

How to kill bacteria

fast

and slow

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## Summary

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Since the discovery of penicillin, antibiotics have been a cornerstone of modern medicine, saving countless lives. This achievement is now under threat, as bacteria have evolved resistance mechanisms against most available drugs. Resistance arises through the acquisition of resistance genes—either via mutations or through horizontal transfer on mobile genetic elements such as plasmids—and is selected for according to the administered drugs, their concentrations, and the pharmacodynamics that link treatment to its effect on the bacterial population.

To curb the spread of resistance, antibiotics should ideally be chosen on the basis of phenotypic information. In emergency situations, however, this is not always feasible. Therapy then relies on predefined treatment protocols rather than tailored data. Chapter 2 examines how these default protocols (treatment strategies) influence the persistence of plasmid-mediated resistance and the emergence of new double resistance. We performed large-scale automated in-vitro experiments that mimic hospital-like transmission dynamics, using two antibiotics in combination with two resistance plasmids derived from clinical isolates.

We found that administering both drugs to every patient (combination therapy) was the most effective strategy in most scenarios. Although theoretical models often predict the success of combination therapy, the clinical literature is less conclusive. This gap has several causes: some drug pairs act synergistically, whereby the combined effect exceeds the sum of single-drug effects, whereas others act antagonistically, reducing overall efficacy.

Drug interactions have so far been studied mainly at concentrations below the minimal inhibitory concentration (MIC), where growth can be tracked conveniently by optical density (OD). OD cannot measure bacterial decline, and probing the clinically relevant super-MIC range—where bacteria are actively killed—requires colony-forming-unit (CFU) assays, which are labor-intensive and unsuited to high throughput.

Chapter 3 therefore evaluates whether a luminescence-based high-throughput method, previously validated for sub-MIC conditions, can also track population dynamics across the entire concentration range. Using bacteria engineered to emit bioluminescent light, we show that this method accurately estimates net growth rates for drugs that do not induce significant filamentation.

Building on these insights, Chapter 4 explores drug interactions for six antibiotics that performed well in the preceding validation. We analysed 15 drug pairs across 144 concentration combinations each, introducing a mathematical framework to describe the pharmacodynamics of combinations. By comparing observed effects to null models of non-interaction, we classified pairs as neutral, synergistic or antagonistic, and demonstrated that interaction types can shift markedly between sub- and super-MIC conditions.

Together, these studies combine experimental and theoretical approaches to elucidate antibiotic effects on bacterial populations and resistance evolution. They deliver robust tools for quantifying population dynamics and bolster the case for combination therapy, while refining our understanding of drug–drug interactions across clinically relevant concentration ranges.



## Zusammenfassung

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Seit der Entdeckung des Penicillins bilden Antibiotika einen Grundpfeiler der modernen Medizin und haben unzählige Leben gerettet. Dieser Erfolg ist jedoch bedroht, da Bakterien gegen die meisten verfügbaren Wirkstoffe Resistenzmechanismen entwickelt haben. Resistenzen entstehen durch den Erwerb von Resistenzgenen – entweder über Mutationen oder durch horizontalen Gentransfer via mobile genetische Elemente wie Plasmide – und werden abhängig von verabreichten Wirkstoffen, deren Konzentrationen und den Pharmakodynamiken selektiert, die Behandlung und Wirkung miteinander verknüpfen.

Um die Ausbreitung von Resistenzen einzudämmen, sollten Antibiotika idealerweise auf Basis phänotypischer Informationen ausgewählt werden. In Notfallsituationen ist dies jedoch oft nicht möglich, sodass auf vordefinierte Behandlungsprotokolle zurückgegriffen wird. Kapitel 2 untersucht, wie diese Standardprotokolle (Behandlungsstrategien) die Persistenz plasmidvermittelter Resistenzen und das Auftreten neuer Doppelresistenzen beeinflussen. Hierfür führten wir groß angelegte, automatisierte In-vitro-Experimente durch, die krankenhausähnliche Übertragungsdynamiken simulieren; zum Einsatz kamen zwei Antibiotika in Kombination mit zwei aus klinischen Isolaten stammenden Resistenzplasmiden.

Die Gabe beider Wirkstoffe an jeden Patienten (Kombinationstherapie) erwies sich in den meisten Szenarien als wirksamste Strategie. Obwohl theoretische Modelle diesen Erfolg häufig vorhersagen, ist die klinische Datenlage weniger eindeutig. Ursachen hierfür sind unter anderem synergistische Wirkstoffpaarungen, bei denen der kombinierte Effekt größer ist als die Summe der Einzeleffekte, sowie antagonistische Paarungen, bei denen er kleiner ausfällt.

Bisher wurden Arzneimittelinteraktionen überwiegend bei Konzentrationen unterhalb der minimalen Hemmkonzentration (MIC) untersucht, da Wachstumsraten dort bequem mittels optischer Dichte (OD) erfassbar sind. OD misst jedoch keinen Bakterienrückgang. Der klinisch relevante Super-MIC-Bereich, in dem Bakterien aktiv abgetötet werden, erfordert koloniebildende Einheiten (CFU), ein arbeitsintensives und wenig durchsatzstarkes Verfahren.

Kapitel 3 prüft daher, ob eine bereits für Sub-MIC-Bedingungen validierte, lumineszenzbasierte Hochdurchsatzmethode auch über den gesamten Konzentrationsbereich einsetzbar ist. Durch gentechnisch veränderte, biolumineszente Bakterien konnten wir zeigen, dass diese Methode die Nettowachstumsrate für Wirkstoffe ohne ausgeprägte Filamentierung präzise erfasst.

Aufbauend darauf untersucht Kapitel 4 Wirkstoffinteraktionen für sechs im Vorversuch bewährte Antibiotika. Wir analysierten 15 Wirkstoffpaare mit jeweils 144 Konzentrationskombinationen und entwickelten einen mathematischen Rahmen zur Beschreibung der Pharmakodynamik von Kombinationen. Durch Vergleich der beobachteten Effekte mit Nullmodellen ohne Interaktion klassifizierten wir Paare als neutral, synergistisch oder antagonistisch und zeigten, dass sich diese Klassifikationen zwischen Sub- und Super-MIC-Bereichen deutlich ändern können.

Insgesamt verbinden diese Arbeiten experimentelle und theoretische Ansätze, um die Wirkung von Antibiotika auf bakterielle Populationen und die Resistenzentwicklung zu beleuchten. Sie liefern robuste Methoden zur Quantifizierung von Populationsdynamiken, stärken die Evidenz

für Kombinationstherapien und verfeinern unser Verständnis von Arzneimittelinteraktionen über klinisch relevante Konzentrationsbereiche hinweg.

## Preface

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about data and stuff...

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1. format frontpage for papers. Do it like niko. Abstract; Authors; Contributions; and manuscript accepted/submitted/in preparation.
2. improve formatting of chapter and section titles

### 0.1 maybe fun to add.

Yuan2000, While many modern drugs originated from plants, unpuri- fied herbal preparations are used extensively in both Europe and Asia. Western herbal medicines are often standardized ex- tracts of single herbs used for particular conditions. For example, valerian and St. John's wort are used for sleep aid and mild depression, respectively. In comparison, traditional Chinese medicines (TCMs) are decoctions of mixtures of up to 20 herbs that are customized for each individual patient. TCMs represent one aspect of Chinese medical philosophy that is characterized by its emphasis on maintaining and restoring balance. This medical approach is much more appropriate to disease prevention and the treatment of chronic diseases without an unacceptably high level of collateral damage. Most Chinese formulations contain a mixture of herbs. There are different methods of classifying the ways in which these can be combined. When combined, two biologically active substances can be observed to have the following effects: mutual accentuation, mutual enhancement, mutual counteraction, mutual suppression, mutual antagonism, and mutual incompatibility. The principal ingredient is a substance that provides the main therapeutic thrust, the second principal ingredient enhances or assists the therapeutic actions of the first. The rest serve one of the following functions: treat accompanying symptoms, moderate the harshness or toxicity of the primary ones, guide the medicine to the proper organs, or exert a harmonizing effect.

