell division as was seen in section 3.3.2. In this model, EPS is capable of consuming antibiotic. This was an attempt to account for the reaction-diffusion barrier that it would offer to the cells in the interior of the biofilm.

3.6 Phenotypic Switching: HQNO Production and Transport

In the current computational model, every *S. aureus* (one of the model bacteria considered) cell in the domain is modeled as either wild-type or as an SCV. In fact, SCVs are treated as if they were an independent cell species since they are phenotypically different from the wild-type. Initially, during colonization, all the *S. aureus* cells are wild-type only. *P. aeruginosa* (another model bacteria), on the other hand, has only one phenotype i.e. the wild-type.

Down-regulated *P. aeruginosa* cells were assumed to produce and release HQNO molecules at a constant rate of $r_{HQNO,d}$. However, up-regulated *P. aeruginosa* cells were assumed to produce and release HQNO molecules at a rate $r_{HQNO,u}$ as given below:

$$r_{HQNO,u} = r_{HQNO,d} + Y_{HQNO,N} * (r_N(C_N, C_B) - mC_B) \tag{3.10}$$

Here, $Y_{HQNO,N}$ is the efficiency at which a part of the nutrient, that had not been consumed for endogenous metabolism, was used for production of HQNO molecules. Note that in the case presented here, $r_{HQNO,u}$, $r_{HQNO,d}$ and $Y_{HQNO,N}$ are valid only for *P. aeruginosa*.

Time evolution of HQNO concentration within the biofilm is given by:

$$\frac{\partial C_{HQNO}}{\partial t} = D_{HQNO} \sum_{i=1}^{3} \frac{\partial^2 C_{HQNO,i}}{\partial x_i^2} + r_{HQNO} - \nabla.(\vec{v} C_{HQNO}) \tag{3.11}$$

Here, r_{HQNO} (= $r_{HQNO,u}$ or $r_{HQNO,d}$) is the production rate of HQNO molecules, C_{HQNO} is the local HQNO concentration, D_{HQNO} is the diffusivity of HQNO in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{HQNO,aq}$) with the relative effective diffusivity $D_{HQNO,e}/D_{HQNO,aq}$, v is the local fluid velocity.

S. aureus cells are allowed to switch between the two phenotypic states, at rates, dependent on the local HQNO concentration.

The transition rate from wild-type to SCV state was taken to be:

$$PS^{+} = \alpha' \left(\frac{C_{HQNO}}{1+\gamma' C_{HQNO}} \right) \tag{3.12}$$

whereas, the transition rate from SCV to wild-type state was taken to be:

$$PS^{-} = \beta' \left(\frac{1}{1+\gamma' C_{HQNO}} \right) \tag{3.13}$$

The probabilities of switching from one phenotypic state to another within a time interval, Δt, was then taken to be:

$$P_{SCV} = PS^{+}\Delta t \quad , P_{WT} = PS^{-}\Delta t \tag{3.14}$$

Here, α' is the conversion rate for SCV-switching, β' is the spontaneous WT-switching rate, γ' is the transition constant, P_{SCV} is the probability of a cell switching from wild-type (WT) to SCV state, P_{WT} is probability of a cell switching from SCV to wild-type (WT) state. During the simulation, a random number is generated and if the probability of switching is greater than the said number, then the cell switches its phenotypic state.

3.7 Antimicrobial Drug Administration and Killing

Antibiotic transport and uptake was modeled within each element of the simulation domain by using a discretized form of the three-dimensional convection-diffusion equation:

$$\frac{\partial C_{Ab}}{\partial t} = -(K_{B,max}\, C_B + K_{E,max} C_E)\left(\frac{C_{Ab}}{K_{Ab}+C_{Ab}}\right) + D_{Ab} \sum_{i=1}^{3} \frac{\partial^2 C_{Ab,i}}{\partial x_i^2} - \nabla.(\vec{v} C_{Ab}) \tag{3.15}$$

Here, the first clubbed term is the volumetric rate of antibiotic consumption by the cells as well as EPS. $K_{B,max}$ is the maximum specific reaction rate of antibiotic with respect to biomass (cells) whereas $K_{E,max}$ is the maximum specific reaction rate of antibiotic with respect to EPS. K_{Ab} is the antibiotic constant (half-saturation coefficient). C_{Ab} represents antibiotic concentration at each discretized element (x,y,z) and at each time point, t. D_{Ab} is the diffusivity of antibiotic in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{Ab,aq}$) with the relative effective diffusivity $D_{Ab,e}/D_{Ab,aq}$, v is the local fluid velocity.

Rate of antibiotic consumption by cells:

$$r_{Ab} = K_{B,max} C_B \left(\frac{C_{Ab}}{K_{Ab} + C_{Ab}} \right) \tag{3.16}$$

Rate of antibiotic consumption by cells at bacterial inhibitory concentration (C_{BIC}):

$$r_{BIC} = K_{B,max} C_{OneCell} \left(\frac{C_{BIC}}{K_{Ab} + C_{BIC}} \right) \tag{3.17}$$

Maximum antibiotic consumption rate by cells:

$$r_{max} = K_{B,max} C_{OneCell} \left(\frac{C_{max}}{K_{Ab} + C_{max}} \right) \tag{3.18}$$

The probability of cell death due to antibiotic consumption is given by:

$$P_{death} = \frac{r_{Ab} - r_{BIC}}{r_{max} - r_{BIC}} \tag{3.19}$$

During the simulation, a random number is generated and if the probability of death is greater than the said number, then the cell dies.

3.8 Model Simulation

The biofilm simulation represents a time march in which the occupancy status of each element is updated at every time step. It begins with the creation of the modelling domain and establishment of parameter values. At t = 0, a small number (n) of colonizers, were randomly placed upon the substratum. The nutrient concentration field was generated by obtaining numerical solutions to partial differential equation (3.1) using a finite-difference

approach, considering very small time steps. The biomass (bacteria and EPS) was assumed to be constant during the transient phase, since the growth of biomass is very slow in comparison with nutrient diffusion and consumption [26]. The same approach was used for other dissolved entities like autoinducer, HQNO and antibiotic (Eqs. (3.8), (3.11) and (3.15), respectively). At each time step, Eqs. (3.3) and (3.9) were solved using the updated nutrient concentration field. The bacteria consumed nutrient, and subsequently grew and underwent division. If any bacterium satisfied any one of the death rules, then it died. Cells that satisfied the detachment requirements were also identified. Both dead and detached cells were removed from the domain and no longer tracked. Quorum sensing and phenotypic switching were performed based on the local concentrations of autoinducer and HQNO, respectively. At the end of each time step, the nutrient reservoir was shifted vertically upwards such that a distance of 18 μm from the topmost solid entity (bacterial cell or EPS) in the biofilm was always maintained.

