The pharmacodynamic inoculum effect from the perspective of bacterial population modeling

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Abstract

The quantitative determination of the effects of antimicrobials is essential for our understanding of pharmacodynamics and for their rational clinical application. Common pharmacodynamic measures of antimicrobial efficacy, such as the MIC and the pharmacodynamic function, fail to capture the observed dependence of efficacy on the bacterial population size — a phenomenon called inoculum effect. We assessed the relationship between bacterial inoculum size and pharmacodynamic relationship and determined the consequences of the inoculum effect on bacterial population dynamics with a mathematical multi-hit model that explicitly describes the interaction between antimicrobial molecules with their targets on the bacterial cells. Our model showed that the inoculum effect can arise from the binding dynamics of antimicrobial molecules to bacterial targets alone. A pharmacodynamic function extended by the inoculum effect on its parameters was able to predict the long-term population dynamics of simple scenrios well. More complex scenarios, however, were only captured with by the mechanistically more explicit multi-hit model. In simulations with competing antimicrobial-susceptible and -resistant bacteria, neglecting the inoculum effect led to an overestimation of the competitive ability of the resistant strain. Our work underpins the importance of including the inoculum effect into quantitative pharmacodynamic frameworks, and provides approaches to accomplish that.

Introduction

Efficacy of antimicrobials against bacteria is commonly quantified using time-kill curves. The slopes of a log-linear function fitted to time-kill curves (Figure 1a) describe the change of the bacterial population over time and are a direct measure of the bacterial net growth rate. The net growth rate and its dependence on the antimicrobial concentration can be captured by the so-called pharmacodynamic (PD) function.[1,2,3] The parameters of this PD function, however, depend on the initial bacterial population size in the time-kill curve experiments.[4,5]

A more widespread, but less informative, way to express efficacy of antimicrobials is the MIC (Figure 1b). Although it is recognized that the MIC might change with bacterial inoculum size (see for example the MIC protocol by Wiegand *et al.*[6]), both the CLSI and the EUCAST recommendations to measure the MIC are based on a single standard bacterial inoculum of 5×10^5 cfu/mL.[7,8] This standardization of the inoculum yields an estimate of the MIC that cannot be extrapolated to other bacterial population sizes.

Changes in efficacy of antibacterial drugs caused by changing the initial bacterial inoculum size are summarized under the term *inoculum effect* (Figure 1). The inoculum effect was first described in the early 1940s,[9] and was observed for a variety of combinations of bacterial species and antimicrobials more recently.[4,10,11,12,13,14,15,16,17,18,19] In addition to its experimental establishment, the inoculum effect has been explored with mathematical models.[4,13,19,20,21,22,23,24] These experimental and theoretical

studies have put forward hypotheses explaining the inoculum effect that fall into two broad categories. The first category attributes the inoculum effect to the binding kinetics of antimicrobials: the higher the bacterial inoculum the more antimicrobial molecules are bound, which leads to a larger reduction of free antimicrobial molecules.[4,21,22] The second category invokes the secretion enzymes at high bacterial inocula, e.g. β-lactamases[9,10,14,15,16,25,26,27] and proteases.[28,29,30]

In this study, we used mathematical models as a tool to investigate these competing hypotheses about the inoculum effect. Specifically, we use the so-called multi-hit model[31] to address if the binding kinetics by itself already leads to an inoculum effect, and to what extent it influences the PD parameters. We have already used this model previously[32] and a similar, albeit more antimicrobial-specific approach has been used to model classic reaction kinetics of antibiotics.[21] Here we present an additional implementation of this model by exploring the effects of the bacterial inoculum conceptually. Additionally, we investigate how the inoculum effect biases the competition between antimicrobial-susceptible and -resistant strains.

Methods

Multi-hit model

We used the multi-hit model[21,31,32] that describes a bacterial population consisting of N_{tot} cells. The key idea of this model is that targets on bacterial cells are "hit", i.e. bound, by antimicrobial molecules, and a bacterial cell

dies if a threshold number of targets, which we call z, has been hit. The total number of hits possible per bacterial cell is called n. Antimicrobial molecules attach to and detach from a bacterial targets with the rates α_i and μ_i, respectively. We denote the number of bacteria that have i antimicrobial molecules adsorbed to cellular targets by N_i. Bacteria die with Bacteria die with a death rate d_i. Formally, the bacterial population in the multi-hit model is structured according to how many antimicrobials have hit the cell (Figure 2). For details of the multi-hit model, see supplement S1.

Variations in the Multi-hit framework

In the basic model (equation 1–3 in supplement S1), we assumed the case of complete multihit,[31] with $z = n$, which maybe best describes antimicrobials that need to hit a large fraction of the existing targets, for example AMPs.[33] Moreover we assumed that the total amount of antimicrobials is stable and that antimicrobial molecules are recycled back into the system when a cell dies.