Chevereau2015 For some of the most worrisome infectious diseases, including tuberculosis, drug resistance evolves mainly via spontaneous mutations that render antibiotics ineffective [4,5]. A possible way of averting the looming resistance crisis is developing novel treatment strategies that use established drugs in ways that minimize resistance evolution [6–9]. To rationally design such strategies, it is crucial to understand the genetic source for MDR tuberculosis:

<https://doi.org/10.1016/j.mcna.2013.03.012>

Gjini2021: - Drug interactions can shape resistance evolution. - Difficult to draw general conclusion (e.g. synergistic combinations always accelerate resistance”)

- Drug interactions are very confusing. - we dont know which interaction we want. we dont know how to define them. and they change over dosing and ratios. it seems very pointless to proceed from here. What we need instead are within and between host pkpd models that we can parameterize with exp data of the kind i generated. based on these we can set goals; like

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minimizing prob that resistance emerges, or maximizing the number of uninfected patients.

# 1

## Introduction

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Antibiotics have nearly eliminated premature death from bacterial infections in most parts of the developed world, to the extent that effective treatment is now often taken for granted. The threat to this achievement posed by antibiotic resistance was recognized early, for instance by [1], and explicitly highlighted by Alexander Fleming in his 1945 Nobel Prize speech. Today, this threat has materialized: antimicrobial resistance (AMR) contributed to an estimated 4.71 million deaths in 2021, with 1.14 million deaths directly attributable to resistant infections [2]. The possibility of returning to a world where antibiotics are largely ineffective underscores the importance of understanding the ecological and evolutionary forces shaping AMR.

Evolution of antibiotic resistance is driven by two key processes: mutation and selection. Resistance genes arise *de novo* through mutations, occurring either on bacterial chromosomes or on extrachromosomal mobile genetic elements that replicate independently, such as plasmids (small circular DNA molecules) or bacteriophages (viruses targeting bacteria). Once established, these genes can spread.

Resistance genes spread in two fundamentally different ways: vertically, when a mother cell passes them to its progeny during division, and horizontally, when previously susceptible bacteria acquire foreign DNA. Horizontal transfer is often mediated by plasmids, many of which can actively initiate their own conjugation by assembling a pilus — a physical tunnel connecting donor and recipient cells — through which plasmid DNA is delivered. However, not all plasmids initiate conjugation: some rely exclusively on vertical inheritance, while others lack conjugation machinery but can opportunistically transfer by exploiting the conjugation apparatus of co-resident plasmids. Plasmid conjugation can facilitate gene exchange across bacterial lineages and even kingdom boundaries [3].

Whether these emerged resistance genes can persist depends on selection. Selection is shaped by the interplay between (i) pharmacokinetics (PK), which describes how drug concentrations

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change over time within the host, and (ii) pharmacodynamics (PD), which describes how these concentrations influence bacterial population dynamics [4].

Each use of antibiotics imposes selective pressure on microbial populations, which can favor the emergence or spread of resistant variants. This is consistent with the widely accepted view that antibiotic use is the main driver of resistance evolution [5, 6]. However, because infections can be life-threatening, antibiotic use is often unavoidable, creating a trade-off between effective treatment and the risk of selecting for resistant bacteria.

This raises the fundamental question of how to best use antibiotics to treat infections; with and without knowledge about the phenotypic properties of the infecting pathogen. Furthermore, this question arises at multiple biological scales: within individual hosts, where antibiotic concentrations and bacterial populations interact dynamically; and between hosts, where treatment strategies influence the transmission and spread of resistance at the population level. Both scales are relevant for evaluating the effectiveness and evolutionary consequences of antibiotic use.

### Treatment Strategies

In an ideal scenario, the treatment of infections is tailored to the resistance phenotype of the infecting pathogen. However, in clinical practice this is often not feasible: in emergency settings, immediate treatment is required and microbiological characterisation often takes 24 to 72 hours [7]; resistance may exist below detection thresholds, rendering diagnostics inconclusive; and in prophylactic contexts such as surgery or treatment of immunocompromised patients, antibiotics are often prescribed preemptively.

In such cases of limited information, clinicians rely on empirical treatment strategies that aim to minimize the risk of selecting for resistance. Commonly discussed approaches include combination therapy (simultaneous use of multiple antibiotics), mixing (random patient allocation to different antibiotics), and cycling (periodic rotation of antibiotics over time). Among these, combination therapy, which was first introduced in agriculture to prevent resistance in plant pathogens [8–10] is often considered the most promising strategy in theoretical models [11–13].

However, concerns persist. One major concern is that combination therapy might accelerate the selection for resistance, given the observed correlation between overall antibiotic consumption and resistance [5, 6], not only regarding the targeted pathogen but also for the microbiome

[14]. Furthermore, combining antibiotics may impose a higher burden on patients, for example through an increased risk of toxicity [15].

In the clinical context combination therapy plays a central role in clinical protocols for rapidly evolving pathogens such as HIV, *Mycobacterium tuberculosis*, and *Plasmodium falciparum* [16]. Nonetheless, the clinical evidence remains inconclusive. A recent meta-analysis found no consistent advantage of combination therapy in preventing resistance across bacterial infections [17]. The discrepancy between theoretical predictions and inconclusive clinical outcomes can stem both from limitations in modelling or clinical study design. On the theoretical side, models often rely on simplifying assumptions and may overlook important biological complexities, such as heterogeneity in patients, pathogens, and treatment responses. On the clinical side, most trials are not designed to detect resistance outcomes and are therefore underpowered for this purpose [17]. Additional variability arises from differences in study design, pathogens, treatment regimens, and clinical endpoints. Moreover, the choice of antibiotic combination itself can increase or decrease treatment success, further contributing to outcome variability.

To bridge the gap between theoretical models and clinical studies, Angst et al. [18] developed an *in vitro* experimental setup mimicking within- and between-host dynamics under different treatment strategies, and showing that combination therapy typically best prevents resistance emergence best.

In Chapter 2, we refine this framework to study plasmid-mediated resistance. Our results confirmed that combination therapy remains the most effective strategy in most scenarios.

### Population Dynamics under Combination Therapy

To better understand the potential of combination therapy, a deeper insight into drug interactions is needed. Drug interaction studies evaluate how the combined effect of two antibiotics compares to a predicted neutral effect derived from their individual actions. Two classical null models are typically used for this purpose: the Loewe additivity model [19] and the Bliss independence model [20]. Numerous studies have investigated drug interactions below the minimum inhibitory concentration (MIC) [21–23], providing valuable insights into sublethal interactions. However, it remains unclear whether these findings extend to the super-MIC range, where antibiotics are expected to kill bacteria. This uncertainty arises from the scarcity of studies exploring

interactions above the MIC.

