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Cover Page Footnote

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Parameter Sensitivity for In Vitro Anthrax Studies

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Abstract

Studies done on interactions between spores and macrophages done *in vitro* show inconsistent results based on experimental protocol and inhibit meaningful extrapolation to *in vivo*. In this study, we perform a sensitivity analysis of a model representing *in vitro* studies of interactions between anthrax spores and macrophages to help address the effects of these inconsistencies. We perform both local and global analyses using Latin hypercube sampling and partial rank correlation coefficients. Our analysis indicates the amount of intracellular bacteria over time is most sensitive to the killing of intracellular bacteria by the macrophages and replication of the bacteria inside the macrophage. Extracellular germination rates are shown to affect the amount of intracellular bacteria only during the incubation period, due to the assumption that any remaining extracellular bacteria is assumed to be washed away after the incubation period.

Keywords: anthrax, germination, *B. anthracis*, *in vitro*, bacterial replication, bacterial killing

1 Introduction

B. anthracis is a gram-positive, endospore-forming bacterium. It is an extremophile producing endospores which allow the bacterium to tolerate extreme heat, cold, and dehydration, making it exceptionally difficult to kill. Living organisms become infected one of three ways: through inhalation of the *B. anthracis* spores, through the skin, or gastrointestinal infection [26]. Inhaling the anthrax spores has been found to be the most lethal, whereas cutaneous and gastrointestinal has an overall higher survival rate among humans [26]. This study will focus on the more lethal form of anthrax infection, inhalation anthrax. If an excess number of spores are inhaled into the host, it is very probable that the infected person, without treatment, will die due to septicemia or swelling of the lymph nodes. Both *in vitro* and *in vivo* models have provided valuable information about disease progression. Previous mathematical models more commonly focus on *in vivo* responses to inhalation anthrax including dose-response curves [15] [9] [27], lung deposition and clearance rates [10] [25], and immune system response [2]. Mathematical models for *in vitro* to *in vivo* extrapolation can be found for other medical conditions such as liver injury [13] and there have been recent efforts to quantify parameter values for inhalation anthrax *in vitro* studies [22]. We intend to expand the current literature by highlighting key differences in the two types of studies and quantify the parameter sensitivity of our *in vitro* model outcomes. Assessing

values that drive disease progression such as intracellular bacteria counts and infected macrophages can be challenging *in vivo* due to the process of extracting cells from lungs of sacrificed animals. Accordingly, *in vitro* experiments can provide insight into parameters driving disease progression, but extrapolation from *in vitro* to *in vivo* is the most useful when variations in experimental protocols are addressed.

1.1 Disease progression

When *B. anthracis* spores are inhaled, they are rapidly engulfed primarily by alveolar macrophages; a process known as phagocytosis. Macrophages are leukocytes that are an essential part of the host immune response. During phagocytosis, macrophages identify and target the invading spores. The macrophages engulf the spores in attempts to fully digest the foreign bodies. Although they are foreign, *B. anthracis* spores are harmless and non-toxic to the cells prior to being engulfed. It is not until the spores are engulfed by macrophages and begin germination and replication that the spores become a threat to the host [28]. As soon as phagocytosis begins, the *B. anthracis* spores begin to germinate and become toxin-producing, vegetative bacteria. As the spores are germinating, the alveolar macrophages continue to migrate from the lung and into the thoracic/mediastinal lymph nodes [2]. If the macrophages are unable to kill the spores early on, the macrophages will become overwhelmed and lyse due to an excess amount of toxic, vegetative bacteria. Once a macrophage has ruptured, the intracellular

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bacteria become extracellular bacteria by releasing into the extracellular environment. *In vivo*, bacteria, in the extracellular environment, will release toxins that will retard the body’s immune response. As toxins increase in concentration, more macrophages will rupture and release additional bacteria, eventually leading to septicemia [23].