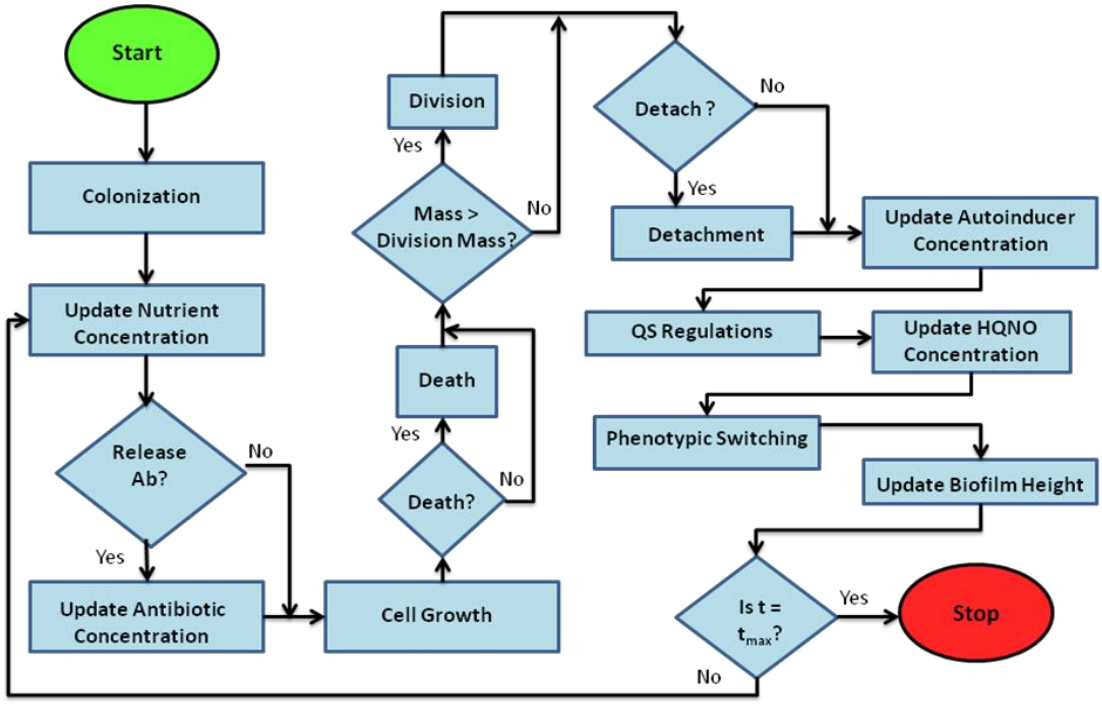


Figure 3.3: Sequence of steps during a typical simulation

Table 3.1: Definition of variables and parameters

Variable/ Parameter	Description	Values	Unit
μ_{max}	Maximum specific growth rate	0.3125 (SA), 0.3125*0.75 (PA), 0.3125/9 (SA-SCV)	h^{-1}
m	Maintenance coefficient	0.036	$g_S\ g_B^{-1}\ h^{-1}$
Y_{BN}	Yield of biomass from nutrient	0.45	$g_B\ g_S^{-1}$
Y_{EN}	Yield of EPS from nutrient	0	$g_{EPS}\ g_S^{-1}$
Y_{HQNO}	Yield of HQNO from nutrient	0 (SA & SA-SCV), 0.01 (PA)	$g_{HQNO}\ g_S^{-1}$
$C_{N,bulk}$	Bulk nutrient concentration	4	$g\ m^{-3}$
$C_{Ab,bulk}$	Bulk antibiotic concentration	7, 9, 11, 13, 15	$g\ m^{-3}$
n	No. of colonizers	6 (SA), 6 (PA)	Unitless
D_i	Diffusivity of component 'i' in biofilm		$m^2\ h^{-1}$
$D_{i,aq}$	Diffusion rate of 'i' in aqueous phase	$2.52*10^{-6}$ (N), $1.44*10^{-6}$ (Ab), $2.52*10^{-6}$ (HQNO), $1.998*10^{-6}$ (aiSA & aiPA)	$m^2\ h^{-1}$
$D_{i,e}/D_{i,aq}$	Relative effective diffusivity	1/3 (N), 1/4 (Ab), 1/3 (HQNO), 1.5/2 (aiSA), 1/2 (aiPA)	Unitless
K_i	Half-saturation coefficient	2.55 (N), 1 (Ab)	$g\ m^{-3}$
v	Local fluid velocity	0	$m\ h^{-1}$
α	Conversion rate for up-regulation (QS)	$7.8*10^{-17}$	$m^3\ h^{-1}\ molecule^{-1}$
β	Spontaneous down-regulation rate (QS)	0.975	h^{-1}
γ	Transition constant (QS)	$7.9589*10^{-17}$	$m^3\ molecule^{-1}$
α'	Conversion rate for SCV-switching (PS)	12	$m^3\ h^{-1}\ g^{-1}$
β'	Spontaneous WT-switching rate (PS)	0	h^{-1}
γ'	Transition constant (PS)	1000	$m^3\ g^{-1}$
$r_{A,u}$	Autoinducer production rate by up-regulated cells	73800	$molecules\ m^{-3}$
$r_{A,d}$	Autoinducer production rate by down-regulated cells	498	$molecules\ m^{-3}$

$r_{HQNO,u}$	HQNO production rate by up-regulated cells		g m^{-3}
$r_{HQNO,d}$	HQNO production rate by down-regulated cells	10^{-8}	g m^{-3}
$K_{B,max}$	Maximum specific reaction rate of anitibiotic with respect to biomass	0.5	g_{Ab} g_B^{-1} h^{-1}
$K_{E,max}$	Maximum specific reaction rate of anitibiotic with respect to EPS	0.25	g_{Ab} g_{EPS}^{-1} h^{-1}
$m_{OneCell}$	Mass of one cell	10^{-12}	g
$C_{OneCell}$	Concentration of one cell	37037.037	g m^{-3}
C_{BIC}	Bacterial inhibitory concentration	0.5 (SA), 0.25 (PA), 4*0.5 (SA-SCV)	g m^{-3}
C_{max}	Antibiotic concentration corresponding to maximum consumption rate	32 (SA), 16 (PA), 4*32 (SA-SCV)	g m^{-3}
$t_{SP,limit}$	Stationary phase limit	24	h
$R_{threshold}$	Minimum C to M ratio	0.15	Unitless
Δt	Time step used for CA	1	h
Δx	Element size	$3*10^{-6}$	m
E_x	No. of elements in x-direction	40	Unitless
E_z	No. of elements in z-direction	40	Unitless
L_{MTBL}	Thickness of MTBL	$18*10^{-6}$	m
E_d	EPS division threshold concentration	33000	g m^{-3}