The reason for this is methodological: to quantify treatment effects, we estimate the exponential change in time-series population curves. Below the MIC, bacterial growth is typically quantified using optical density (OD), which allows for high-throughput measurement. However, OD does not distinguish between live and dead cells, making it unreliable for estimating net growth rates above the MIC. At super-MIC concentrations, colony-forming unit (CFU) assays remain the gold standard, but they are labor-intensive, costly, and can become unreliable at high drug levels.

To address this gap, Chapter 3 evaluates luminescence as a high-throughput alternative to CFU counting and tests how reliably each method captures bacterial dynamics at inhibitory drug levels. We find that luminescence assays tend to track biomass more closely than viable cell numbers; which can cause divergence for estimated rates when cells filament.

Using this validated approach, Chapter 4 systematically characterizes pharmacodynamic interactions for multiple antibiotic pairs, examining whether interaction classifications are consistent across sub- and super-MIC concentration ranges.

### 1.1 Thesis outline

In this thesis we develop experimental and analytical methods to quantify how antibiotic-induced selection pressure shapes the evolution and transmission of resistance at both within-host and between-host scales.

In Chapter 2, we use an in-vitro hospital-ward model, to compare three multidrug strategies (combination, mixing, cycling), two monotherapies (Mono A, Mono B) and an untreated control. We show that combination therapy suppresses plasmid-borne double resistance most efficiently under the majority of conditions, but also find that in certain scenarios, mixing, cycling, or even monotherapy can perform equally well. Surprisingly, we find that omitting treatment entirely can accelerate the emergence of multi-resistance—a phenomenon specific to plasmid-mediated resistance dynamics.

In Chapter 3, we assess the applicability of luminescence assays as a high-throughput method for estimating net growth above the MIC, benchmark them against CFU counts, and develop a mathematical framework that explains when each read-out reflects biomass, viable cell number, or neither. We find that light intensity correlates more closely with biomass than with viable

cell number, which leads to discrepancies in growth-rate estimates under conditions that induce filamentation. However, we encountered two well-documented yet commonly ignored limitations of CFU counting — antibiotic carry-over and loss of culturability — which make rate estimates unreliable at high drug concentrations.

In Chapter 4, we build on the validated luminescence workflow, and use it to map net-growth surfaces for 2160 drug–drug–concentration combinations, using six antibiotics. From these data, we derive interaction parameters for each pair and compare them to the expectations under Loewe additivity and Bliss independence. We find that the classification of drug interactions can shift between sub- and super-MIC ranges, implying that interaction types at sub-MIC levels do not necessarily generalise to inhibitory concentrations. Furthermore, we extend the pharmacodynamic formalism to be applicable for drug combinations, allowing us to analyse how pharmacodynamic parameters of individual drugs combine under co-treatment.

We conclude the thesis in Chapter 5 with a synthesis of our findings and a discussion of their implications for optimising antibiotic use.

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# 2

## The Impact of Treatment Strategies on the Epidemiological Dynamics of Plasmid-Conferred Antibiotic Resistance

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### 2.1 abstract

The issue of antibiotic resistance is a critical concern for public health, prompting numerous investigations into the impact of treatment strategies on preventing or slowing down the emergence of resistance. While existing studies have predominantly focused on chromosomal resistance mutations, the consequences of often clinically more relevant plasmid-conferred resistance remain insufficiently explored.

To address this gap, we conducted three extensive in vitro experiments utilising a liquid-handling platform. These experiments evaluated the efficacy of five distinct treatment strategies using two antibiotics (tetracycline and ceftazidime) along with two horizontally transmissible clinical resistance plasmids conferring the respective resistances.

Among the experimentally investigated treatment strategies, combination therapy proved to be the most effective in preventing the emergence of double resistance while minimising the number of infections.

To verify the reliability of these findings, we constructed a computational model of our experiments that we parameterised using the experimental data. We employed this model to augment the experimental data by conducting an *in silico* parameter sensitivity analysis. The sensitivity analysis corroborated our experimental results, demonstrating that combination therapy consistently outperformed other treatment strategies across a range of parameter values.

### 2.2 Introduction

In light of the growing threat of antimicrobial resistance (AMR) to human health, various multidrug strategies are being considered to improve the sustainability of antibiotic use. These approaches include combination therapy (simultaneous use of multiple antibiotics), mixing therapy

## 2. THE IMPACT OF TREATMENT STRATEGIES ON THE EPIDEMIOLOGICAL DYNAMICS OF PLASMID-CONFERRRED ANTIBIOTIC RESISTANCE

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(randomly assigning patients to receive different antibiotics), and cycling therapy (alternating between multiple antibiotics over time).

Combination, originally proposed alongside cycling therapy to prevent biocide resistance in plant pathogens [1–3], was later adopted in human medicine. Combination therapy proved its effectiveness in preventing resistance evolution in highly adaptable pathogens such as HIV, *Mycobacterium tuberculosis*, and *Plasmodium falciparum* [4]. However, a recent meta-analysis investigating the effect of combination therapy on resistance across various bacterial infections and antibiotic combinations found no evidence for a difference in the risk of resistance acquisition [5]. Also, a comprehensive cluster-randomised crossover study comparing mixing and cycling by van Duijn et al. [6], spanning nearly two years across eight ICUs, found no significant differences in outcomes.

A review of the available model literature by Uecker et al. [7] reveals the complexity and context-dependent efficacy of treatment strategies such as combination, cycling or mixing strategies. Yet, theoretical models often identify combination therapy as the best strategy to prevent new resistance [8, 9]. It remains unclear whether the inconclusive results regarding the effectiveness of multidrug treatment strategies in the literature are due to the theoretical models failing to account for key processes, or if clinical studies lack statistical power, as suggested by Siedentop et al. [5]. This lack of power may be caused by patient and bacterial strain heterogeneity, stochasticity in infection dynamics, and other unknown factors that make it difficult to isolate single effects.

We recently started experiments to make a foray into the large gap between theoretical models and clinical trials. In an in vitro experiment mimicking the epidemiological scenario of transmission in a hospital ward, Angst et al. [10] investigated the effect of cycling, mixing, and combination therapy on resistance evolution and showed that for chromosomal resistance mutations combination therapy outperformed the other strategies. One potential reason why combination therapy succeeded in that study and tends to be superior in mathematical models is that it increases the genetic barrier to resistance by requiring the acquisition of multiple mutations in the same background.

Here, we explore the effect of horizontal gene transfer (HGT) on resistance evolution under treatment by conducting three large-scale in vitro experiments. The experiments mimic epi-

demiological transmission dynamics of symptomatic infections by a focal strain in an intensive care unit (ICU) and include patient discharge and admission, infection between patients, and treatment. We use two antibiotics, ceftazidime (A) and tetracycline (B), along with two clinical resistance plasmids [11] we call  $p_A$  and  $p_B$ , conferring resistance to the corresponding antibiotics. The plasmids are compatible, can conjugate, and were isolated from clinical samples collected and characterised in a study at University Hospital Basel [12]. We model patients as wells in a 384-well microtiter plate filled with LB medium. These “patients” can be infected with a mixture of bacteria, which may carry up to two resistance plasmids. Depending on the presence of bacteria and resistance, we assign each “patient” a resistance profile: uninfected ( $U$ ), sensitive infected ( $S$ ), single-resistant ( $A_r$ ,  $B_r$ , or  $(A_r \& B_r)$ ), or double-resistant ( $AB_r$ ).