We considered variants of the multi-hit model by relaxing some of the assumptions above: (i) not all targets need to be hit $z < n$, which leads to non-effective binding, (ii) non-reversible binding, (iii) unspecific binding (i.e. binding to non-targets) in combination with non-reversible binding, leading to molecules attaching non-reversibly to targets and non-targets alike, and (iv) enzymatic degradation. For details and model equations, see Supplement S2.

Pharmacodynamic and Pharmacokinetic function

To investigate how the efficacy of antimicrobials changes with the inoculum in our multi-hit model simulations, we used the PD function[1,2,3] as a measure of antimicrobial efficacy. Specifically, we calculated the bacterial net growth rate ψ on basis of the antimicrobial concentration A at the start of the simulation ($A(t = 0) = A_0$), with

$$\psi(A_0) = \psi_{max} - e(A_0) \tag{1}$$

In this common conceptualization of PD effects, the net growth rate ψ is divided into the growth rate of the bacteria in absence of the antimicrobials ψ_{max} and an effect $e(A_0)$ that depends on the concentration of the antimicrobials. Note that we use the concentration A_0, because throughout the simulation, A varies due to binding and degradation.

The effect $e(A_0)$ is further parameterized by the concentration, MIC_{PD}, that leads to a zero net growth rate, $\psi(A_0 = MIC_{PD}) = 0$, the minimum net growth rate that can be obtained with antimicrobials $\psi_{min} = \psi(A_0 \to \infty)$, and a parameter that characterizes the steepness of the pharmacodynamic relationship, κ:

$$\psi(A_0) = \psi_{max} - \frac{(\psi_{max} - \psi_{min})(A_0/MIC_{PD})^{\kappa}}{(A_0/MIC_{PD})^{\kappa} - \psi_{min}/\psi_{max}} \tag{2}$$

Here, the MIC_{PD} approximates the MIC if the antimicrobial is concentration independent.[34]

To estimate the PD parameters, we simulated time-kill curves for which we varied the antimicrobial concentration (Figure S1). In a first step, the net growth rate was calculated based on the simulated time-kill curves using the relationship:

$$\psi(A) = \frac{log(N_{tot}(t_1)) - log(N_{tot}(t_0))}{t_1 - t_0} \quad (3)$$

The time window, t_1-t_0, is typically chosen to be on the order of hours[1,32,35]**Error! Reference source not found.**,[36] to allow the measurement of potentially steep declines of the bacterial population size at high antimicrobial concentrations. For example, Tobramycin decreases the bacterial population faster at high concentrations than Ticarcillin[35]. Thus, to estimate the PD parameters, a shorter time window needs to be chosen for Tobramycin than for Ticarcillin. Additionally, for wider time windows, more complexities arise, such as persister populations that are detectible within a few hours.[37,38,39,40] For the sake of simplicity, we set the time window that we use to calculate the growth or decline of the bacterial population in our simulations to 1 hour. The time window can be set to be narrower or wider when, instead of aiming for a generic understanding of the inoculum effect, a specific bug-drug combinations is being investigated.

The determined net growth rates were then used to estimate the PD parameters. The steepness parameter κ was calculated (see supplement S3). The estimates of the PD function parameters differed by inoculum. This

136 dependence of the PD parameters were captured by an extended PD
137 function, $\psi(A_0, N_{inoc})$. Specifically, the inoculum effect was quantified as slope
138 m of
139 the function

140 $$A_{0,\psi=y}(N_{inoc}) = mN_{inoc} + c, \quad (4)$$

141 with $y = 0$ and $y = E_{max}/2$ in the case of $\psi(A_0 = MIC_{PD})$ and $\psi(A_0 = A_{50})$,
142 respectively.

143 Note that the pharmacodynamic function does not capture evolutionary
144 processes. However, evolved bacterial strains can be incorporated into this
145 pharmacodynamic modeling framework as subpopulations with their own
146 set of PD parameters.[36,41]

147 The pharmacokinetic function we used is described in Regoes *et al.*[1] In
148 short, the initial antimicrobial concentration A_0 is assumed to decay with a
149 rate k_A, and reset to A_0 every 8 hours.