* SA = *S. aureus*, PA = *P. aeruginosa*, SA-SCV = *S. aureus* SCV,
N = Nutrient, Ab = Antibiotic, HQNO = 4-hydroxy-2-heptylquinoline-N-oxide,
aiSA = Autoinducer of *S. aureus*, aiPA = Autoinducer of *P. aeruginosa*

Chapter 4

Results and Discussion

A cellular automata model has been used to investigate the role of presence of small colony variants (SCVs) in the antibiotic susceptibility of a mixed-species biofilm comprising of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Initially, simulations were run for both QS+ (with quorum sensing) and QS- (without quorum sensing) biofilms, at a nutrient concentration of 4 g/m^3 without any sort of antibiotic treatment and were used as control runs. EPS production was kept as zero for all the simulations that were performed. Additionally, reversion of SCVs back to WT or WT-like variants were not considered (i.e. $\beta'=0$). Using the procured control runs, simulations were resumed from a specific time point (time at which population of *P. aeruginosa* attains a maximum value minus 12 hours; differs for each control run) following which the biofilm was exposed to antibiotic for 12 continuous simulation hours. Resumptions were done at different antibiotic concentrations of 7, 9, 11, 13 and 15 g/m^3. Due to stochastic nature of the model, running multiple simulations with the same parameters never really gave identical results but they were comparable. Since it is not possible to present all of the simulation results, here we are presenting only a few. MATLAB was used as a tool to analyze the data that was obtained from the simulations.

4.1 Antibiotic Concentrations Below the Effective Concentration Prolong the Life of a Biofilm

The polymicrobial biofilm was subjected to a range of antibiotic concentrations varying from 7 to 15 g/m^3 (Fig. 4.1). At an antibiotic concentration of 13 g/m^3, the entire biofilm (except *S. aureus* SCVs) was exterminated within 11 hours of treatment. This specific concentration of antibiotic was termed as "effective concentration" and was defined as the minimum antibiotic concentration at which wild-type variants of both the species get eradicated. Moreover, at an antibiotic concentration of 11 g/m^3 (just below the effective

concentration), all the wild-type cells couldn't be obliterated within the 12 hour treatment period and the biofilm sustained; therefore this specific concentration of antibiotic was termed as "threshold concentration" and was defined as the maximum antibiotic concentration which the biofilm (specifically, the wild-type population) can tolerate.

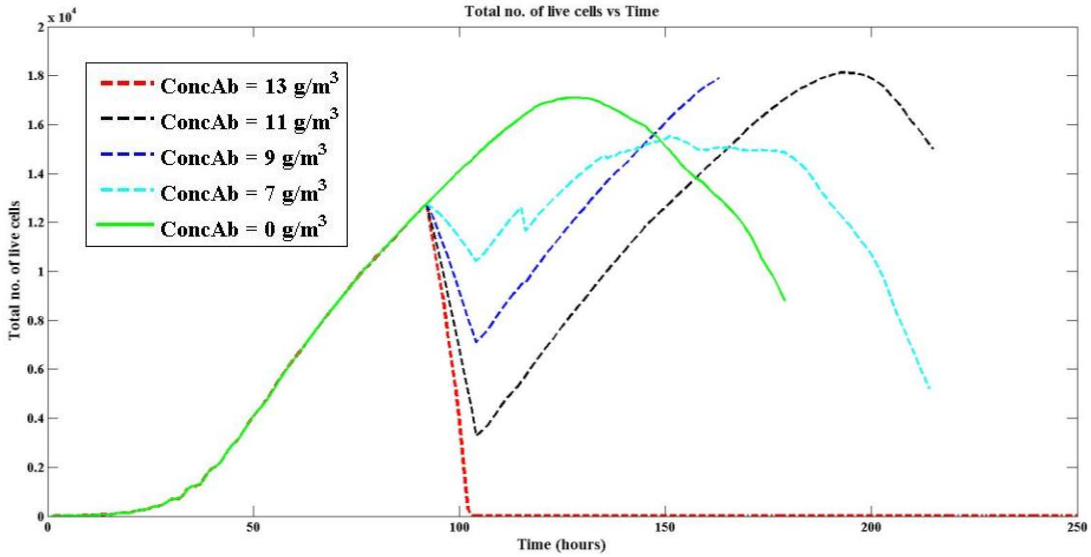


Figure 4.1: Total number of live cells versus time at different antibiotic concentrations; Effective antibiotic concentration=13g/m^3, Threshold antibiotic concentration=11g/m^3

Interestingly, these profiles indicate that using any concentration of antibiotic below the effective concentration prolongs the life of the biofilm.

4.2 Threshold Concentration of Antibiotic Impedes the Transformation of Polymicrobial Biofilm Into an Exclusively Mono-Species Biofilm

S. aureus is much faster growing (greater μ_{max}) and therefore is in direct competition with *P. aeruginosa* for the growth-limiting nutrient and living space. This interaction has key implication for the population dynamics and distribution of both the species. Figure 4.2 illustrates that *S. aureus* is the dominant species in this polymicrobial biofilm.

In the absence of antibiotic (control run), the number of *P. aeruginosa* cells drops down to an extent that the entire population transforms into an exclusively mono-species biofilm after a certain time point (beyond t = 185h) comprising of only *S. aureus* species (both wild-type and SCVs). Thus, *S. aureus* is able to outcompete *P. aeruginosa*.