In each experiment, we model six hospital wards to assess the ability of five treatment strategies (mixing, cycling, combination therapy with two antibiotics and two monotherapies with each antibiotic alone) and one control (no antibiotics) to contain the spread of plasmid-borne resistance and prevent the emergence of double resistance. All patients in each ward are treated daily according to the assigned strategy. A schematic of the experimental setup is shown in [Figure S2.1](#). Each of the three experiments addresses a different scenario (Table 2.1), varying in patient turnover probability (admission/discharge), infection probability, and the distribution of resistance profiles for incoming patients (sampling proportions). The prevention scenario addresses a situation with low levels of pre-existing single and no double resistance brought into the hospital ward from the community. The containment scenario focuses on the ability of treatment strategies to contain pre-existing double resistance and in the maximum-emergence scenario, we maximised the opportunities for emerging double resistance through HGT by admitting single-resistant patients only.

Alongside our experiments, we created a computational model that mimics the experiment and is parameterised but not fitted using the experimental data. We used the model to assess the robustness of our findings to the randomisation of the experimental decisions and conducted an in silico sensitivity analysis to augment the experimental data.

### 2.3 Results

In each of our three experiments, we simulated the transition dynamics across six hospital wards on six 384-well plates. Each 384-well plate simulates four replicate hospital wards, with each replicate comprising 96 wells representing 94 patients and two negative controls. We replace each assay plate daily to renew the treatment and medium ([Figure S2.1](#)). Based on the turnover probability  $\tau$ , we randomly decide if a patient stays. If this is the case, we inoculate the well on the new plate from the same well on the old plate. Else we replace this patient with a new incoming patient by inoculating the well on the new plate from a strain plate containing all resistance profiles. The resistance profile of the incoming patient is randomly selected based on predefined probabilities (sampling proportions  $c_\phi$ ). Based on the infection probability  $\beta$ , we randomly decide if a patient will infect another randomly chosen patient. These infections are then simulated in vitro by passing a drop to the infected well on the new plate. All inoculations are carried out using the same pintoool.

**Multidrug strategies keep the overall number of infections lowest and best suppress single resistance.** The prevention scenario is characterised by a moderate proportion of single-resistant admissions to the hospital ward, the absence of pre-existing double resistance, and a moderately spreading infection dynamic ( $R_0 = 1.5$ , [Equation S2.1, SI Methods](#)). In this scenario, there are no differences between combination, mixing, and cycling on the frequency of uninfected, single-resistant-infected and double-resistant-infected wells ([Fig. 2.1A](#), time series in [Figure S2.2](#)).

However, all multidrug strategies are significantly better at suppressing single resistance and increasing the number of cleared wells than the single-drug strategies and the control without treatment ([Fig. 2.1A](#)). In all scenarios, combination therapy was one of the most successful treatment strategies in minimising single-resistant and overall infections. At the same time, we

Table 2.1 Parameter sets and  $R_0$  used in the three experiments:  $c_\phi$  is the proportion of admitted patients with resistance profile  $\phi$ ,  $\tau$  denotes the probability that a patient is replaced with a new patient sampled from the community and  $\beta$  denotes the infection probability.

scenario	$c_S$	$c_{Ar}$	$c_{Br}$	$c_{ABr}$	$c_U$	$\tau$	$\beta$	$R_0$
prevention	0.75	0.05	0.05	0	0.15	0.20	0.30	1.5
containment	0.58	0.11	0.11	0.05	0.15	0.20	0.35	1.75
maximum-emergence	0	0.50	0.50	0	0	0.50	0.25	0.5

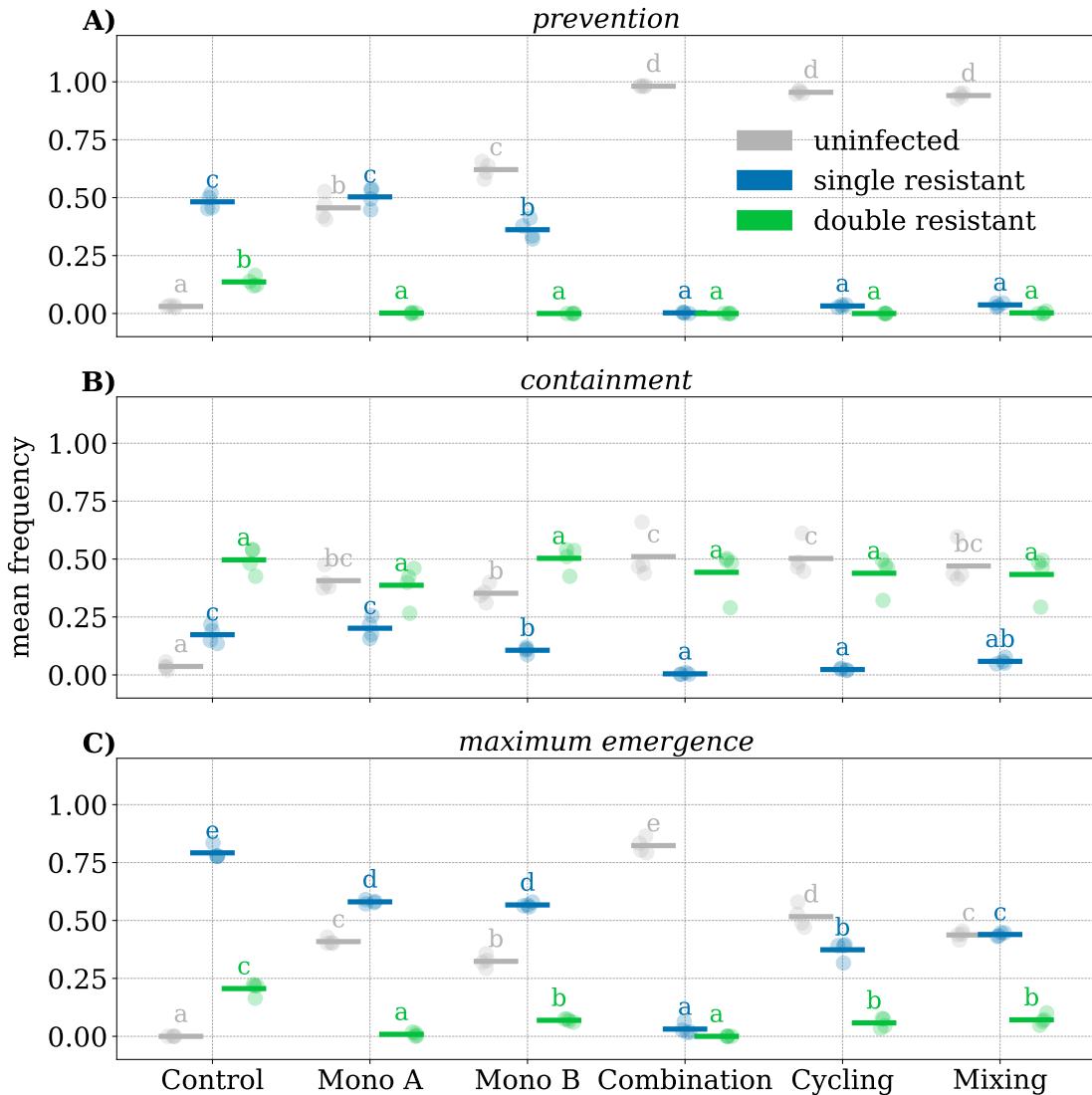


Fig. 2.1 Panels A–C show the mean frequency of uninfected (grey), single-resistant infected (blue), and double-resistant infected wells (green) during the last four transfers of the three scenarios. Circles represent replicates ( $n = 4$ ), and bars represent means. Within resistance categories, bars not sharing a letter are significantly different (pairwise Tukey post hoc test,  $p < 0.05$ ; ANOVA tables and all p-values can be found in Table S2.34 – S2.50).

observed most single and double resistance in the untreated control. All strategies (but not the control) were able to clear sensitive infections effectively with clearance probabilities of 97% for drug A, 73% for drug B, and 86% for AB (Table S2.8).