1.2 Comparisons of *in vitro* and *in vivo*

There exists variability among and between *in vivo* and *in vitro* studies and general inconsistencies in methodologies among anthrax studies that need to be considered. *In vitro* studies are not able to perfectly mimic the conditions that occur *in vivo*. Typically, during *in vivo* studies, the *B. anthracis* spores are observed within live specimen, primarily mammals whereas *in vitro* studies house their experimentations primarily on petri dishes within a medium. Anthrax spores will not germinate *in vivo* prior to being taken up by a phagocyte, while mediums used in *in vitro* studies often provide an optimal environment for the germination of spores. Additions to the medium can also cause the germination rates of the spores to be accelerated or decelerated. For instance, it was observed that 96% of Sterne strain anthrax spores germinated within the first hour in a Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% fetal bovine serum (FBS), but only 2% of the same strain germinated in DMEM + 10% horse serum within the same time frame [28]. Additionally, bacteria that germinate in the medium prior to being phagocytosed may have a higher probability of being killed by the macrophage [8]. After an initial incubation time to allow phagocytosis of spores by macrophages, some studies will wash away any extracellular spores and bacteria that were not phagocytosed [23, 28]. After this washing, studies focus on the interaction between macrophages and spores once internalized. The effective multiplicity of infection may be altered from the original plating after this washing. In contrast, *in vivo*, spores lie dormant within the body until they are phagocytosed by the macrophages. An additional variation between *in vitro* and *in vivo*, is that *in vivo* the bacteria, after the germination stage, will secrete toxins that will restrict the host immune response making *B. anthracis* difficult to destroy [6]. The toxins are composed of a receptor-binding subunit protective antigen (PA) and two enzymatic subunits known as edema factor (EF) and lethal factor (LF). Any one of the subunits alone will result in no morphological change in the host cells [4], however, when PA is combined with either LF or EF, the newly formed complex results in two active toxins, lethal toxin (PA + LF) and the edema toxin (PA + EF) [3].

We summarize in Table 1 factors that have been shown to affect parameter rate of *in vitro* studies.

Parameter	Affected by	Cite
Phagocytosis (p)	anti-PA (+)	[28]
	macrophage strain	[14, 21, 24]
Germination (extracellular (g_e))	anti-PA (-)	[28]
	medium	[8, 14, 16]
	spore strain	[8]
Germination (intracellular (g_i))	(k)anti-PA (-)	[28]
	spore strain	[14]
Killing rate (k)	anti-PA (+)	[28]
	germination (+)	[8]
	macrophage strain	[8]
	spore strain	[14]
Spores per macrophage (M)	macrophage strain	[11, 19]

Table 1: Factors affecting parameter values. A plus sign (+) indicates documented increase in the parameter, minus sign (-) indicates documented decrease in the parameter, no sign indicates the parameter can be positively or negatively affected.

2 Model Description

We create a model to capture the dynamics of a typical *in vitro* model. We assume extracellular spores (S_e) and healthy macrophages (M_h) are plated together in a medium and allowed to incubate for one hour, as indicated by $1 - u_1(t)$ term where $u_c(t) = u(t - c)$ is the Heaviside step function. During that one hour, extracellular spores (S_e) are phagocytosed at a rate p by healthy macrophages, M_h , using a mass action model (pS_eM_h) and become intracellular spores (S_i) creating infected macrophages (M_I). Once inside the macrophage (M_I), spores begin germinating at a rate g_i and become intracellular bacteria (B_i). Spores can also germinate extracellularly at a rate g_e , becoming extracellular bacteria (B_e) and then be phagocytosed at the same rate p as spores. After the first hour of incubation, we assume all extracellular spores (S_e) and bacteria (B_e) are washed away. We assume intracellular spores (S_i) continue to germinate at the same rate as in the first hour (g_i). Qualitative support for bacteria killing (k) and replication (r_i) are more abundant in the literature than quantitative support, therefore we make a simplifying assumption that killing of bacteria at a rate k does not commence until after the first hour of incubation [8, 14, 17]. Ruthel [24] found replication of bacteria (r_iB_i) visible at 5–6 hours, therefore we have a delay in replication at a rate r_i of bacteria to commence at 5 hours after the initial incubation period. It is possible

that macrophages would burst in the time period we consider, but we do not track bursting macrophages and assume any bacteria that would escape from a macrophage and continue to replicate is accounted for in the intracellular bacteria count (B_i). Lab experiments are able to show macrophages infected by spores and visually inspect plates for germinated spores, both intra- and extracellularly as elongated bacteria [28], therefore we track all macrophage, spore, and bacteria counts as populations.

$$\begin{aligned}
 S'_e(t) &= \overbrace{(1 - u_1(t))}^{\text{first hour}} \left(\underbrace{-pS_eM_h}_{\text{spore phagocytosis}} - \underbrace{g_eS_e}_{\text{extracellular germination}} \right) \\
 S'_i(t) &= \overbrace{(1 - u_1(t))}^{\text{first hour}} \left(\underbrace{-pS_eM_h}_{\text{spore phagocytosis}} - \underbrace{g_iS_i}_{\text{intracellular germination}} \right) \\
 B'_e(t) &= \overbrace{(1 - u_1(t))}^{\text{first hour}} \left(\underbrace{g_eS_e}_{\text{extracellular germination}} - \underbrace{pB_eM_h}_{\text{bacteria phagocytosis}} \right) \\
 B'_i(t) &= \overbrace{(1 - u_1(t))}^{\text{first hour}} \left(\underbrace{pB_eM_h}_{\text{bacteria phagocytosis}} + \underbrace{g_iS_i}_{\text{intracellular germination}} \right. \\
 &\quad \left. - \underbrace{u_1(t)k B_i}_{\text{bacteria killing starting at hr 1}} + \underbrace{u_5(t)r_i B_i}_{\text{bacteria replication starting at hr 5}} \right) \\
 M'_h(t) &= \overbrace{(1 - u_1(t))}^{\text{first hour}} \underbrace{(-p(S_e + B_e)M_h/M)}_{\text{spore and bacteria phagocytosis}} \\
 M'_i(t) &= \overbrace{(1 - u_1(t))}^{\text{first hour}} \underbrace{(p(S_e + B_e)M_h/M)}_{\text{spore and bacteria phagocytosis}} \tag{1}
 \end{aligned}$$