150 Population model

151 To investigate the agreement between the bacterial population dynamics
152 predicted by the common parameterization of antimicrobial efficacy with
153 PD functions and those predicted by the more detailed multi-hit model, we

154 incorporated the extended PD function and the pharmacokinetic function
155 into a population model:

156 $$\frac{dN_{tot}}{dt} = \psi_{max} N \left(1 - \frac{N}{K}\right) - e(A_0, N_{inoc}) N \quad (5)$$

157 Here, K is the carrying capacity. We used this framework before in.[42]

158 Competition

159 To describe competition between an antimicrobial-susceptible and an
160 antimicrobial resistant strain, we extended the basic multi-hit model with a
161 second strain. The number of susceptible and resistant cells are denoted by S
162 and R, respectively. In our simulations, strain R is assumed to have a 10 times
163 lower attachment rate, which could correspond to surface modification on
164 resistant bacterial cells — a common resistance mechanism against AMPs.[28]
165 For more details and model equations, see Supplement S4.

166 Implementation

167 The multi-hit model was implemented in R[43] (www.r-project.org; version
168 3.4.0). We solved the deterministic multi-hit model numerically with the
169 package deSolve.[44] For plotting, we used the following packages: sfsmisc[45]
170 and RColorBrewer.[46] The code is available upon request.

171 Results

172 We developed a mathematical model to describe the interaction between a
173 bacterial population and antimicrobials. The model is based on the multi-hit

174 concept of antimicrobial action,[31] according to which bacteria are killed as
175 soon as a certain number of antimicrobial molecules hit the cell. This
176 modeling framework describes the interaction between antimicrobial
177 molecules and bacterial cells in more detail than common population
178 biological models, and thus allowed us to investigate the determinants of
179 the inoculum effects and predict quantitatively how the inoculum affects the
180 pharmacodynamics.

181 Binding kinetics alone generates an inoculum effect

182 First, we investigated if an inoculum effect emerges simply as a result of
183 concentration-dependent interactions between bacteria and antimicrobials
184 and their binding kinetics. To that end, we used the basic multi-hit model
185 that assumes reversible binding of antimicrobial molecules to target sites
186 on
187 the bacterial cells.
188 We found that an inoculum effect emerged. In our simulations, a
189 bacterial population starting at a high inoculum grew over time, while a
190 smaller initial population exposed to the same amount of antimicrobials
191 declined (Figure 3a). The explanation of the effect can be found in the
192 concentration of free antimicrobial molecules that is sensitively affected by
193 the bacterial inoculum (Figure 3b), resulting in a lower efficacy if the
194 inoculum is high. Consistent with this explanation, the inoculum effect was
195 abrogated if the free antimicrobial concentration was assumed to be
196 constant independent of any binding processes (Figure S2). Thus, only the

binding of antimicrobial molecules — even though it was assumed reversible — caused an inoculum effect. Allowing for degradation of antimicrobials by extracellular enzymes in our model exacerbated the inoculum effect (Figure 3) but was not necessary.

Inhibitory concentrations increase linearly with inoculum, but with a proportionality constant < 1

This inoculum effect can be quantitatively captured. Specifically, we studied how the inoculum affects the parameters of the *pharmacodynamic function* We considered the effect of the inoculum on the pharmacodynamic function that describes relationship between the net growth rate of bacterial population and the antimicrobial concentration (see Methods). Performing *in silico* time kill experiments, we estimated the pharmacodynamic functions for different inocula (Figure 4a) and found that the efficacy across all antimicrobial concentrations is lower for larger inocula.

The most important efficacy parameter, the pharmacodynamic parameter MIC_{PD} increases linearly with the inoculum (Figure 4b). However, the proportionality constant is less than one, meaning that a doubling of the inoculum does not lead to a doubling of number of antimicrobial molecules required to halt bacterial population from growing, and thus does not lead to a doubling of the MIC_{PD}. We found a similar linear increase of another common efficacy measure, the A_{50} (see Methods for definition and Figure 4c), which is interestingly also linearly affected by z.[32] We also found that these

220 two efficacy parameters increased linearly with the number of hits required
221 to kill a bacterial cell, z (Figure 4b,c) and that the increase was steeper when
222 we allowed for degradation (Figure S3). Furthermore, the steepness
223 parameter κ did not increase linearly, but saturated (Figure 4d). These
224 results hold for different modes of binding (Figure S4).

225 Formally, we found the following relationship between the efficacy
226 parameters and the inoculum N_{inoc}:

$$MIC_{PD}(N_{inoc}) = m_{MIC_{PD}} N_{inoc} + c_{MIC_{PD}} \quad (6)$$

$$A_{50}(N_{inoc}) = m_{A_{50}} N_{inoc} + c_{A_{50}} \quad (7)$$

227 Estimates for the intercept and slope parameters, c and m, of these linear
228 relationships are shown in Table 1. With these two parameters, we can also
229 describe how many more antimicrobial molecules we need to achieve the
230 same pharmacodynamic efficacy when we increase the bacterial inoculum
231 x-fold:

232 $$\frac{MIC_{PD}(xN_{inoc})}{MIC_{PD}(N_{inoc})} = \frac{mxN_{inoc} + c}{mN_{inoc} + c} < x \text{ for } c > 0 \quad (8)$$

233 The same relationship holds for the A_{50}, but, of course, with A_{50}-specific
234 estimates for c and m. Thus, the increase in antimicrobials required to
235 achieve the same effect on an x-fold increased inoculum is, according to our
236 model, less than x-fold and thus the proportionality constant is less than
237 one.