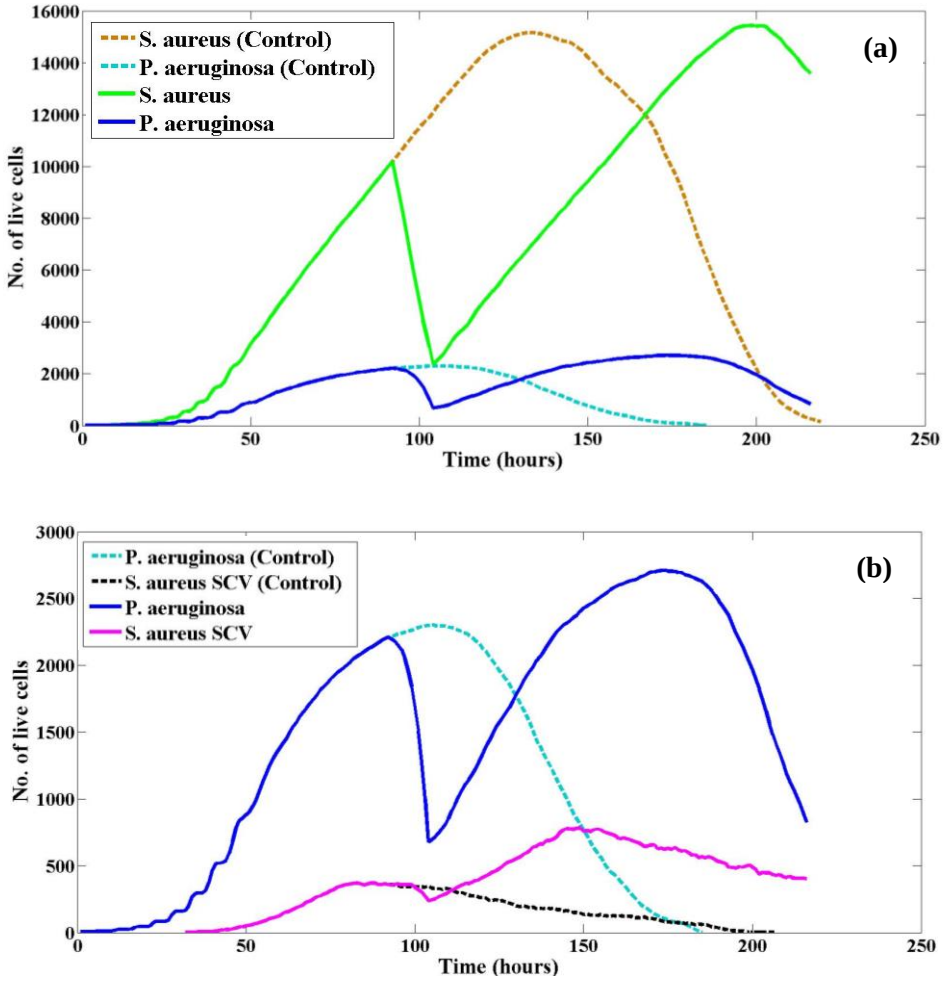


Figure 4.2: Number of live cells (species-wise) versus time at threshold antibiotic concentration. (a) *S. aureus* & *P. aeruginosa* (b) *P. aeruginosa* & *S. aureus* SCVs

However, when the biofilm is exposed to antibiotic, the *P. aeruginosa* subpopulation is able to withstand for a longer time. One might assume that this may be because the interspecific competition (a form of competition in which members of different species compete for the same resource) between the two species is affected. In order to explore this further, spatial distribution during the course of antimicrobial treatment, temporal evolution of death fractions and effect of concentration of antibiotic on the cell division rate of the two species were analyzed.

Moreover, Fig. 4.2b also manifested the increase in the number of *S. aureus* SCVs as a stress response.

4.3 When the Concentration of Antibiotic is Below the Effective Concentration, Dominant Species (*S. aureus*) Shields the Minor Subpopulation (*P. aeruginosa*) by Hindering Antibiotic Infiltration

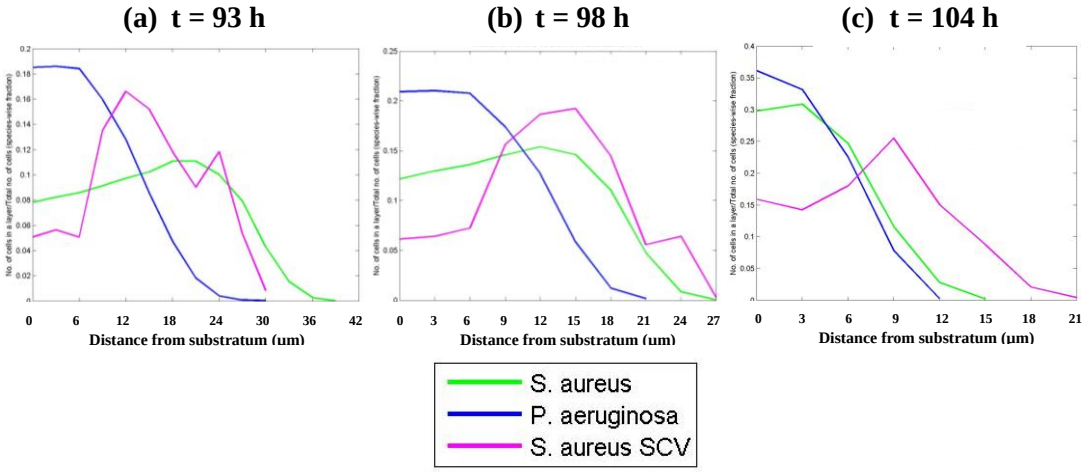


Figure 4.3: Species-wise fraction of live cells (Number of live cells of a particular species in a layer/ Total number of live cells belonging to that species) versus distance from the substratum at three different time points: (a) start of antibiotic treatment, t = 93h (b) midway of antibiotic treatment, t = 98h (c) end of antibiotic treatment, t = 104h.

Figure 4.3 depicts that at all times, during the course of antibiotic treatment, most of the individuals of *P. aeruginosa* subpopulation are concentrated at the bottom of the biofilm i.e. nearer to the substratum (*S. aureus* partly "blankets" *P. aeruginosa*; an effect of the interspecific competition). At the start of the treatment process, the *S. aureus* SCVs are randomly distributed within the interior of the biofilm and the wild-type *S. aureus* cells are, more or less, evenly distributed across the entire biofilm. However, since the top layers of the biofilm are dominated by wild-type variants of *S. aureus* and the antibiotic starts diffusing from the top, it seems reasonable to reckon that they are the ones more prone to death.