2.1 Baseline parameter values

While *in vitro* studies can vary greatly in their quantitative conclusions that can help direct parameter value choice, we strive to pick initial parameter values that represent many of the studies. We initially consider values from experiments using the Sterne spore strain and mouse primary macrophages cell lines including RAW 264.7 with medium DMEM +10% FBS at a multiplicity of infection (MOI) of 1:1 ($S_e(0) = S_0 = M_h(0) = 10^6$). Gut [8] found that the uptake of spores is consistent between germinating and non-germinating environments, therefore we assume the same phagocytosis rate (p) for both extracellular spores and bacteria such that 80% of the initial spores plated are internalized within one hour [14] with an average of 3.38 bacteria per macrophage (M) [24].

Germination rates vary greatly with extracellular germination (g_e) ranging from 0 to 100% in one hour depending on medium [8]. We select those consistent with the experiment protocol of choice with .976 for extracellular germination (g_e) [8] and .366 for intracellular germination (g_i) [16]. We initially use Pantha's [22] parameter values for a model fitted to data from Kang [17] for the killing (k) and replication rate (r_i).

3 Sensitivity Testing

Anthrax develops when *B. anthracis* spores are able to germinate and survive to replicate inside the host cell and eventually escapes into the lymph nodes and enters the blood stream [12]. Intracellular bacteria counts for *in vitro* studies can provide insight into both the time to disease development, and factors that allow bacteria to escape killing and proliferate. Intracellular germination and phagocytosis are well documented in the literature [28][21][5][1][14] and our primary interest is the affects of extracellular germination and phagocytic capacity on killing and replication rate, therefore we focus our analysis analysis on extracellular germination (g_e), phagocytic capacity (M), macrophage rate of killing bacteria (k), and bacteria replication rate (r_i).

3.1 One at a time sensitivity

First we vary each parameter of interest and estimate the derivative at either the 1-hour time point, 12-hour time point, or both using finite differences [30]. In Figure 1, we show graphs of the intracellular bacteria, B_i over time for different extracellular germination rates and corresponding estimates for the derivative of B_i with respect to the germination parameter. For the first hour, we normalize with respect to the initial spores load, S_0 , then, for time points after one hour, we normalize with respect to the intracellular spore and bacteria count at $t = 1$, due to the assumed washing of all extracellular material after 1-hour of incubation. We show similar graphs for spores/macrophage, killing, and replication in Figures 2 and 3. Since we assuming no killing before the 1-hour time point, and no replication before the 5-hour time point, we adjust those graphs accordingly, and only show the derivative estimate at $t = 12$.

We notice the greatest amount of sensitivity to the killing rate k and intracellular replication rate r_i in Figure 3. Figure 3(a) shows the amount of intracellular bacteria at a given time point as a percent of the number of intracellular spores and bacteria when the washing occurred at hour one ($B_i(t)/(B_i + S_i)(1)$), showing multiple time series graphs for varied killing rate, k values. We notice with a killing rate k close to zero, the amount of extracellular nearly doubles within 6 hours after washing

Parameter	Value	Units	Description	Minimum	Maximum	Citation
p	1.88×10^{-6}	/macrophage-hr	Phagocytosis rate	0	1×10^{-4}	[14]
g_e	0.976	/hr	Extracellular germination rate	0	1	[8]
g_i	0.36	/hr	Intracellular germination rate	0	1	[16]
M	3.68	spores/macrophage	Phagocytic capacity	1	8	[24]
k	2.0398	/hr	Killing rate	0	3	[22]
r_i	1.3424	/hr	Replication rate	0	3	[22]

Table 2: Baseline parameter values and range for sensitivity analysis.