238 The parameters characterizing the net bacterial growth rate in absence
239 of antimicrobials is independent of the inoculum (Figure S5a). Also, the
240 maximum effect that can be achieved with an antimicrobial, E_{max}, and the

related minimum net growth rate, ψ_{min}, are independent of the inoculum (Figure S5b-c). This is a consequence of the fact that these parameters are not affected by the binding of antimicrobial molecules to targets on the bacterial cells.[32]

The PD function ψ(A0) has been used to predict the long-term bacterial population dynamics from antimicrobial effect on the initial net growth rate.[1,4,5,35,42,47,48,49] However, if we include the inoculum effects on MIC_{PD}, $A50$, and κ into the PD function, we found that this extended PD function therefore captured only the short-term bacterial population dynamics while only the mechanism-based multi-hit model could describe more complex population dynamics (see supplement S5, Figure S6, Figure S7).

Inoculum effect alters the outcome of bacterial competition

To shed light on the effects of the inoculum on the competition between bacterial strains, we extended the multi-hit model framework by an additional resistant strain R. We assume that strain R carries a fitness cost and is hence not able to outcompete strain S at low antimicrobial concentrations (Figure 5a). At intermediate concentrations, R outcompetes strain S (Figure 5b). Lastly, at high concentrations, both strains are killed (Figure 5c). The antimicrobial concentrations that mark the transitions between these three dynamical outcomes define the so-called *mutant selection window* (MSW).[50,51]

To investigate how the inoculum affects the size of the MSW, we conducted simulations across a range of inocula. In these simulations we also included a time frame of 7 days and repeated doses of the antimicrobial, to mimic antimicrobial usage in a treatment scenario. We therefore use the term" treatment MSW". The difference between the MSW and the treatment MSW is that the MSW is based on time-kill curves with a single input of antimicrobials, while the treatment MSW is based on time-kill curves in treatment regimes.

We find that the treatment MSW shrinks and even vanishes for increasing inocula (Figure 5d). Qualitatively similar results hold for simulations based on the extended PD function (Figure S8). Simulating bacterial competition with a model that does not capture the inoculum effect, in contrast, left the MSW unaffected (Figure 5e).

Discussion

In this study, we used a mathematical model to understand how an inoculum effect can arise and to investigate how the bacterial inoculum affects the pharmacodynamics of antimicrobials. With our model simulations, we were able to derive predictions for the inoculum effect not only on the MIC, but on multiple pharmacodynamic parameters. While the insights from our study are generic and conceptual, they can be adapted to any specific antimicrobial-bacterial pathogen combination. Our predictions give rise to many experimentally testable hypotheses that we detail below.

Udekwu *et al.*[4] previously showed how the inoculum effect on the MIC can be integrated into the PD function. We extended these efforts to the

287 additional pharmacodynamic parameters: the antibiotic concentration at
288 which 50% of the maximum effect is obtained, A_{50}, and the parameter that
289 describes the steepness of the pharmacodynamic relationship, κ. We found
290 that the inoculum affected the PD parameters MIC_{PD} and A_{50} linearly, and the
291 parameter κ non-linearly, resulting in an inoculum effect that does not
292 simply scale with the number of bacterial cells and antimicrobial molecules
293 in the system. For example, to obtain the same effect on a, say, 100-fold
294 increased bacterial population, we require less than 100-fold increase in the
295 number antimicrobial molecules. This finding directly contradicts the
296 argument that the inoculum effect is an artifact[27] arising from simple
297 scaling. We could show that to achieve the same antimicrobial effect for a
298 higher inoculum always requires a smaller increase in the number of
299 antimicrobial molecules than a simple scaling would suggest. How much
300 smaller depends on the specific parameters of the bacteria, antimicrobials,
301 and assay system. Interestingly, this means that, on a per-molecule level,
302 antimicrobials become more effective with increasing inoculum.