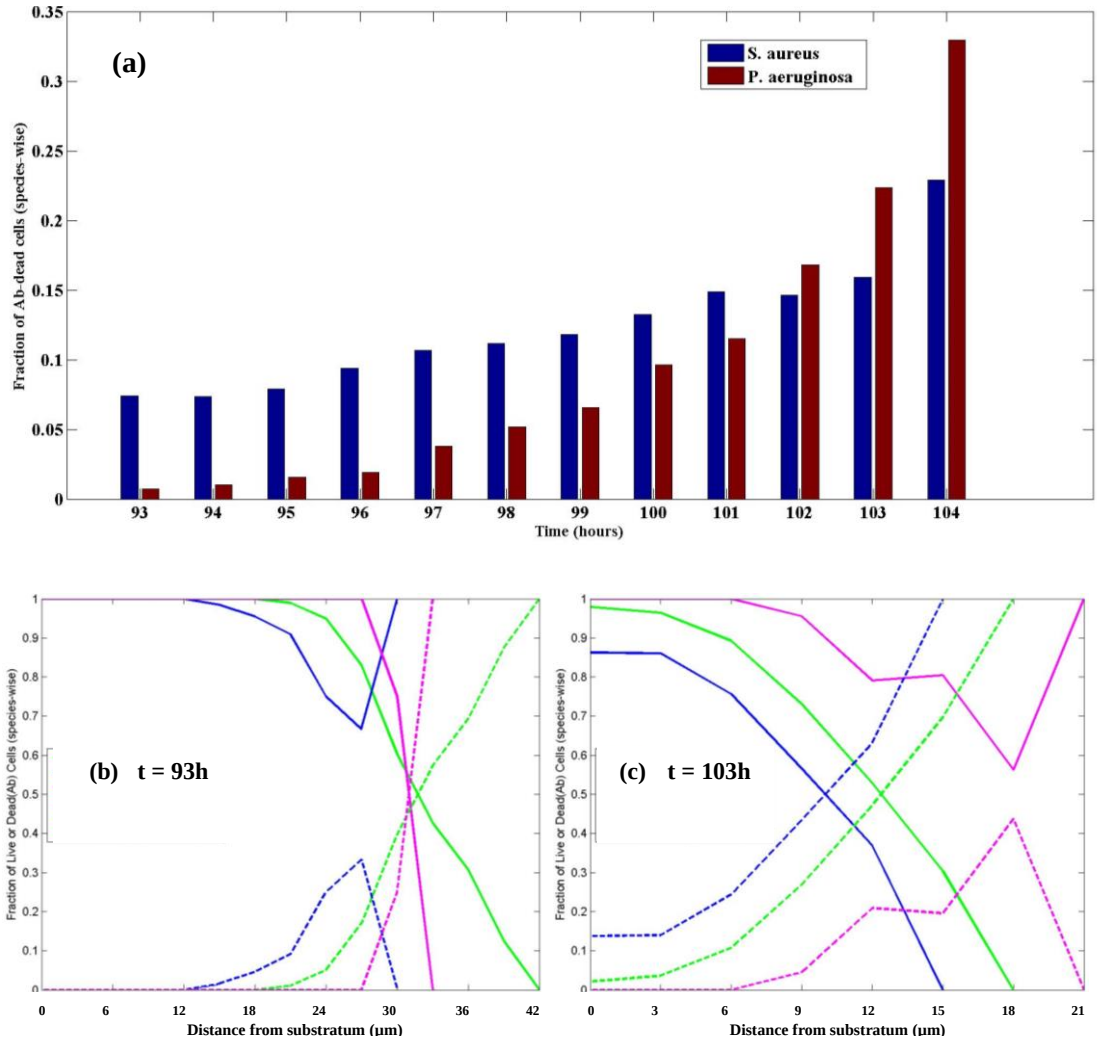


Figure 4.4: (a) Species-wise fraction of dead cells due to antibiotic (Number of dead cells due to antibiotic of a particular species/Total number of live and dead cells of that species) versus time. Species-wise fraction of live and dead cells (Number of live (or dead due to antibiotic) cells of a particular species in a layer/Total number of live and dead cells of the same species in the same layer) versus distance from substratum at (b) t = 93h (c) t = 103h.

With the commencement of antimicrobial dosing, wild-type *S. aureus* variants were most afflicted (Fig. 4.4) as was anticipated based on the spatial distribution of the two species. In the early stages of the treatment, *S. aureus* (WT) hindered the diffusion of antibiotic into the depths of the biofilm by consuming it; thereby shielding *P. aeruginosa* from the direct action of the antimicrobial agent to a certain extent. However, after a while, the viability of *P. aeruginosa* decreased sharply as the permeation of the antibiotic outpaced the "neutralization" by *S. aureus* (WT).

Subsequently, the impact of concentration of antibiotic on the proliferation of the two species was investigated (Fig. 4.5).

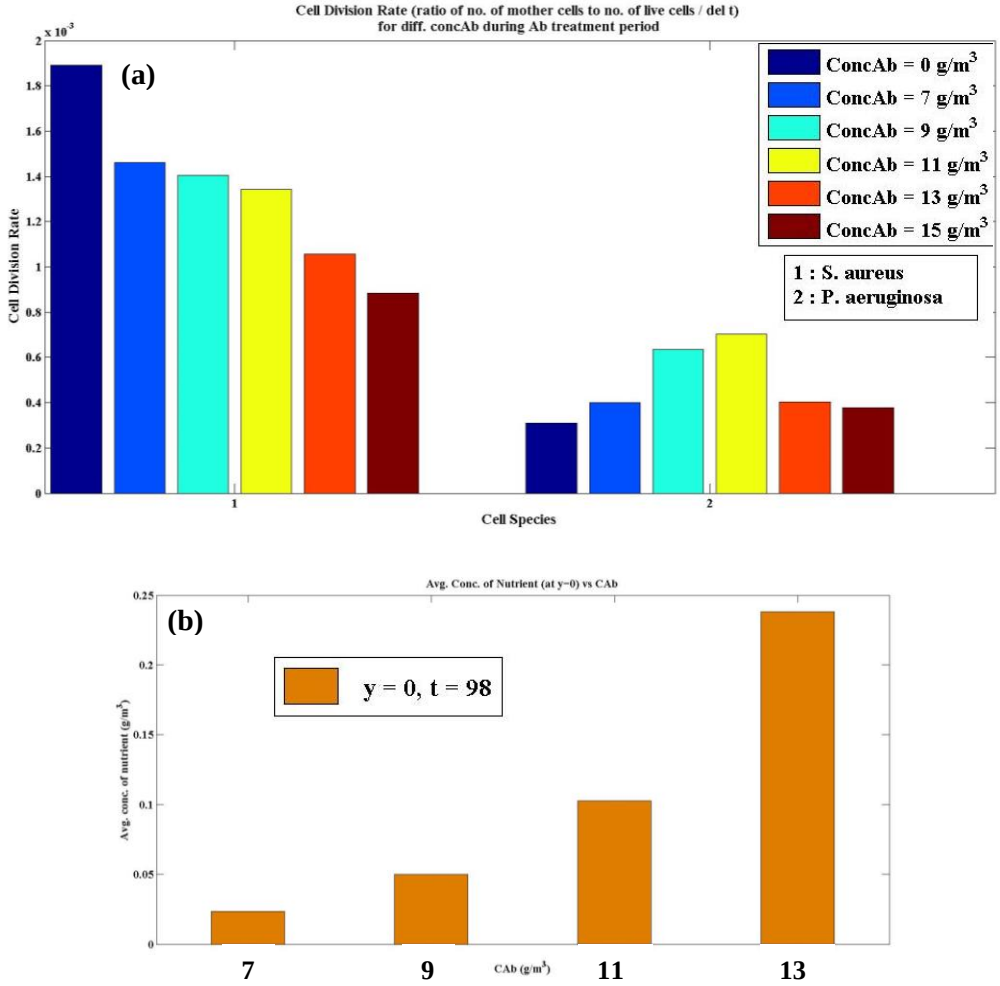


Figure 4.5: (a) Cell division rate (ratio of number of mother cells to number of live cells/delta t) versus antibiotic concentration during the antibiotic treatment period for wild-type *S. aureus* and wild-type *P. aeruginosa*. (b) Average concentration of nutrient at the substratum versus concentration of antibiotic.