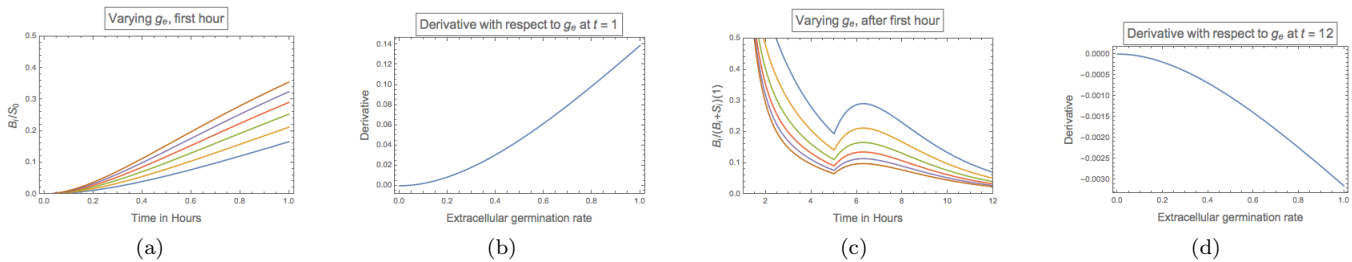


Figure 1: (a) B_i time course for the first hour with varied g_e values in range 0 to 1. (b) Finite difference estimate of change in B_i/S_0 with respect to g_e at $t = 1$. (c) B_i time course after the first hour with varied g_e values in range 0 to 1. (d) Finite difference estimate of change in $B_i/(S_i + B_i)(1)$ g_e at $t = 12$.

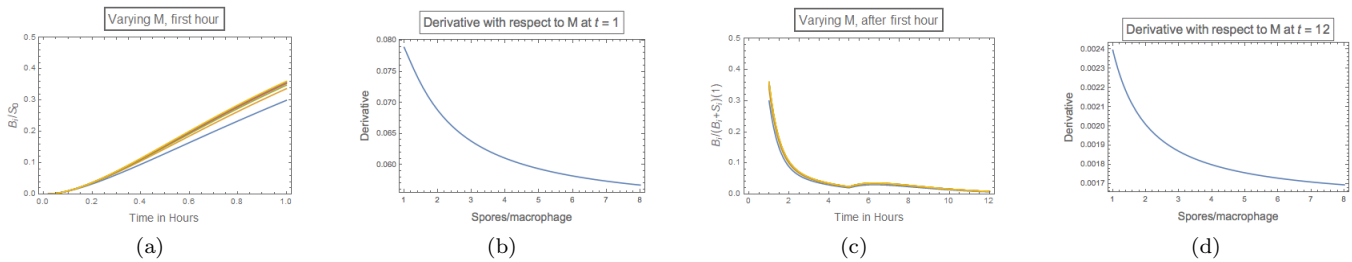


Figure 2: (a) B_i time course for the first hour with varied M values in range 1 to 8. (b) Finite difference estimate of change in B_i/S_0 with respect to M at $t = 1$. (c) B_i time course after the first hour with varied M values in range 1 to 8. (d) Finite difference estimate of change in $B_i/(S_i + B_i)(1)$ with respect to M at $t = 12$.

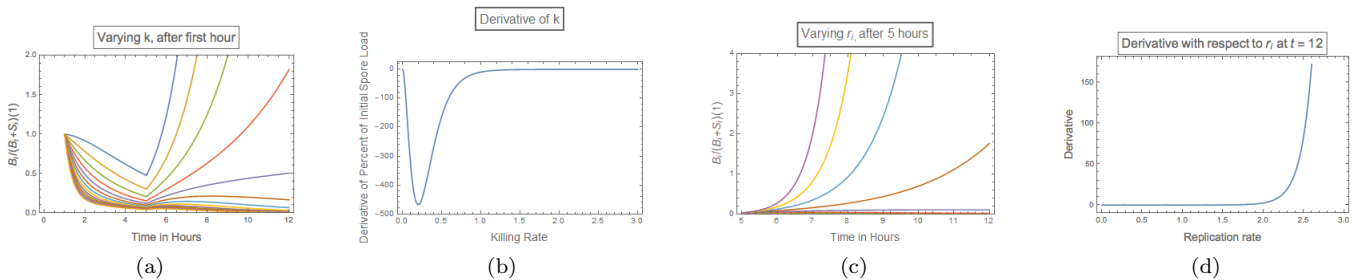


Figure 3: (a) B_i time course after the first hour with varied k values in range 0 to 3. (b) Finite difference estimate of change in $B_i/(S_i + B_i)(1)$ with respect to k at $t = 12$. (c) B_i time course after the five hours with varied r_i values in range 0 to 3. (d) Finite difference estimate of change in $B_i/(S_i + B_i)(1)$ with respect to r_i at $t = 12$.

as seen by the blue uppermost curve in 3(a). In contrast, at higher killing rates, the bacteria growth is much more contained as shown by the purple curve and lower curves that indicate bacteria is decreased to less than half of the value at one hour. Similarly, we see in Figure 3(c), when the replication rate r_i is close to zero, bacteria levels are kept low, as shown by the lower time series graphs, but quickly escape containment when replication increases as seen by the higher blue, yellow, and purple curves.