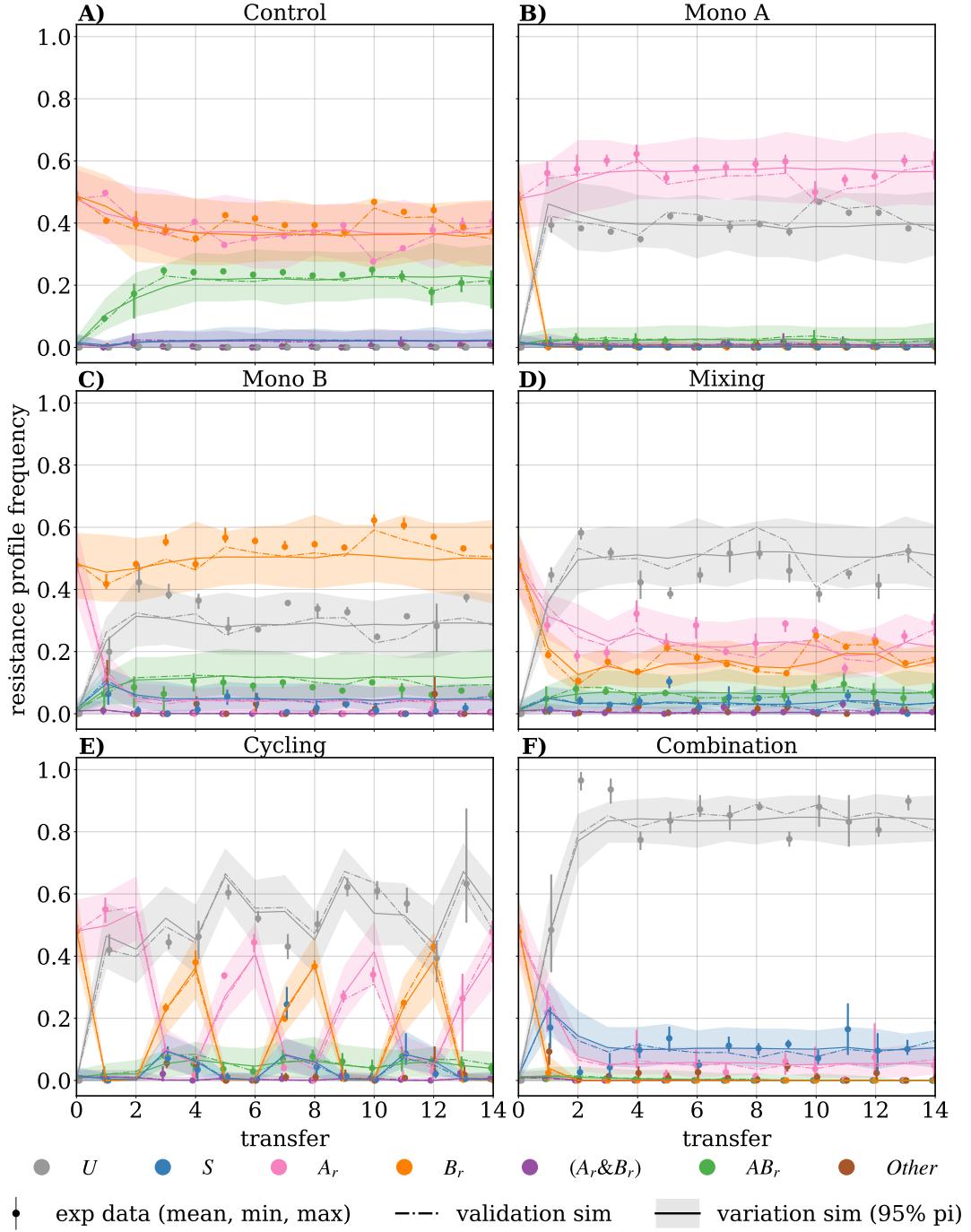


Fig. 2.2 Frequencies of resistance profiles over time during the maximum-emergence scenario. Panels (A–F) show the six tested strategies. Dots and bars show the mean and min/max interval of the four replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment (validation simulation). The solid line shows the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment (variation simulations). The shaded error band indicates the 95-percentile interval between the variation simulations.

**All treatment strategies fail to contain pre-existing double resistance.** The containment scenario explores a situation in which patients infected with double-resistant bacteria are continuously admitted to the hospital. No strategy was able to contain the spread of double resistance, resulting in increased frequencies of double resistance ( $> 40\%$ ) in all treatment arms at the conclusion of the experiment (Fig. 2.1B).

**Treatment strategies affect the emergence of double resistance.** In our experiments, double resistance primarily emerges in wells inoculated with both single-resistance plasmids via HGT, as the evolution of de novo resistance (e.g. by point mutations) to high drug concentrations ( $> 50\times\text{MIC}$ ) is unlikely. As the inoculum volumes for turnover, infection, and passage are identical in our experiments, we do not distinguish between wells containing A-resistance ( $A_r$ ) infecting wells containing B-resistance ( $B_r$ ) or vice versa and simply refer to these events as superinfections. During the prevention and containment scenario, we could not identify differences in the strategies' abilities to suppress the emergence of double resistance. We attribute this to a lack of statistical power because we observed only a few instances of double resistance emerging, mostly in the untreated control. To address this, we selected parameters for the maximum-emergence scenario designed to maximise superinfection opportunities between wells carrying complementary resistance. To this end, all admitted patients carried bacteria with only one of the two resistance plasmids (at equal proportions). In addition, we set the probability of admission and discharge to  $\tau = 0.5$  and the infection probability to  $\beta = 0.25$ , resulting in a basic reproduction number  $R_0 = 0.5$  (Equation S2.1). An  $R_0 < 1$  makes double resistance more likely to be replaced by newly admitted patients than to spread, thus maintaining a high potential for emergence. We implement this scenario to explore emergence under a magnifying glass, being aware that it does not reflect a likely clinical situation. In this scenario, combination therapy and monotherapy with drug A lead to the lowest frequency of double resistance during the last four transfers (Fig. 2.1C, Fig. 2.2).

For the maximum-emergence scenario, we observed that combination therapy, cycling, and monotherapy with drug A were most effective in preventing newly emerging double resistance. Combination was the only strategy in which we did not observe a single case of emerging double resistance after the first transfer (Fig. 2.3A). Furthermore, combination therapy is the most successful treatment strategy in minimising the number of both single-resistant and overall infec-

tions, while the control leads to the highest number of double- and single-resistant, and overall infections.

**Combination therapy suppresses the emergence of double resistance by preventing superinfections.** Treatment strategies can impact the emergence of double resistance by suppressing superinfections. The number of superinfections  $n_{\mathcal{S}}$  is dependent on the abundance of the single resistance carrying wells  $A_r$  and  $B_r$ . Hence, we expect the highest number of superinfections and most opportunities for emerging double resistance when both single resistances are unaffected by the treatment and the fewest if the treatment successfully suppresses both single resistances. Our measurements confirmed these expectations during the maximum-emergence scenario. Here  $n_{\mathcal{S}}$  is highest in the control group (no treatment) and lowest under combination therapy (Fig. 2.3B).

