Antibiotics Alternative: Investigating the Effect of Bacteriophage Titer on Bacterial Populations

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Abstract

Bacteriophages, viruses that recognize and attack bacteria, have the potential to be used in a variety of different practical settings such as treatment of localized infections and environment disinfection. A major question in this field of research involves why phages are so often ineffective, and one such reason is biofilm, adherent microorganisms surrounded by extracellular polymeric substances that acts as protection. This project investigates the effect of phage titer and pattern of phage introduction on the elimination of bacteria and biofilm in a controlled environment. It was hypothesized that a medium phage titer and a pattern of small clusters would maximize the intended result. A mathematical model of the interaction of phages, bacteria, and biofilm is constructed in `NetLogo`, a computer programming language for agent-based modeling, and data is produced and analyzed in `Wolfram Mathematica`. It was concluded that a specific pattern and titer would optimize the elimination of bacteria and biofilm. This has the potential to have many applications to ensure that the phage are effective in industrial and medical settings.

1 Appendix

1.1 Data Analysis

1.1.1 Experiment 1

The number of phages introduced is tested in this experiment. The numbers of phages tested were 0, 300, 600, 900, 1200, 1500, 1800, and 2100. 0 phages was used as a control, to see on average how much bacteria would grow if phages are not preying on them. The average value was 132.2. The first row of graphs, Figure 4, shows the quantity of phages over time for each trial within each variable. The phages decline in a oscillating pattern because the bacteria lyse and make sharp increases in the population. The second row of graphs, Figure 5, shows the quantity of bacteria killed over time for each trial within each variable. It is observed that most bacteria is killed at the beginning and the rate slows as time progresses. It is also interesting to note that the trial results seems to be more compact as the phage titer increases. This may be due to less randomness in behavior as the titer increases.

Figure 6 graphs the average phage population and bacteria killed over time for each initial phage titer variable value. This exhibits that the increased phage titer kills more bacteria before phage extinction. In all cases, the majority of the phage population is destructed before 50 ticks/minutes. Additionally, most of the bacteria killed by phage die in this time period as well.

The average final values of bacteria killed, ticks, and bacteria remaining at the time of phage extinction are presented in Figure 7 and Table 3. The average number of bacteria killed increases as initial phage population size increases; this measure is the best indication of phage effectiveness, as the results are mostly consistent. A linear model was generated from the bacteria killed data in Figure 8, given by the equation. The R-Squared value of 0.928885 indicates that the approximately 93% of the bacteria killed counts can be explained by the initial phage population size, proving to be an strongly correlated model. The average number of ticks passed was determined to be not an accurate measure of phage effectiveness, since it seems to not show a direct effect from the initial phage population size. This may be due to the high phage death rate that makes the phage population diminish quickly. The average quantity of remaining bacteria is a decent measure of phage effectiveness, and shows a somewhat consistent decreasing pattern, but the correlation is not as strong as the bacteria killed quantity. It is also observed that the increased titer of phage indeed makes the bacterial population smaller than the population would be if unaffected by phage, the control. The smaller initial phage quantities had a greater number of remaining bacteria, but this is likely

due to the control being performed for a longer period of time.

1.1.2 Experiment 2

The number of clusters the initial phage population is divided into is tested in this experiment. The values tested as quantity of phage are 0, 1, 2, 3, 4, 5, and 6 where 0 clusters indicated random dispersion like that of Experiment 1. The first row of graphs, Figure 9, shows the quantity of phages over time for each trial within each variable. With the clusters, the phage population has a much sharper population decline at the beginning than without clusters. The second row of graphs, Figure 10, shows the quantity of bacteria killed over time for each trial within each variable. The curve follows the same shape as that with no clusters, where most bacteria is killed at the beginning, but there is a much more shallow increase. These two observations are likely due to the fact that the clusters cannot cover as much surface area to prey on bacteria and reproduce.

Figure 7 graphs the average phage population and bacteria killed over time for each initial phage titer variable value. This graph shows that the phages were able to survive better and kill more bacteria when there were no clusters involved. The shape of the curves are very different between the two distribution types of random and clustered, as described previously.