While the percent of extracellular spores that became intracellular bacteria within the first hour varied with extracellular germination rates g_e from 0.16 to 0.35 as seen in Figure 1(a), the variation seems to be restricted to the incubation period as similar variation is not shown after the first hour in Figure 1(c). This may be misleading, though, since Gut [8] indicates killing rate may be increased in germinating mediums, therefore the sensitivity of B_i to k may be an indirect result of an increased g_e . Our sensitivity analysis indicates, that while the impact of extracellular germination needs to be considered when using *in vitro* models to inform *in vivo* studies, the results after the initial incubation period are not especially sensitive to varied extracellular germination rates. Additionally, Ruthel [24] indicates killing may also be reduced with increased macrophage spore load, M . Therefore sensitivity due to varied killing rate may also be an indirect result of varied spore loads, even though our results indicate very little change in intracellular bacteria counts with varied spores per macrophage as seen in Figure 2(a).

3.2 Latin hypercube sampling and partial rank correlation coefficient analysis

In order to better understand the interactions among the parameters, we perform a Latin hypercube sampling (LHS) procedure with partial rank correlation coefficient (PRCC) analysis [7, 18, 20]. For the LHS, we pick our parameters from a uniform distribution, with minimum, maximum, and baseline values for each parameter listed in Table 2. We perform 200 simulations to create a parameter sample space. Figure 4 shows the monotonicity plots for the parameters of interest at $t = 12$. We notice the relationship between B_i and the parameters of interest is monotonic, therefore the PRCC analysis is valid. Monotonicity plots for the other time points in Table 3 are shown in the Appendix.

For each parameter set created by the LHS scheme, we convert the raw parameter values to ranked values and find two linear regression models. One model is between the parameter of interest and the other parameters. The other regression model is between the outcome measure B_i at a given time point and the parameters other than the one of interest. We then find the Pearson Correlation

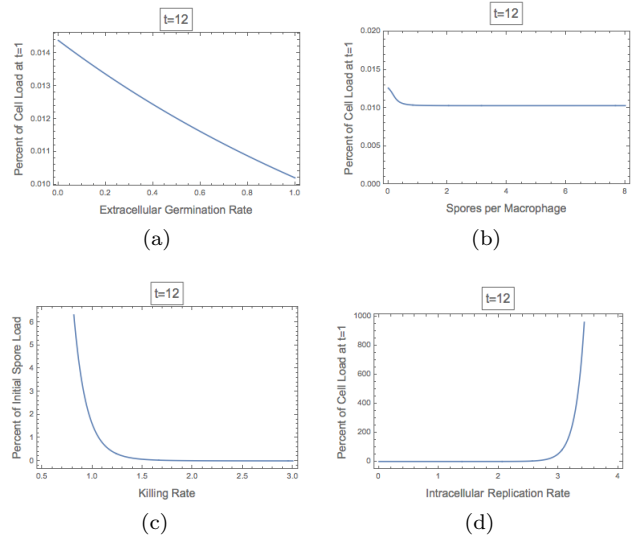


Figure 4: Monotonicity plots of $B_i(12)$ v. parameters

Coefficient between the residues of the two linear regression models. We summarize the results in Table 3. The given values for the first hour are based on intracellular bacteria counts relative to the initial amount of spores plated, $B_i(1)/S_0$. After one hour, we assume all extracellular spores and bacteria are washed away, therefore, for later time points we consider the amount of intracellular bacteria relative to the amount of intracellular spores and bacteria at time $t = 1$.

The significant relationships are listed in blue. We notice, as anticipated, B_i has a strong negative correlation with killing rate k after the initial incubation period and strong positive correlation with replication rate r_i once bacteria start replicating after five hours. Additionally, the rate of extracellular germination within the first hour is correlated with higher intracellular bacteria at one hour. All analyses and graphs were done in Mathematica [29].

4 Conclusion and Further Work

The biggest challenge for creating a meaningful model for anthrax infection are the inconsistencies in data available for parameter estimation and variation in experiment protocol. Extrapolation between *in vitro* and *in vivo* studies can be challenging and even unreliable if parameter sensitivity is not properly assessed. For instance, extracellular germination rates (g_e) were shown by Gut [8] to vary wildly different based on the plating medium, and the study showed viability of macrophages in non-germinating mediums. Therefore it was suggested the use of non-germinating mediums for studies so as to min-