303 The mechanistic underpinning of the inoculum effect in our simulations
304 is that an increase in the bacterial inoculum results in higher depletion of
305 free antimicrobials. We call this the *numbers game*: Binding, even if it is
306 reversible, takes molecules out of the pool of free antimicrobial molecules,
307 which in turn means that there are fewer molecules available for binding in
308 the next time step. For larger bacterial inocula, the antimicrobial depletion
309 by binding is increased, resulting in the inoculum effect. Thus, for a given
310 antimicrobial-bacterial pathogen combination, we expect an inoculum effect

if the numbers game is at play. This numbers game results in an inoculum effect has already been observed in mathematical modeling studies.[21,31] Experimental studies that investigate the possibility and extent of an inoculum effect arising from the numbers game alone[4,18,21], however, are rare. Often, the inoculum effect is measured in systems in which additional effects, e.g. non-targeted binding[52] or enzymatic degradation[10,11,12,14,15,16,25,53,54,55], occur. These effects strongly increased the complexity of the population dynamics in our simulations. We therefore suggest conducting experimental studies that address the inoculum effect first in systems that are governed only by a simple binding kinetics of antimicrobials to their targets. These experiments should be conducted in a regime of declining free antimicrobial molecule concentrations with increasing bacterial inoculum sizes, because, in the numbers game scenario, this decline causes the inoculum effect. Such studies will form the basis of a quantitative understanding of the inoculum effect and will provide valuable baselines for studies of systems with more complexity.

In addition to determining if the binding kinetics of antimicrobials alone results in an inoculum effect, we suggest experiments that assess the more qualitative prediction of the multi-hit modeling framework we presented here. For example, we showed that the more targets are needed to be hit to kill a cell (z), the more pronounced the inoculum affect was. In the case of antimicrobial peptides, z varies from 80-800 in the case of protegrin[56] to 10^6 -10^7 in the case of PMAP-23.[33] With everything else being equal, we therefore expect a more pronounced inoculum effect for PMAP-23 than for

335 protegrin. Similar predictions can be easily derived for other antimicrobials
336 if quantitative parameters characterizing their mechanisms of action are
337 known. Generally, the quantitative relationships between the mechanisms
338 of action of an antimicrobial, its pharmacodynamics, and the size of the
339 inoculum effect represent a promising area of future theoretical and
340 experimental studies.

341 The inoculum effect has been demonstrated experimentally for a number
342 of bacterial strains and antimicrobials. [4,10,11,12,14,15,16,17,18,19,21,53,54,57] In most
343 cases, the MIC was used to determine and quantify the inoculum
344 effect.[4,10,11,12,14,17] Our comprehensive analysis of the inoculum's effect on all
345 pharmacodynamic parameters showed that the steepness parameter κ
346 increases. Therefore, the inoculum effect is stronger for A_{50} than for MIC_{PD}
347 and determining the effect on A_{50} represent the most sensitive measure of
348 the inoculum effect. Based on this observation, we recommend to not only
349 quantify the MIC, but the whole pharmacodynamic relationship, and to use
350 OD measurements with caution because they do not allow the estimation of
351 the pharmacodynamic relationship for antibiotic concentrations above the
352 MIC_{PD}.

353 The absence of the inoculum effect has been reported repeatedly.[27,57]
354 Our results shed light on the conditions under which an inoculum effect is
355 expected to arise. First, in our simulations the inoculum effect did not
356 manifest itself in certain regions of the PD plane: for very small and very
357 high antimicrobial molecule numbers, the inoculum effect was absent.
358 Second, in our simulations, the inoculum effect essentially depends on a

359 decline in free antimicrobial concentrations. Therefore, we expect the
360 inoculum effect not to arise in any system, in which the antimicrobial
361 concentration does not decline with increasing bacterial inoculum. Third,
362 the establishment of the inoculum effect on the MIC is impaired because of
363 the uncertainties arising from the doubling of the antimicrobial
364 concentration that is required according to the standard protocol for MIC
365 determination. To give a specific example: according to the standard
366 protocol, "real" values of the MIC of 9 and 15 *mg/L* would be both estimated
367 as 16 *mg/L*; therefore, an inoculum effects increasing the MIC from 9 to 15
368 *mg/L* would not be detected. Lastly, in many studies an arbitrary threshold
369 of an 8-fold increase of the MIC for a 100-fold higher bacterial inoculum is
370 used to define the inoculum effect[6,10,14,15,16] clouding inoculum effects of
371 smaller extent. Rather than setting such a cutoff, we suggest determining
372 the MIC_{PD} or, even better, the A_{50} from adequately powered time-kill
373 experiments.