**Treatment strategies influence the emergence of double resistance within superinfected wells.** We observed the highest average frequency of superinfections developing double resistance ( $\frac{n_{\mathcal{S}}}{n_{\mathcal{S}}}$ ) in antibiotic-free medium and in medium treated with antibiotic B (tetracycline). In contrast, superinfections resulting in double resistance rarely occur in medium treated with antibiotic A (ceftazidime) or both drugs (Fig. 2.3C). We think the impact of treatment on cell densities within superinfected wells (both in infected and infecting wells) can best explain these findings.

Firstly, applying a drug affects the in-well population dynamics of superinfected wells. Reducing the cell density for one or both single-resistant strains within a superinfected well reduces the probability of bacteria with complementary resistance to encounter and conjugate (see [SI Results](#)). As drug A (bactericidal) decreases the cell density faster than drug B (bacteriostatic), more conjugation opportunities occur in wells treated with drug B.

Secondly, the treatment strategies influence the number of transferred single-resistant bacteria that inoculate superinfections by curbing the bacterial density within the infecting wells (see [SI Results](#)).

Due to the differences in the abilities of drugs A and B to prevent conjugation, there are times (cycles) and places (beds) during cycling and mixing where using drug B offers increased opportunities for the emergence of double resistance, which is never the case with combination therapy.

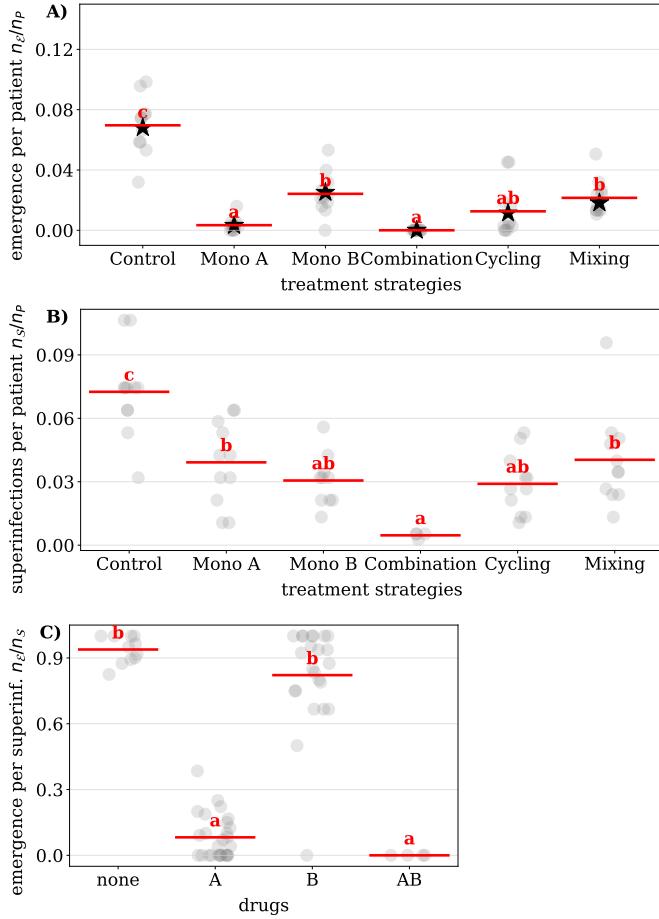


Fig. 2.3 Analysis of the emergence of double resistance in vitro and superinfection between single resistant  $A_r$  and  $B_r$  wells during the maximum-emergence scenario, from transfer four onwards. Each dot corresponds to data from a single plate, with each plate representing a distinct treatment arm, encompassing 376 patients for one transfer. Mean values are represented by red bars. Bars not sharing a letter are significantly different ( $p < 0.05$ , ANOVA tables and pairwise Tukey post hoc results can be found in Table S2.51 – S2.56). A) Number of newly emerged cases of double resistance per plate ( $n_d$ ), normalised to the total number of patients ( $n_p = 376$ ). B) Number of superinfections per plate ( $n_s$ ), normalised to  $n_p$ . C) Proportion of superinfected wells treated with A, B, AB, or none that develop double resistance.

**Computational model corroborates the robustness of experimental outcomes.** The experiments are conducted by a liquid handling platform that carries out predefined instructions, specifying which infections occur and who is admitted or discharged. The instructions are randomized based on parameter sets we defined for each scenario, including the overall infection and turnover probability as well as the distribution of the resistance profiles of admitted patients. We call the entirety of all instructions that come up during one experiment an ‘instruction set’. Due to the scale and technical complexity of the experiments, it was not feasible to carry out individual instruction sets for each replicate, so we opted to apply the same instruction sets for all replicates. This raises the question of whether the experimental results are a consequence of a specific instance of this random process and whether they are robust to the randomisation in the instruction set. To address this, we developed a discrete-time stochastic model comprising

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94 individual in silico patients mimicking the epidemiological dynamics of the experiment ([SI Computational Model](#)). The model was parametrised, but not fitted, with transition probabilities ([Table S2.18–S2.25](#)) that we estimated based on the transition frequencies measured in vitro. We used the same transition probabilities in the simulations for all scenarios.

First, we validated the model by averaging 100 validation simulations, each employing the identical instruction sets used in vitro. The aim of the validation simulations is to recreate the experiments in silico ([Figure S2.3B](#)). We found that the simulation results are in good agreement with the experimental data, indicating that the model reflects the dynamics observed in the in vitro experiments well (see Fig. 2.2, [Figure S2.2](#), and [Figure S2.4](#)). One exception is the spread of A-resistance during the prevention scenario in control and Mono A. This could indicate an increased number of contaminations at the beginning of the prevention scenario. We also observe some discrepancies for the spread of double resistance during the prevention scenario, which we attribute to contamination artefacts in the transition probabilities (see [SI Computational Model](#)).

Second, we averaged 100 variation simulations to assess the robustness of the experimental outcomes against variations in the instruction sets. In these variation simulations, each of the 100 instruction sets was randomized based on the same three parameter sets used in vitro ([Figure S2.3C](#)). Differences between the validation and variation simulations indicate differences in outcome due to the randomization of the instruction sets. For instance, with a turnover probability  $\tau = 0.2$  and an admission probability  $c_A = 0.05$ , we expect  $0.94 A_r$  admissions per transfer. However, random fluctuations can result in either more (or fewer)  $A_r$  admissions, leading to a temporarily higher (or lower) frequency of  $A_r$  in the validation simulations, creating a temporary spread between the validation and variation simulations. We observed that the validation simulations fluctuate around the variation simulations and never diverge far (see Fig. 2.2, [Figure S2.2](#), and [Figure S2.4](#)), indicating robustness of the experimental results to the randomisation of the instruction sets.

***In silico* sensitivity analysis indicates that the superiority of combination therapy is robust.** Given that the validation simulations agreed well with the experiments, we used the model to perform an in silico parameter sensitivity analysis of the experimental results ([Figure S2.3D](#)). To this end, we ran ten simulations for each of 20,000 randomly generated parameter sets by varying

the turnover and infection probability and the five sampling proportions for incoming patients:  $(\tau, \beta, c_S, c_{A_r}, c_{B_r}, c_{AB_r}, c_U)$ . For half of the parameter sets, we forced the frequency of incoming patients with double resistance ( $c_{AB_r}$ ) to zero.