Time	g_e	M	k	r_i
1	0.86(1.7×10^{-60})	-0.01(0.39)	0.018(0.80)	-0.04(0.56)
2	0.20(0.005)	0.08(0.23)	1(1.14×10^{-356})	0.07(0.33)
4	-0.40(7×10^{-9})	-0.03(0.71)	1(1.68×10^{-260})	-0.01(0.89)
6	-0.08(0.29)	0.003(0.97)	-0.98(4.31×10^{-150})	0.93(1.11×10^{-89})
8	-0.07(0.34)	0.03(0.70)	-0.98(3.05×10^{-146})	0.97(2.42×10^{-117})
10	-0.04(0.55)	0.03(0.66)	-0.98(4.46×10^{-147})	0.97(8.79×10^{-124})
12	-0.02(0.76)	0.032(0.66)	-0.98(1.25×10^{-146})	0.97(5.33×10^{-126})

Table 3: PRCC (p -value) for outcome measure B_i at various time points.

imize the affect of extracellular germination on the study outcome. Our analysis indicates that extracellular germination (g_e) alone may not be the primary cause of variability in study outcomes. We suggest the parameters most affecting bacterial counts are the killing rate (k) and replication rate (r_i) and accurately assessing these parameters are paramount to being able to properly determine if an infection will be able to be established. Since extracellular germination (g_e) may be linked to an increased killing rate (k), in further study we would like to consider more closely, possible quantitative relationships between the killing rate (k) and both extracellular germination (g_e) and phagocytic capacity (M). This would best be done through either an agent-based model or stochastic model where macrophages with lower spore loads would be more likely to kill germinated bacteria. Pantha [22] considered newly germinated bacteria to be more susceptible to killing by macrophages. Using a similar path, we may consider bacteria that germinate before phagocytosis as more susceptible to killing as well. Additionally, we would like to consider if spore load affects the replication rate directly, or just affects if spores survive to replicate.

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Appendix

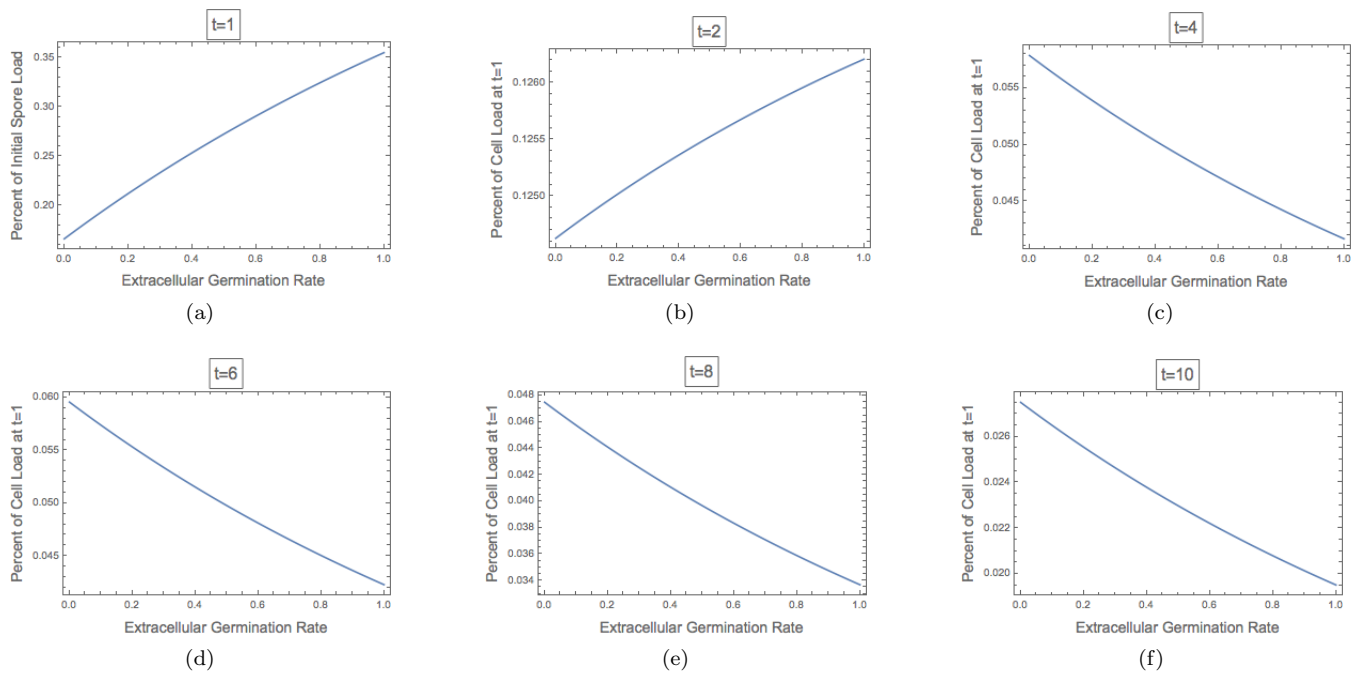


Figure 5: Monotonicity plots of B_i versus g_e at various time points.