374 Most surprisingly, we found that the inoculum effect can have
375 unexpected and wide-ranging consequences for the evolutionary dynamics
376 of bacterial populations under drug treatment. Our simulation of bacterial
377 competition between drug-susceptible and -resistant strains revealed that
378 the competitiveness of drug-resistant strain is overestimated when the
379 inoculum effect is not considered. We found that, in our simulations, the
380 "treatment MSW" — an estimation of the MSW[50,51] under drug treatment —
381 shrinks for higher bacterial inoculum sizes. Although the treatment MSW is
382 an idealized concept, that does not capture all complexities of bacterial

evolution and competition, a negative effect of higher inocula on the emergence of resistant strains should be observable in competition experiments. In competition experiments between drug-susceptible and -resistant bacterial strains, such as those presented in e.g.[51], our analysis predicts the selection coefficients to decrease with inoculum. Hence the inoculum effect is generally expected to impact bacterial survival and evolution *in vitro*, within infected hosts, and potentially even on the epidemiological level.

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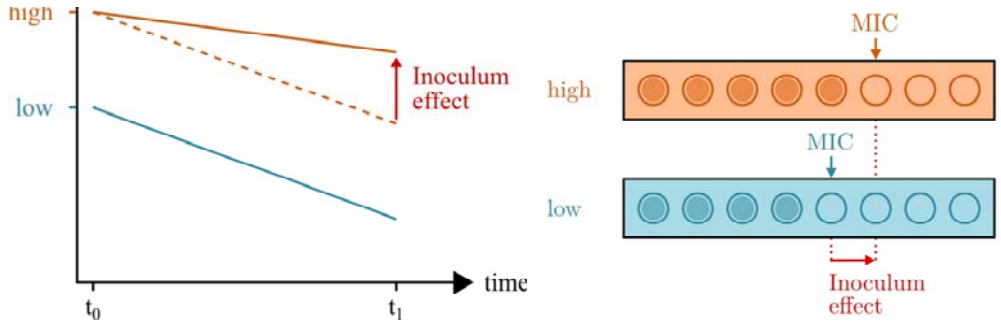
Figures and Tables

Table 1: Results of fitting linear function $x = m_x N_{inoc} + c_x$ with $x = MIC_{PD}$ and $x = A_{50}$ to the simulated data. Reversible binding with z = 1,2,10 is also plotted in Figure 4b-d.

			MIC_{PD}		A_{50}	
z	n	binding	$c_{MIC_{PD}}$	$m_{MIC_{PD}}$	$c_{MIC_{PD}}$	$m_{A_{50}}$
1	1	reversible	2514516	0.167	8284221	0.258
2	2	reversible	6234471	0.728	14665506	0.812
10	10	reversible	37675418	6.508	61533236	6.577
10	39	reversible	37368958	6.672	61618874	6.902
10	10	non-reversible	36111621	9.081	62435986	9.944
10	10	non-reversible, non-targeted	57313826	9.416	111901448	11.08



(a) (b)

Figure 1: The inoculum effect changes efficacy of antimicrobials. Two common methods to quantify the efficacy of antimicrobials are (a) time-kill curves and (b) MIC measurements. (a) Time-kill curves illustrate the change in population size over time in presence of antimicrobials. The bacterial inoculum is the bacterial population size N at $t0 = 0h$ ($N_{inoc} = N(t = 0)$). The slope of the curve results from fitting a log-linear function to the population dynamics. Here, only the log-linear curves and not individual measurements are shown. The slope of the time-kill curve representing dynamics of bacteria starting at a high inoculum size (orange) is shallower than dynamics of bacteria starting at a low inoculum (blue). In the absence of an inoculum effect, the slope is independent of the inoculum (dashed orange line). (b) Alternative to measuring time-kill curves, efficacy is typically determined with the MIC. An inoculum effect is observed if increasing the inoculum size from low (blue) to high (orange) increases the MIC.