We used the frequency of uninfected in silico patients to measure treatment success. Using this criterion, the control strategy (no treatment) always performed worst, and accordingly, we excluded this treatment arm from the following analysis. Strategies were then classified as (i) 'single winners' if they are significantly better than all other strategies; (ii) 'winners' if they are not outperformed by any other strategy; (iii) 'losers' if they do not outperform at least one other strategy; or (iv) 'single losers' if all other strategies outperform them.

In parameter sets with and without pre-existing double resistance, combination therapy ranks most often as one of the best strategies (87% and 98%, respectively). It is the single best strategy in 55% of the tested parameter sets with pre-existing double resistance and in 93% of cases without pre-existing double resistance (Fig. 2.4, [Table S2.14](#), [Table S2.15](#)).

In some situations (for example, when one strategy is much worse than all others), it is more important to avoid the worst strategy than selecting the very best strategy among the good ones. Our analysis finds that combination therapy is almost never among the worst strategies, while usually one of the two monotherapies performs worst. As expected, single-drug strategies perform particularly poorly when there is a high frequency of pre-existing single-resistance to the applied drug ([Table S2.11](#), [Table S2.13](#)).

Cycling and mixing lose substantially less than the monotherapies but are rarely the single best strategy.

## 2.4 Discussion

In our study, multi-drug strategies, particularly combination therapy, outperformed monotherapies in reducing overall infections and the emergence of double resistance across most scenarios, while we observed most emergence of double resistance in the untreated control. Interestingly, the effectiveness of combination therapy does not stem from an increased efficacy associated with higher doses. This is because an asymmetrical antagonism exists between the bactericidal antibiotic ceftazidime (drug A) and the bacteriostatic antibiotic tetracycline (drug B), resulting in a lower clearance rate for the combination A+B compared to drug A alone ([SI Results](#)). This

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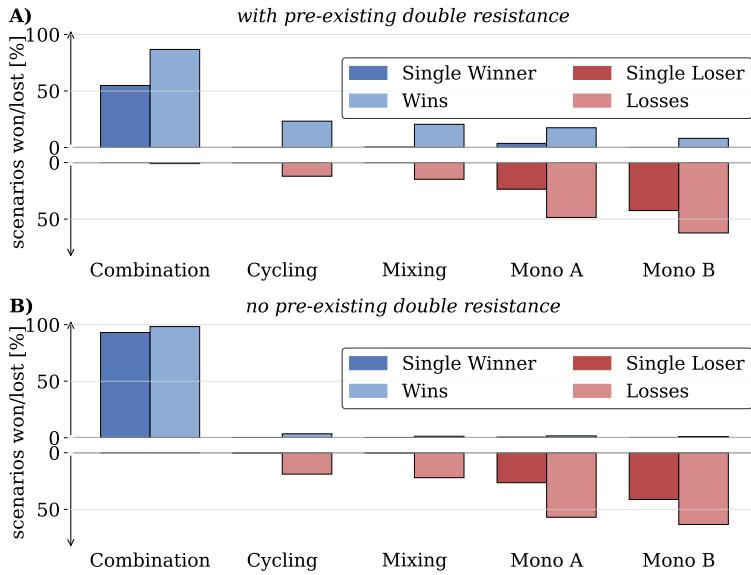


Fig. 2.4 Effectiveness of the five treatment strategies in maximising the frequency of uninfected individuals across randomly generated parameter sets. Strategies not significantly better than any other are marked as losers (pastel red), and those significantly worse than all others as single losers (dark red). Strategies not significantly worse than any other are classified as winners (pastel blue), and those significantly better than all others as single winners (dark blue). Strategies without significant differences were excluded.  
 (A) 10,000 parameter sets with pre-existing double resistance. 606/10,000 sets yielded no significant difference between the strategies.  
 (B) 10,000 parameter sets without pre-existing double resistance. 100/10,000 sets yielded no significant difference between the strategies.

observation implies that combination therapy may be even more advantageous when drugs are neutral or synergistic towards each other.

Why does the absence of treatment lead to worse outcomes, and why is combination therapy preventing the emergence of double resistance so effectively?

First, we measured the presence, not the density, of resistant bacteria in wells by assessing if small aliquots of the liquid culture could grow on treated agar plates. This approach quantifies the number of wells hosting a specific resistance but can not quantify the frequency of resistance in the in-well population. The information about presence/absence alone yields important information about potential treatment success and is used in analogous clinical diagnostic methods, such as disk diffusion tests [13].

We would only recognize a loss of resistance (in the experiments and clinical samples) if the

resistant strain were fully outcompeted. This was not observed during the containment scenario in the untreated control. Such an outcome was expected due to the short average patient stay of 2–5 days in our experiments and 5–6 days in clinical situations [14]. For the same reason, we would not expect an eradication of resistance but only a shift in resistance density, even if there were more substantial costs of resistance or higher segregational loss. In our experiments, we found no evidence of a cost of resistance (see [SI Methods](#), [Figure S2.6](#), and [Table S2.3](#)) or segregational loss (see [SI Methods](#) and [Table S2.4](#)).

Second, in our experiments, the emergence of double resistance requires conjugation, which relies on superinfection between hosts with complementary resistance profiles. As demonstrated in Fig. 2.3B, the lowest number of superinfections occur in combination therapy, where both single-resistant strains can be cleared. Conversely, without treatment, the abundance of single resistance is highest resulting in the highest number of superinfections.

Third, the applied antibiotics affect the frequency of superinfections leading to double resistance, likely by influencing the growth dynamics within the superinfected well and potentially the conjugation rate [15]. However, our experimental data are unsuitable for supporting or rejecting the impact on conjugation rates. We observed the least emergence of double resistance in superinfected wells treated with both drugs and most in untreated wells, contributing to the superiority of combination therapy and the high rates of double resistance in the absence of treatment (Fig. 2.3C). This effect on the in-well dynamics may be a property of the chosen drugs and concentrations, and we expect better results for cycling and mixing if both drugs were equally effective in suppressing double resistance or worse results for combination therapy if the combination of both drugs was less effective.

Fourth, we observed that the number of single-resistant bacteria inoculating superinfections impacts the frequency of emerging double resistance (see [SI Results](#), [Table S2.1](#)). In our setup, superinfected wells receive two inocula, with at least one inoculum transferred from the previous plate (by infection) that has already undergone treatment for one day. When prior treatment led to a low bacterial density in the source wells, we did not observe any cases of double resistance emerging. This could magnify the effectiveness of combination therapy, where all potential single-resistant inocula transferred from the previous plate contain low bacterial densities due to effective treatment. On the one hand, this may be more a characteristic of our experimental

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setup due to the fixed length of the treatment interval and high clearance probabilities. On the other hand, we indeed expect fewer cases of emergence in superinfected patients if the infecting inocula are small.

In our experiments and simulations, combination therapy showed superior results in minimizing infections and preventing double resistance. This advantage may partly result from assumptions and simplifications, including the chosen strain, drugs, plasmids, and inoculum size, the discrete setup with fixed treatment durations, colonization-independent infection and turnover probabilities, and the absence of an immune system and microbiome. Also, treating all patients irrespective of colonization diverges from clinical reality in two ways: i) in a clinical setting, some untreated patients may serve as a sanctuary for resistance and a potential source of double resistance and ii) treating all patients, regardless of infection status, contrasts with clinical efforts to promote targeted antibiotic use. However, since patients as we model them in our in vitro experiments lack a microbiome, treating uninfecteds should have no impact on the resistance dynamics.