The average final values of bacteria killed, ticks, and bacteria remaining at the time of phage extinction are presented in Figure 12 and Table 4. Again, the bacteria killed is the most representative of phage effectiveness. No clusters and random distribution proved to be the most effective in killing bacteria as compared to those using clusters. If using clusters, the most clusters possible, six, was killed the most bacteria. As the quantity of clusters increased, the bacteria killed increased for the most part. The results for two clusters is likely an outlier, since it does not follow the quantitative pattern. A linear model was generated from the bacteria killed data in Figure 13 given by the equation: $2.013 + 1.834x$. The R-squared value was 0.764749 indicating that it is a moderately correlated model, which is not as strong as the model for initial phage population. As with the first experiment, tick count is not a representative value for phage effectiveness, so it is ignored in analysis. As with before, the average quantity of remaining bacteria is a decent measure of phage effectiveness. The remaining bacteria of the random dispersion is lower than all the values for the clustered introduction, indicating that the initial random distribution is more effective than initial clustered distribution. The results for two clusters do not fit the pattern of decline within those that are clustered likely due to the fact that the simulation had a longer run time, tick count, before extinction.

1.2 Simulation Procedure

1. Setup-Clear: Set up all the initial conditions and reset the simulation to start anew
    - Setup-World: Sets shape of phage and bacteria
    - Create-Some-Bacteria: Creates bacteria according to num-bacteria at random locations, also setting their size, color, and a random age
    - Create-Some-Phage: Creates phages according to num-phage, if clusters? is on, the population will be split into groups of num-clusters and placed into specific xy coordinates
    - Reset-Ticks: Ticks are the unit of time in `Netlogo`, this procedure resets the tick counter to 0
2. Go: this series of procedures occur at a specified time or repeatedly at every tick
    - Ask-Phage
        (a) Move-Phage: Rotate in a random direction, if the patch ahead by phage-in-speed has no biofilm, then allow the phage to move at the phage-out-speed, otherwise destroy the biofilm
            – Destroy-Biofilm: Destroy layer of biofilm ahead; probability determined by biofilm-destroy-rate
        (b) Eat-Bacteria: Prey on bacteria if in same patch, according to lyse-probability, the prey will lyse with the phage-lyse and burst-size parameters (phages do not actually "eat" bacteria)
        (c) Kill-Phage: Naturally deteriorate over time; phage-death-rate determines probability of destruction
    - Ask-Bacteria
        (a) Reproduce-Bacteria: Bacteria reproduce through binary fission, producing two identical daughter cells once their age reaches split-time
        (b) Move-Bacteria: Rotate in a random direction and move forward at the bacteria-speed
        (c) Kill-Bacteria: Die according to bacteria-death-rate; this value was chosen as the minimum for the bacterial population to grow and not naturally die out without phage present
        (d) Regrow-Biofilm: Grow biofilm as bacteria moves according to biofilm-growth-rate

- Tick: ticks are the measure of time within a `NetLogo` Simulation, so after all procedures are completed, tick will move time forward
- Stop: The simulation will automatically stop when the phage population reduces to 0

Control: The number of bacteria after the average number of ticks (129) with no phage is 132.2

All pictures, charts, and graphs were created by myself, Jessica Shi, using `Wolfram Mathematica` and `NetLogo`.

Bacteriophages or phages are viruses that only infect and kill bacteria. They are unable to exist on their own; instead using bacteria as a vector for reproduction [2]. When a lytic phage or virulent phage lands on a bacterial cell, it injects its genetic information into the bacterial cell and uses the cell's interior mechanisms to make copies of itself. Eventually, the cell will lyse, and the phages will diffuse [14].

Biofilm is developed when microorganisms, like bacteria, attach to surfaces. The cells form an extracellular polymeric surface (EPS) matrix, which is a slimy substance used for protection [4]. They may cause problems like in pipes or on medical implants and are also the cause of many human infections and plant infections [5]. In fact, they account for 80% of all bacterial infections in the body, still little is known about them. Antibiotics have also proven to be widely ineffective in biofilm-associated infections [16].