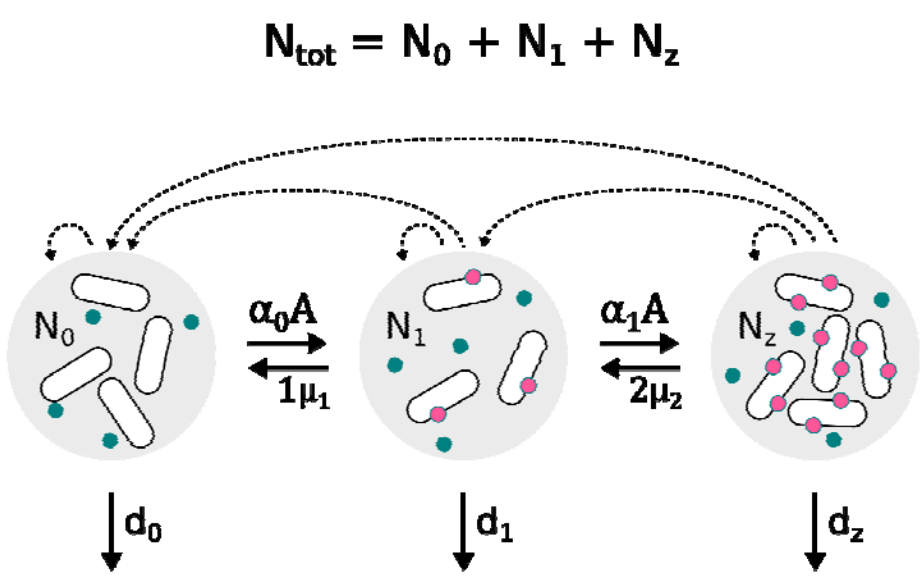
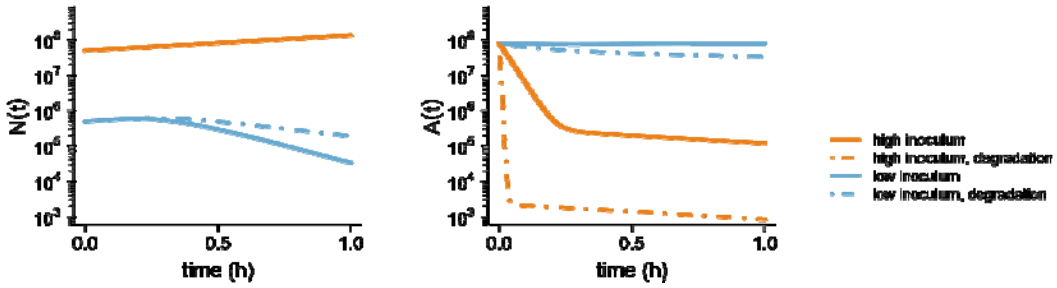


Figure 2: Schematic representation of the basic multi-hit model with three classes (n = 2). At every time point the bacterial population N_{tot} can be divided in n subpopulations based on the number of antimicrobials that have hit the bacterial cells. A hit is the mathematical simplification of adsorption and subsequent downstream processes connected to binding of one antimicrobial molecule to a bacterial target site and is described with the rate α. The parameter μ is the

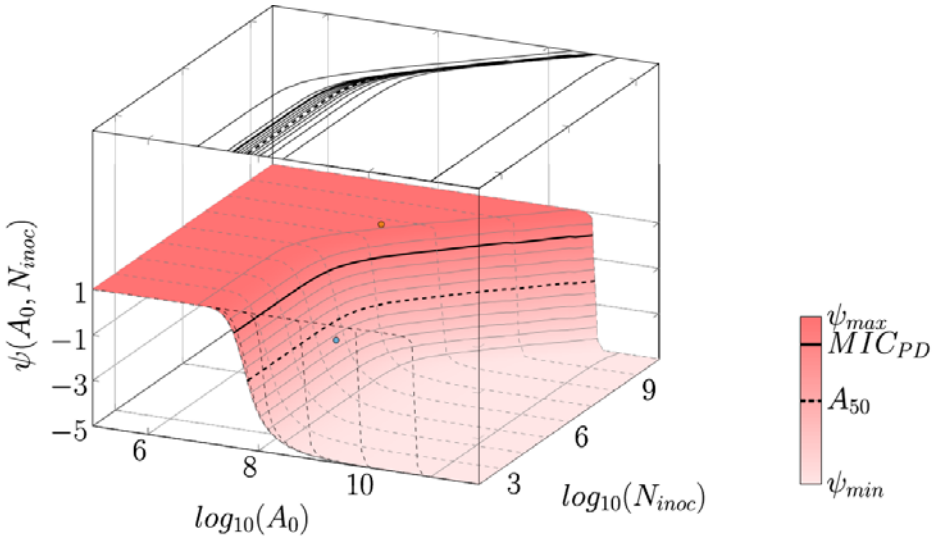
detachment of an antimicrobial from the bacterial target. We assumed that finding the bacterial cell is the rate limiting step for an antimicrobial molecule.[59] This is appropriate if the number of targets are in abundance, e.g. when antimicrobial peptides adsorb to the cell membrane of bacterial cells. For antimicrobials that act within the cell, a hit rate dependent on the number of free targets is more appropriate (see[21]).

Each class N_i gains bacterial cells from the class N_{i-1} with the term $\alpha_{i-1}AN_{i-1}$ and a bacterium dies with an increased death rate d_z if two antimicrobial molecules have hit the cell, i.e. $z = 2$. Therefore, $N_{tot} = N_0 + N_1 + N_2$, with $N_2 = N_z$. A is the number of free antimicrobial molecules, here indicated in blue, antimicrobials that have hit a cell are indicated in pink. The rates of the ODE (see supplement S1 for equation) are indicated with arrows and bacterial replication with dashed arrows.