Rate of cell division of *S. aureus* (WT) species decreases with increasing concentration of antibiotic. In other words, cell death of *S. aureus* wild-type variants has overwhelmed their cell division and the effect enhances with increasing antibiotic concentration.

In the absence of antibiotic, the dominating wild-type variants of *S. aureus* deprived the *P. aeruginosa* species from growing up to its full potential by depriving it of the growth-limiting nutrient. On the other hand, in presence of antibiotic, as the cell count of *S. aureus*

(WT) declines, the biofilm loses its compact structure and becomes porous. Subsequently, nutrient diffuses more effectively into the deeper layers of the biofilm (Fig. 4.5b). As a result, *P. aeruginosa* at the bottom gains ample access to the common nutrient and is able to thrive (up to the threshold antibiotic concentration). But beyond the threshold antibiotic concentration, cell death rate of *P. aeruginosa* overpowers the cell division rate.

It was discerned in section 4.2 that at threshold antibiotic concentration *S. aureus* SCVs also increased as a stress response along with the *P. aeruginosa* subpopulation. And here it was observed that increasing antibiotic concentration (up to the threshold antibiotic concentration) favors *P. aeruginosa*. Thus, in order to inspect whether *S. aureus* SCVs followed a similar trend, fractions of SCVs were plotted against time at different antibiotic concentrations.

4.4 Production of SCVs is Potentiated in Response to Antibiotic Treatment, Indicating That SCV Production Acts as a Defense Mechanism Against External Stresses

For the control run (without antibiotic treatment), the *S. aureus* SCV fraction diminishes with time (Fig. 4.6). This is because, in the absence of any antimicrobial agent, the wild-type cells proliferate when compared to SCVs which are slow-growing; the SCVs are unable to compete with the wild-type cells for the common nutrient and as a consequence get killed. On the other hand, in the presence of antibiotic, nutrient diffusion into the biofilm increases as time progresses (Fig. 4.5b) and the SCVs gain more access to it, just like in the case of *P. aeruginosa* species.

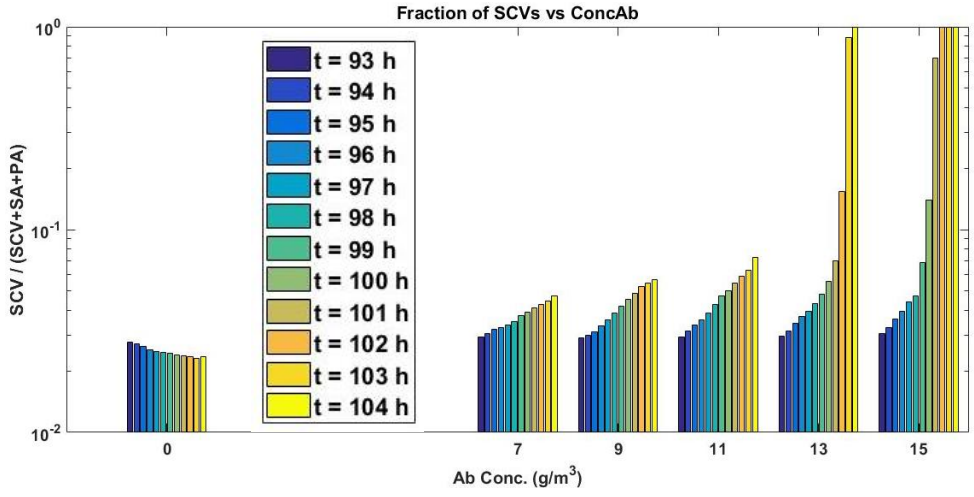


Figure 4.6: Fraction of *S. aureus* SCVs versus time at different antibiotic concentrations

As the *P. aeruginosa* subpopulation is being shielded by the wild-type variants of *S. aureus* and its cell division rate increases with increasing antibiotic concentration (observed in section 4.3), it continues to produce HQNO, in turn improving the probability of phenotypic switching which is dependent on the local HQNO concentration. Consequently, the fraction of SCVs increases with the concentration of antibiotic (Fig. 4.6).

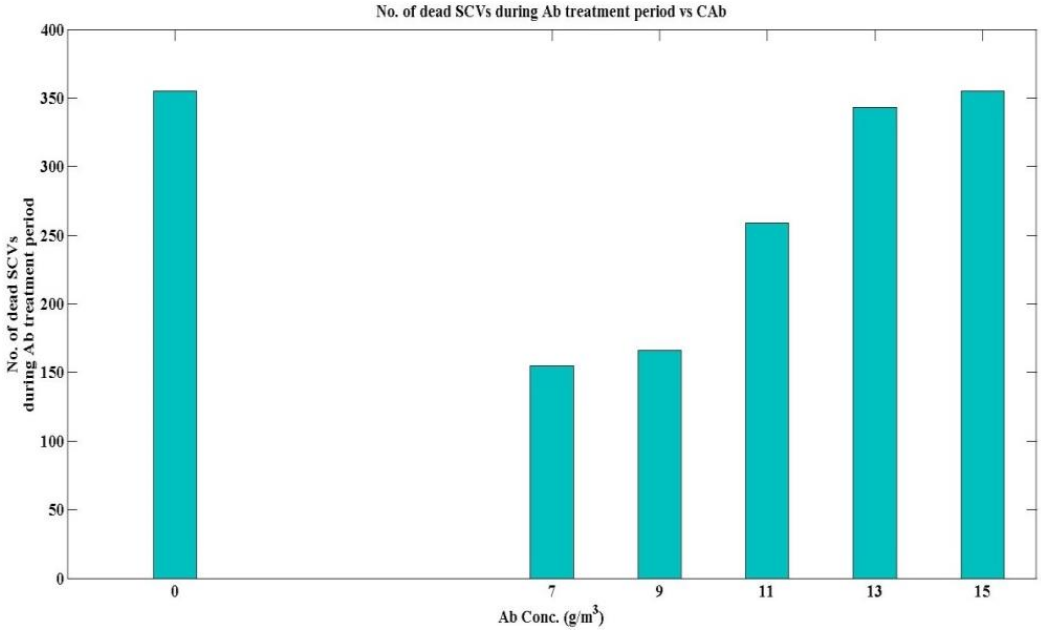


Figure 4.7: Number of dead *S. aureus* SCVs versus antibiotic concentration

Additionally, Fig. 4.7 depicts that SCV-death is lesser in the presence of antibiotic (up to threshold antibiotic concentration) as compared to that in the absence of antibiotic (control run). Hence, in simple words, it can be said that SCV production is potentiated only when a defense mechanism is required.