Phages have huge potential in a medical application through phage therapy, the treatment of bacterial infections using phages. Phages can target specific species of bacteria, allowing it to avoid falsely attacking the beneficial microbe that is essential to our health. They also have the ability to attack antibiotic-resistant bacteria, which has become a prevalent and growing issue in global health [8]. Phages have also shown potential in removing infectious biofilms. Some phages carry surface enzymes that can degrade bacterial polysaccharides, but there is still much impracticality of the methods. In previous studies, bacterial counts were reduced by the phage, but not completely eliminated [12].

NetLogo provides a programmable environment for agent-based modeling. It can support multiple agents, that are stationary patches, mobile turtles, and the observer. Patches are lattice sites, specifically two-dimensional squares on a continuous plane. In most simulations, turtles move from patch-center to patch-center. Agents can interact with each other. For instance, the observer issues instructions for the patches and turtles, and the turtles can interact with the patches [15]. Agent-based modeling is particularly useful in modeling change in complex systems over time [13]. NetLogo also has an integrated user-interface, which allows one to create buttons, sliders, monitors, plots and other objects that allows the observer to watch and modify the setup.

Previous models have been created to show the interaction of biofilm and phages. In 2018, researchers created an agent-based model to study this relationship and concluded there were three possible outcomes: biofilm death, coexistence and phage extinction. They found that three key parameters had the greatest effect on the phage infection: environmental nutrient concentration, phage burst size, and the relative diffusivity of phages within biofilms [11].

2 Problem

What is the effect of phage titer and introduction pattern on bacterial populations in biofilm?

One factor that may affect the effectiveness of phages in biofilm is how and how much phages are introduced. In this project, both the titer or amount of phage and the pattern of introduction, specifically, small clusters of phage, are tested.

3 Hypothesis

If phage are introduced in a biofilm, then the most effective pattern would be clustered in several groups and at a high titer. The clustered groups would increase the probability that a phage comes in contact with a bacteria, therefore increasing the chance of lysis and phage reproduction. The more groups, to a practical maximum, and the higher the titer, the more effective the phage will be in eliminating bacteria.

Figure 5: Bacteria killed-time plot for Experiment 1

parameter	definition	values	units	reference
num-phage	The number of phage at start	Variable	phages	
cluster?	Whether or not the phage are introduced in clusters or randomly	On or Off		
num-clusters	Number of clusters the phage are split into at start	Variable $1-6$	clusters	
num-bacteria	Number of bacteria at start	100	bacteria	
death-rate-phage	If random number (1-100) $<$ this value, phage is destroyed	10	%	
biofilm-growth-rate	If random number (1-100) $<$ this value, biofilm grows in patch of bacteria	50	%	[3]
biofilm-destroy-rate	If random number (1-100) $<$ this value, phage will destroy layer of biofilm in patch ahead	30	%	[6]
death-rate-bacteria	If random number (1-100) $<$ this value, bacteria dies	3	%	
lyse-probability	If random number (1-100) $<$ this value, when phage contacts bacteria, bacteria lyses	40	%	
burst size	Distance phage travels when the bacteria first lyses	2	patches	
split-time	Number of ticks the bacteria will split/reproduce	20	minutes	[1]
phage-lyse	Number of phages that are produced from lyse	100	phages	[11]
bacteria-speed	Distance traveled by bacteria in one tick	1	patches	
phage-out-speed	Distance traveled by phage in one tick when patch ahead lacks biofilm	0.6	patches	[11]
phage-in-speed	Distance traveled by phage in one tick when patch ahead is biofilm	0.3	patches	[11]

Table 1: Parameters in simulation model

Experiment	Independent variable	Value 1	2	3	4	5	6	7	Additional Constants
Experiment 1	Initial phage population size	300	600	900	1200	1500	1800	2100	Clusters?: Off
Experiment 2	Number of clusters	0 (random dispersion)	1	2	3	4	5	6	num-phage: 2100

Table 2: Experiment and Variable Description

Initial Phage Population Size	300	600	900	1200	1500	1800	2100
Average # of Bacteria Killed	8.2	20	28.4	34.6	48	44.4	50.6
Average # of ticks passed	118	161.7	149	120.8	140.8	114.8	147.8
Average # of remaining bacteria	166.8	158.3	150.8	102.2	106.8	80.6	83.4