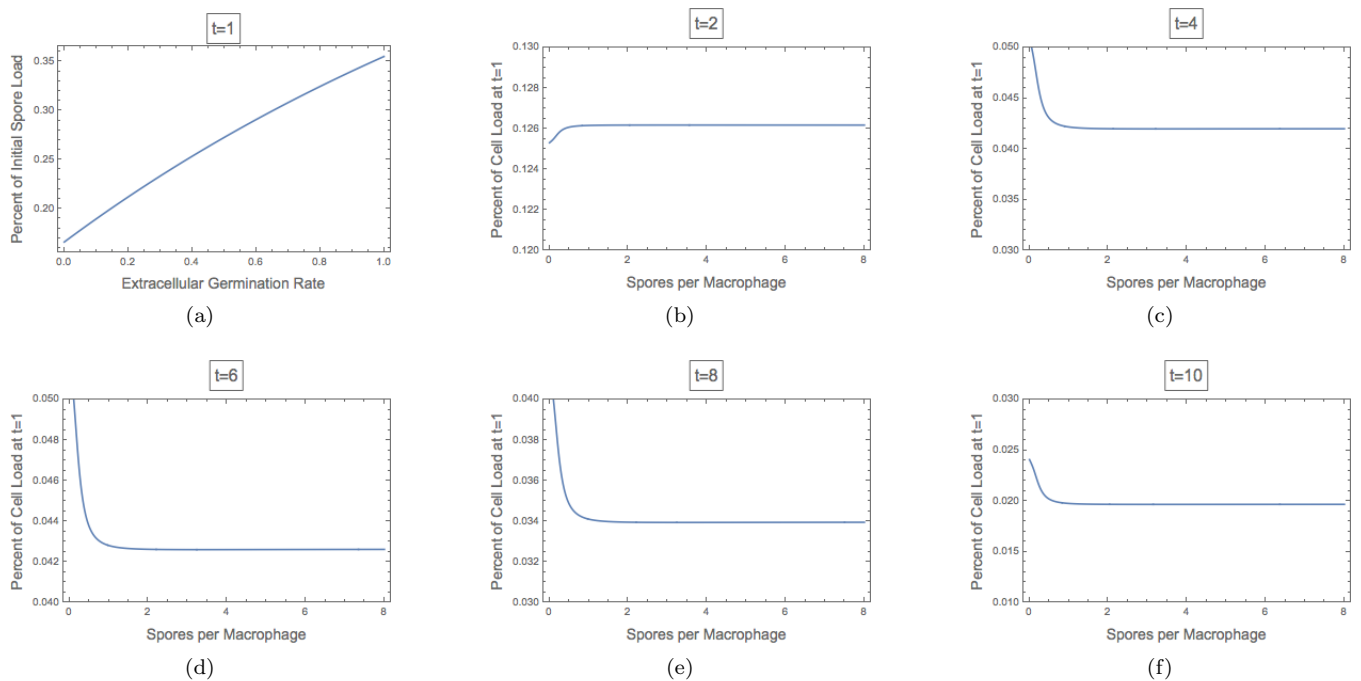


Figure 6: Monotonicity plots of B_i versus M at various time points.