As the antimicrobial treatment starts, bacteria are killed first near the biofilm-bulk fluid interface. With time, the killing front (defined as the location in the biofilm where the live and dead cell concentrations are equal) progresses inward towards the substratum (Fig. 4.8a). The killing front divides the entire biofilm into two zones: above the killing front, effect of antibiotic is more pronounced whereas below the killing front, cells that survive are more in number compared to those that die.

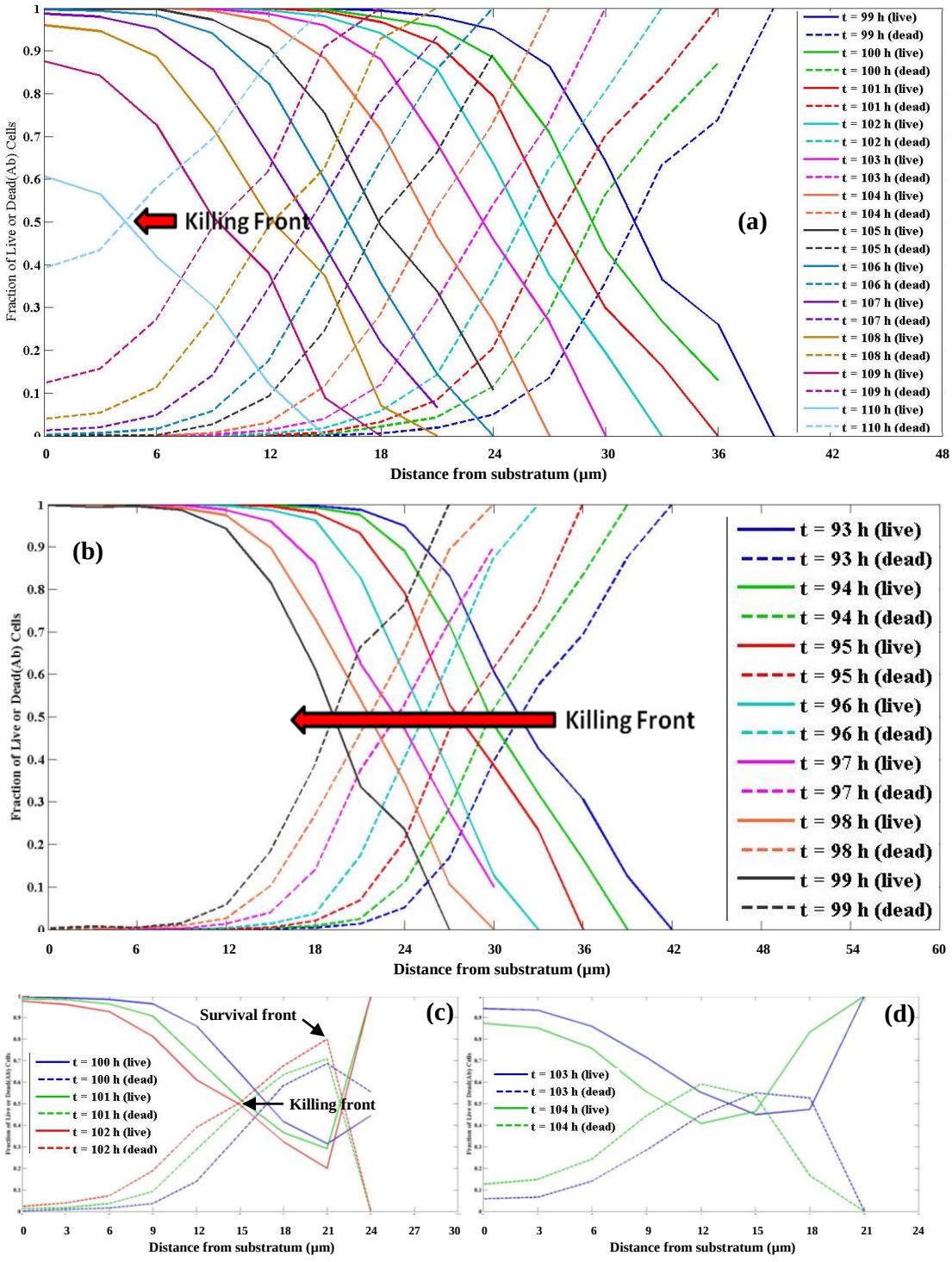


Figure 4.8: Fraction of live and dead (antibiotic) cells versus distance from substratum, (a) in the absence of SCVs, antibiotic treatment from 99 to 110h; (b)-(d) in the presence of SCVs, antibiotic treatment from 93 to 104h

In the presence of SCVs, similar trend was observed for the first few hours (up to 7 hours) of antibiotic treatment (Fig. 4.8b), following which the biofilm was stratified into three zones: (i) below killing front, (ii) above killing front but below survival front, and (iii) above survival front; as opposed to just two in the case with the absence of SCVs. The survival front is characterized by an SCV-dominated zone above it and is the location from where biphasic patterns were encountered (Fig. 4.8c&d). This was because the SCVs that were earlier present within the interior of the biofilm got exposed at the top as the cells above them got killed (Fig. 4.9) and since the SCVs are refractory (higher BIC than wild-type), they don't die immediately.