Table 3: Final Results for Initial Phage Population Size

Initial Cluster Quantity	0	1	2	3	4	5	6
Average Bacteria Killed	48	2.8	8.2	75.2	9.4	12.6	12.2
Average of ticks passed	140.8	101.2	160.6	109.6	121	139.4	131.6
Average of remaining bacteria	106.8	150.4	244	162	140.6	150.6	137.8

Table 4: Final Results for Initial Cluster Quantity

4 Model

The space tested is approximately 80 x 40 μm, relative to the size of the bacteria and phage, which are estimated to be 0.5 x 2 μm and 200nm respectively. [10] There are three agents in this model: the phages, bacteria, and biofilm EPS. The EPS, the patches, exhibits a characteristic of 4 layers each with a value of .1 and a shade of yellow. If the patch is a brighter yellow, there is more EPS present, and if the patch is black, no EPS is present. The bacteria used follow the parameters of E. Coli. They move, reproduce through binary fission, produce more EPS, and naturally die. It is assumed that the phages are lytic, move slower within biofilm, and contain enzymes that can destroy polysaccharides. The phages in the model move, destroy biofilm, reproduce using the lytic cycle killing bacteria, and naturally die.

5 Experiments & Procedure

To test our variables, there are two experiments. In each trial, the simulation is run until the phage population is extinct. The simulation is repeated five times for each variable. As NetLogo is running, Wolfram Mathematica records the data at each tick for the size of the biofilm EPS, bacterial population, phage population, and bacteria killed. This information is used for data analysis, as bacteria killed, time until phage extinction, and remaining bacteria at extinction may be useful to determine the effectiveness of the phage. Additional parameters used in the simulation and the values chosen to remain constant are within Table 1. The independent variables are pattern and quantity of phage introduction, and the values tested are shown in Table 2.

Labels

Figure 1: Interface of phage-biofilm simulation in NetLogo

Figure 2: Population dynamics of example simulation

Figure 3: Bacteria lysing and reproducing phage in simulation

Figure 4: Phage-time plot for Experiment 1

Figure 6: Phage-bacteria killed-time plot for Experiment 1

Figure 7: Final results bar charts for Experiment 1

Figure 8: Linear model for bacteria killed in Experiment 1

Figure 9: Phage-time plot for Experiment 2

Figure 10: Bacteria killed-time plot for Experiment 2

Figure 11: Phage-bacteria killed-time plot for Experiment 2

Figure 12: Final results bar charts for Experiment 2

Figure 13: Linear model for bacteria killed in Experiment 2

6 Results

6.1 Experiment 1: Initial phage population size/Titer

6.2 Experiment 2: Initial phage introduction cluster quantity

An agent-based simulation model was constructed to measure the effectiveness of phages to combat infectious bacterial biofilm. Initial phage titer or population size and initial clustering pattern were tested to determine their effect on the bacteria killed, time passed, and bacteria remaining at phage extinction. When testing initial phage titer or population size, the more phage introduced, the more bacteria were killed and less bacteria remained at phage extinction. This supports the hypothesis that the higher titer amount will improve phage effectiveness. Due to the increased quantity, the phages cover more surface area and are at a greater quantity, increasing the probability that the phages will contact a host bacterium and proceed to reproduce itself. Then, the experimental results from the random initial phage distribution simulation was compared to the one that the initial phage concentration is divided into clusters. The random dispersion produced significantly higher quantities of bacteria killed, proving that random dispersion is more effective than a clustered distribution. This disproves the hypothesis that the clusters would be the most effective. When comparing the tests between the cluster quantities, the initial configuration with most clusters killed the most bacteria, which agrees with the hypothesis that more clusters is more effective.

As this is a computer simulation and the behavior of agents is artificial; there are several other variables occurring in real life that may affect results. Some of the factors that were not considered in experimentation are listed below:

- All the other variables that were included in the simulation, but held constant, listed in Table 1 may have an effect on the results and can vary depending on situation.
- During the lytic cycle there is an incubation period after phage infection before lysis occurs [11].
- Quorum sensing occurs in bacteria and regulates gene expression in response to changes in population density. This is a way that bacteria can communicate with each through autoinducers affecting bacterial reproduction [7].
- Bacterial phage resistance through spontaneous mutations, restriction modification systems, and adaptive immunity may increase in occurrence with increased phage titer [9].
- Nutrient concentration affects bacterial division and growth and can vary by location. In addition, bacteria in this model grow in an uncontrolled exponential model, where in real-life they are more likely to grow in a logistic model with a carrying capacity due to limited resources [11].