(a) (b)

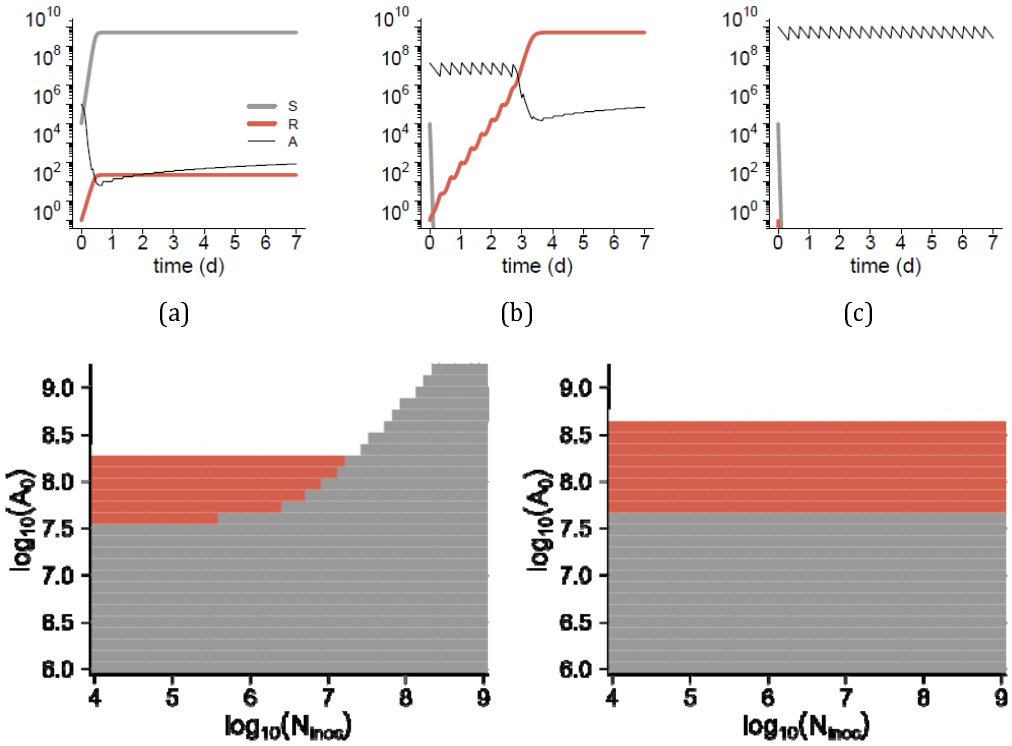
Figure 3: Increasing the bacterial inoculum size from $5 \cdot 10^5$ (blue) to $5 \cdot 10^7$ (orange) changes the efficacy of the antimicrobial due to reduction of the number of free antimicrobials. Simulations with reversible binding is shown with solid lines, dashed lines indicate simulations with degradation. (a) The bacterial inoculum *N(t)* is affected differently by antimicrobials depending on bacterial inoculum size and binding (b). Time course of free antimicrobial molecules. The range of the decline of free antimicrobials is consistent with other modelling studies[19] , and *in vitro*[33] and *in vivo* observations[35]. Parameters not mentioned here are listed in Table S1.



(a)

(b) (c) (d)

Figure 4: Inoculum size affects pharmacodynamics. (a) We determined the efficacy for varying inoculum sizes (N_{inoc}) with z = 10, resulting in a PD plane (note that in contrast to (b)-(d), the inoculum effect here is illustrated on a log_{10}-log_{10} scale). In the PD plane, solid gray contour lines show combinations of antimicrobials and inoculum sizes with the same net growth rate, both in the PD plane and projected above the PD plot. MIC_{PD} and A_{50} are indicated as bold solid and dashed line, respectively. For several inoculum sizes, we plotted the PD curve as dashed contour lines along the antimicrobial molecule number axis and again projected the contour lines, this time in front of the plot. The slopes of Fig. 3a (solid lines). are marked as dots in the corresponding colors. In (b)-(d) we plotted the PD parameters MIC_{PD}, A_{50}, and κ against the bacterial inoculum for varying number of hits needed to kill a bacterial cell (z). The bold black line in (b) corresponds to the bold solid line in (a) and the bold black line in (c) correspond the bold dashed lines in (a). Parameters not mentioned here are listed in Table S1.



(a) (b) (c)

(d) (e)

Figure 5: The inoculum effect influenced competition outcomes. Antimicrobial doses A_0 changed the outcome of competition: (a) S won when exposed to low antimicrobial doses, (b) R won at intermediate antimicrobial doses and (c) bacteria went extinct at high antimicrobial doses. (d) In an additional dimension, the inoculum effect changed the outcome of competition (gray: S is more abundant, red: R is more abundant, white: extinction ($S,R < 1$) at the end of the simulation). (e) For comparison, we used the PD function to simulate competition without inoculum effect.