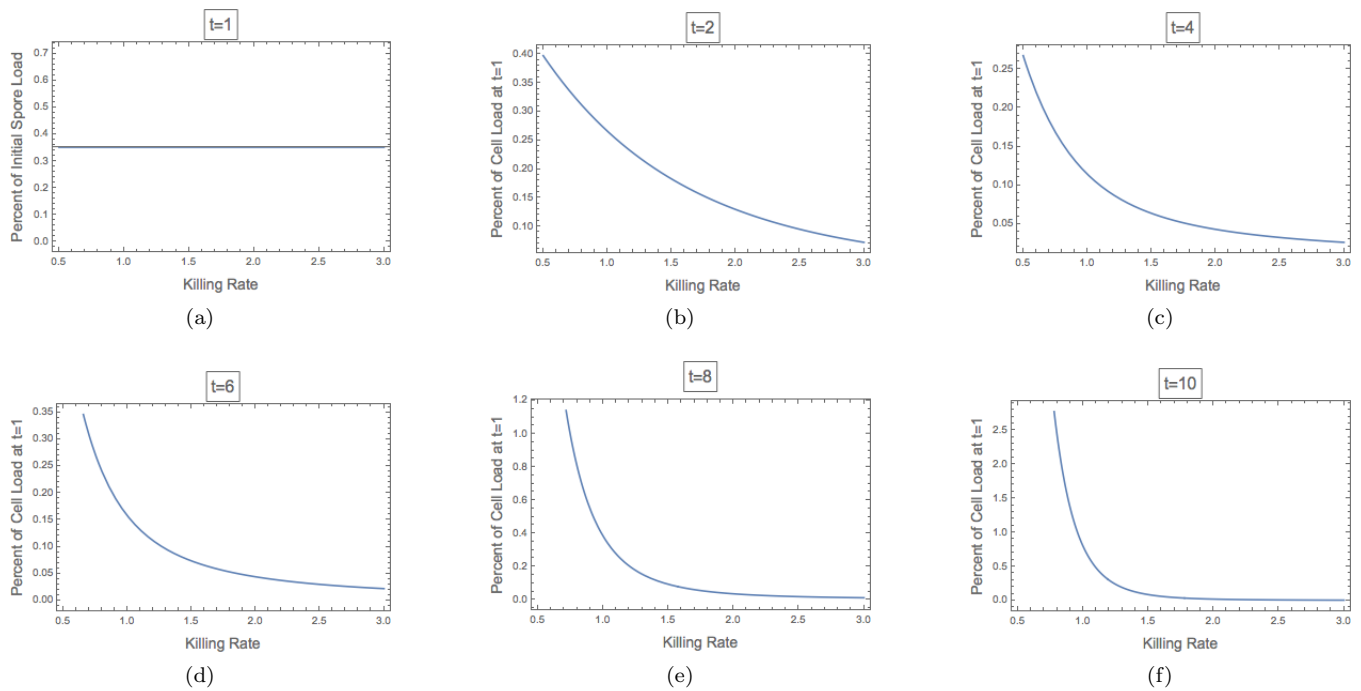


Figure 7: Monotonicity plots of B_i versus k at various time points.

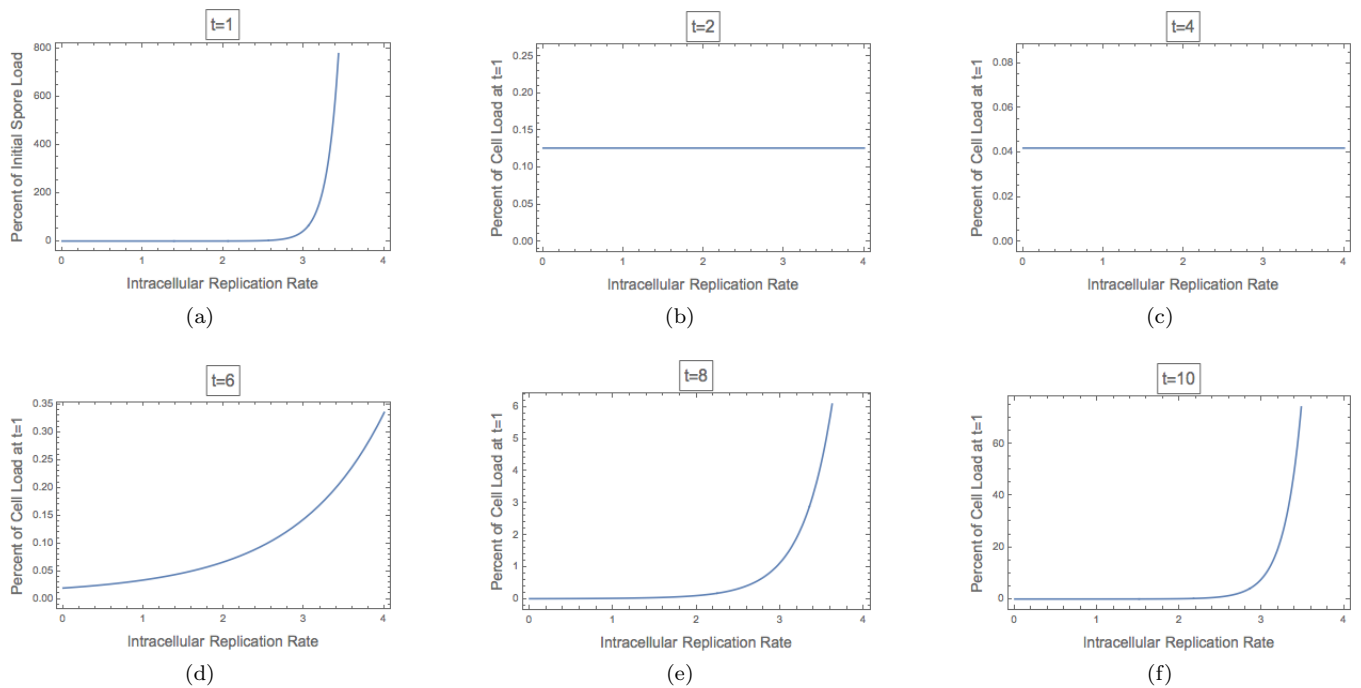


Figure 8: Monotonicity plots of B_i versus r_i at various time points.