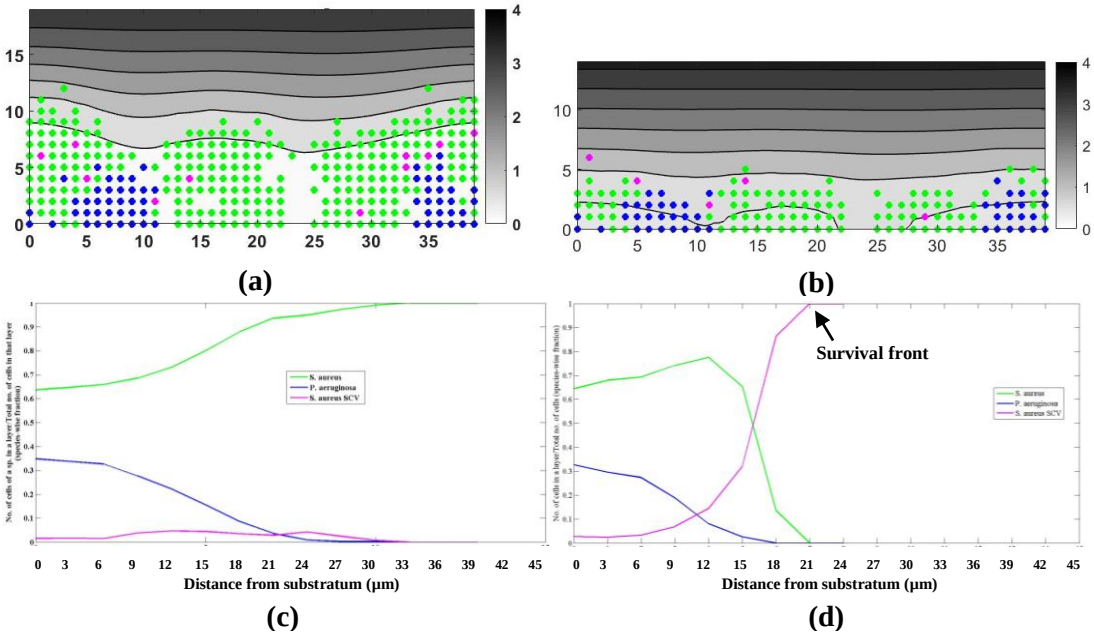


Figure 4.9: 2-D Visualization of live cells [*S. aureus*-WT (green), *P. aeruginosa*-WT (blue), *S. aureus*-SCV (pink)] at (a) 93h, (b) 102h. The isolines indicate the nutrient concentration distribution across the domain. Species-wise fraction of live cells versus distance from substratum at (c) 93h, (d) 102h.

In the absence of SCVs, killing front moves at a velocity of approximately 1.8 μm h^{-1} whereas when the SCVs are present, the velocity is 2.275 μm h^{-1}. The rate at which the killing front advances appears to be determined by the rate at which the antibiotic penetrates into the biofilm (Fig. 4.10); hence this is purely a physical phenomenon. Thus, SCV production retards antibiotic infiltration to some extent.

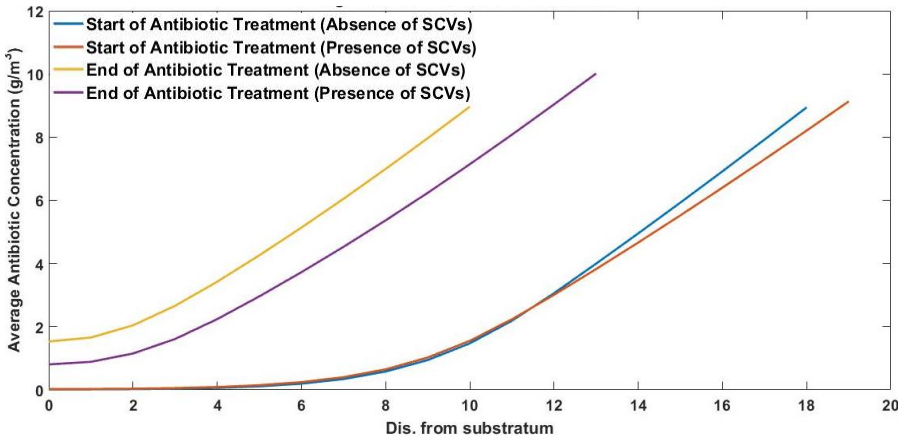


Figure 4.10: Average antibiotic concentration versus distance from the substratum at the start and end of antibiotic treatment for two cases: in the presence and absence of *S. aureus* SCVs

As a result, even after 12 hours of antimicrobial treatment, the death fraction at the bottom of the substratum is lower in the presence of SCVs (12.5%, Fig. 4.8b) when compared to the biofilm without SCVs (40%, Fig. 4.8a).

Chapter 5

Conclusion

A 3-D cellular automata computational model was developed as a tool to investigate the role of small colony variants (SCVs) in the antibiotic susceptibility of polymicrobial biofilms. In general, this model could be utilized for any bacterial population that switches between two different growth phenotypes in response to a molecular signal.

Our investigation revealed that treating a biofilm with antibiotic concentration less than the effective concentration allows the biofilm to regrow and therefore prolongs its lifetime. Interspecific competition plays a significant role in the population dynamics and distribution of species within the biofilm community. For example, faster growing species divest others from growing up to their full potential by depriving them of the growth-limiting nutrient. However, external stress can influence the interspecific competition; for instance, faster growing cells which would have been the dominant species within the biofilm, die faster in the presence of antibiotic. When treated with antibiotic, if the concentration of antibiotic is below the effective concentration, dominant species (S. aureus) shields the minor subpopulation (P. aeruginosa) by hindering antibiotic infiltration up to a certain extent. An added effect to this is improved diffusion of nutrient into the depths of the biofilm enabling the minor subpopulation to grow effectively. However, beyond the threshold antibiotic concentration, cell death rate overpowers cell division rate. Production of SCVs is potentiated in response to antibiotic treatment, indicating that SCV production acts as a defense mechanism against external stresses. The SCVs retard antibiotic permeation due to their refractory nature (higher BICs). As a result, even after using same antimicrobial treatment, the fraction of dead cells at the bottom of the substratum is lower in the presence of SCVs than when compared to a biofilm without SCVs. Thus our simulations collectively demonstrate that presence of SCVs within a biofilm is a plausible mechanism for the antibiotic tolerance of mixed-species biofilms.

Chapter 6

Future Work

Stewart et al. (2005) demonstrate that splitting of bacteria is functionally asymmetric, even in symmetric dividers [65]. Upon division, one progeny inherits the effects of age while the other does not. The hypothesis is that older cells are more tolerant than younger cells to antimicrobial challenge [66]. Thus age structure could be incorporated in the model to test the persistence of biofilms due to senescence.

Another focus of research could be to study the influence of biofilm morphology on antibiotic tolerance. It would involve commencing simulations with pre-determined morphologies- varied shapes, porosities, diffusion distances etc., followed by subjecting the biofilm to external stresses like antimicrobial treatment. The role of different spatial distributions (separate microcolonies, coaggregation or layering) could also be analyzed.

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