This simulation and experimentation has potential applications in many fields that use phages. For instance, it can be used in deciding how much and in what manner phage therapy would be the most effective in treating infections. Similarly, evidence from computer simulation would be helpful in making decisions on phage use in food, industrial, and other medical applications.

7 Spatial Model of Phage-Bacteria-Biofilm Interaction

A reaction-diffusion model was created to describe the dynamics of phage ($P(x,t)$), bacteria ($B(x,t)$) and biofilm ($F(x,t)$). The density functions $P(x,t)$, $B(x,t)$ and $F(x,t)$ are defined over a two-dimensional bounded spatial domain Ω and time interval $[0,T]$, so $x=(x_1,x_2)$ is a 2D location. For simplicity, one may choose $\Omega=(0,a)\times(0,b)$, a rectangle.

- Phage dynamics: Phages are generated by the cell lysis initiated by bacteria, so it is assumed that the lysis rate is proportional to both the phage and bacteria densities $+k_1PB$, and the phage decays at a rate $-d_PP$. Phage particle also diffuses with a diffusion coefficient D_P, and D_P depends on the density of biofilm at the location: more biofilm slows down the movement of phage.
- Bacteria dynamics: Bacteria grows following a logistic model $+b_BB\left(1-\frac{B}{N}\right)$, where b_B is the growth rate per capita and N is the carrying capacity (maximum bacteria density allowed by the space); the bacteria is killed by the phage at a rate $-k_2PB$ (this process does not affect phage population), and the bacteria also has a natural death rate $-d_BB$. Bacteria also moves diffusively with diffusion coefficient D_B.
- Biofilm dynamics: Biofilm also grows following logistic rule $b_FBF\left(1-\frac{F}{M}\right)$, where b_F is the growth rate per capita and M is the biofilm carrying capacity (maximum biofilm density allowed by the space); the biofilm is killed by the phage at a rate $-k_3PF$ (this process does not affect phage population), and the biofilm also has a natural death rate $-d_FF$. The biofilm does not move.

These processes are summarized in the equation (1), with all variables and parameters in (1) are listed in Table 5 and Table 6 respectively.

$$\begin{cases} \dfrac{\partial P}{\partial t} = \underbrace{D_P(F)\Delta P}_{\text{phage diffusion}} \quad \underbrace{+k_1PB}_{\text{phage generated by lyse}} \quad \underbrace{-d_PP}_{\text{death}}, & x\in\Omega,\ t>0, \\ \dfrac{\partial B}{\partial t} = \underbrace{D_B\Delta B}_{\text{bacteria diffusion}} \underbrace{+b_BB\left(1-\frac{B}{N}\right)}_{\text{bacteria growth}} \underbrace{-k_2PB}_{\text{bacteria killed by phage}} \quad \underbrace{-d_BB}_{\text{death}}, & x\in\Omega,\ t>0, \\ \dfrac{\partial F}{\partial t} = \underbrace{b_FBF\left(1-\frac{F}{M}\right)}_{\text{biofilm growth}} \underbrace{-k_3PF}_{\text{biofilm killed by phage}} \quad \underbrace{-d_FF}_{\text{death}}, & x\in\Omega,\ t>0. \end{cases} \tag{1}$$

variable	meaning	units
$x = (x_1, x_2)$	space point	μm
t	time	s
P	phage density	$mg/\mu m^2$
B	bacteria density	$mg/\mu m^2$
F	biofilm density	$mg/\mu m^2$

Table 5: Variables in Model (1)

parameter	meaning	units	values	reference
b_B	bacteria split rate	s^{-1}		
b_F	biofilm growth rate	$s^{-1}mg^{-1}\mu m^2$		
d_P	phage death rate	s^{-1}		
d_B	bacteria death rate	s^{-1}		
d_F	biofilm death rate	s^{-1}		
k_1	lyse rate			
k_2				
k_3				
N	bacteria carrying capacity			
M	biofilm carrying capacity			
D_P	phage diffusion coefficient			
D_B	bacteria diffusion coefficient			

Table 6: Parameters in Model (1)

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