Despite the numerous differences between our experiments and a real clinical situation, we argue that the relative effectiveness of combination therapy in suppressing double resistance would likely translate to real patients. The reason is that the emergence of double resistance hinges on two critical processes: 1) preventing superinfections between patients carrying bacteria with complementary resistance plasmids and 2) the probability that superinfected hosts develop double resistance. We think that combination therapy offers a strategic advantage in addressing both processes.

Our results complement the findings by Angst et al. [10], who observed similar outcomes in the context of chromosomal resistance. We believe that such in vitro experimental models, which explore admittedly idealised and simplified epidemiological scenarios, can help to bridge the divide between mathematical models and randomised clinical trials. However, ultimately the evidence for or against the benefits of combination therapy must be confirmed by rigorous clinical trials with sufficient statistical power to support or challenge the effectiveness of combination therapy.

## 2.5 Methods

**Drugs and Media.** In all experiments, we used LB (Sigma L3022) with  $25 \mu\text{g}/\text{ml}$  (prevention scenario) or  $5 \mu\text{g}/\text{ml}$  (containment and max-emergence scenario) chloramphenicol as a liquid medium and the same LB and drugs with 1.5% agar as a solid medium. Chloramphenicol was added to prevent external contaminations. We could not measure any significant growth effects of the chloramphenicol concentrations on the chloramphenicol-resistant strains (see [Table S2.5](#)). We used  $80 \mu\text{g}/\text{ml}$  ceftazidime as drug A and  $40 \mu\text{g}/\text{ml}$  tetracycline as drug B, with identical concentrations for liquid and solid media.

**Strains and Plasmids.** We used two compatible plasmids  $p_A$  and  $p_B$  derived from samples ESBL9 and ESBL25 from a clinical transmission study [12]. Samples were kindly provided by Adrian Egli and sequenced and analysed by Huisman et al. [11]. Plasmids  $p_A$  and  $p_B$  provide (among other resistances) resistance against drug A and drug B, respectively. We used these plasmids and the chloramphenicol-resistant host MDS42-YFP [16] (sensitive to drugs A and B) to create three additional strains by conjugation ([Table S2.2](#)) (i) A-resistant, containing  $p_A$ ; (ii) B-resistant, containing  $p_B$ ; and (iii) AB-resistant, containing both plasmids (see [SI Methods](#)).

**Treatment arms.** We simulated the epidemiological dynamics of six hospital wards in vitro, with each ward exploring a different treatment arm: (i) control with no treatment, (ii) monotherapy with ceftazidime (mono A), (iii) monotherapy with tetracycline (mono B), (iv) cycling therapy (A, A, B, B, ...), (v) mixing therapy (treatment A and B are randomly assigned daily, without knowledge of prior treatment), and (vi) combination therapy (treating all patients with both drugs, each at full concentration).

**Assay plates.** Each hospital ward was simulated in vitro on a 384-well microtiter plate (Greiner 781186). Wells are interpreted as beds in four replicate hospital wards with 94 beds each. The remaining wells contained only growth medium and remained untouched, acting as sentinels for contamination. Across all experiments and treatment arms, 2752 control wells were used, 67 of which became contaminated. Wells with growth medium but no bacteria represent uninfected patients, whereas wells with growth medium and (resistant or sensitive) bacteria represent infected patients.

**Experimental procedure.** Experiments were performed using a Tecan Evo 200 automated liquid handling system (Tecan) with an integrated, automated incubator (Liconic STX100, Liconic), a Tecan Infinite F200 spectrophotometer (Tecan), and a camera (Pickolo, SciRobotics).

Every day new assay plates were filled with 45  $\mu$ l fresh medium and 5  $\mu$ l antibiotic stock, according to its designated treatment strategy (see [Figure S2.1](#)). At each of these transfers, we simulate patients staying overnight in the hospital (passage), the admission and discharge of patients (turnover), and infections between patients (infection). Passage, turnover and infections were all done by inoculating the new plate using a pintoool with retractable pins, as detailed below, carrying  $\approx 0.3 \mu$ l drops between wells ( $\approx 1 : 150$  dilution) leading to an approximately 6-8 hours exponential phase. The assay plates were then incubated at 37°C and 95% relative humidity. The incubation duration varied due to variations in the time it takes to set up a new transfer and occasional transfer repetitions made necessary because of machine errors or user mistakes. The mean incubation duration was 27 hours.

We initiated the experiment by inoculating one 384-well plate from fresh overnight cultures representing patients from an outside community. We assume that this community is sufficiently large to be unaffected by interactions with the hospital ward. Incoming patients are either uninfected or carry one of the four strains (sensitive, each single resistant or double resistant) and are sampled according to predefined sampling proportions, defining the probability of a resistance profile being admitted to the hospital. (Table 2.1). This initial plate remained untreated and was used as the initial population for all six treatment arms.

**Turnover.** Every transfer, each patient has a turnover probability  $\tau$  to be discharged from the hospital and replaced by a newly admitted patient. Wells representing staying patients were passed from the previous to the new assay plate using the pintoool. Here, the pins for discharged patients are retracted. Vacant beds on the assay plate were then reoccupied by patients from the community analogous to the initial setup.

**Infections.** To simulate infections, each well has an infection probability  $\beta$  to infect another randomly chosen well on the next assay plate during the transfer. Therefore, each patient can infect at most one other patient per transfer, but several patients could potentially infect the same patient.

**Resistance Profiles.** To assess the resistance profile of each well, we spotted the previous assay plate onto four agar plates, using the pintool. Three plates were treated with antibiotics (A, B, or AB), while one was untreated (none). After incubation at 37 °C and 95% relative humidity, images of the agar plates are taken and analysed using the Pickolo package (SciRobotics, Kfar Saba, Israel). The software automatically detects the presence of colonies at each well position, which we also manually verified. The growth pattern on the four agar plates allowed us to determine the resistance profile of a well, which reflects how the well would behave if treated.

By default, we distinguish six resistance profiles ([Table S2.6](#)). The wells may either be 1) uninfected ( $U$ ), 2) exclusively infected with sensitive bacteria ( $S$ ), 3) infected with A-resistant bacteria ( $A_r$ ), 4) infected with B-resistant bacteria ( $B_r$ ), 5) infected with AB-resistant bacteria ( $AB_r$ ), 6) or be infected with a mixed population containing A-resistant and B-resistant bacteria, but no AB-resistant bacteria ( $(A\&B)_r$ ). The way we classify the resistance profiles of the bacterial population in a well leads to the dominance of resistance, in the sense that a predominantly sensitive population harbouring a resistant minority would be classified as resistant (see [Table S2.7](#)). Any observed growth pattern not corresponding to the six resistance profiles mentioned above is classified as 'other'. The resistance profile 'other' primarily occurs when bacterial densities are low (see also [SI Methods](#)).

**Scenarios.** We conducted experiments for three scenarios (prevention, containment, and maximum-emergence) with 14 to 27 transfers each. Each experiment was defined by a different parameter set consisting of (i) the infection probability  $\beta$  within the hospital, (ii) the turnover probability  $\tau$  and (iii) the sampling proportions  $c_\phi$  of patients with resistance profile  $\phi \in \{U, S, A_r, B_r, AB_r\}$  (see Table 2.1).

The prevention scenario ([Figure S2.2](#)) addresses how the treatment strategies perform with a moderately resistant community and a moderate infection regime in the hospital ward and how well they are able to prevent the upcoming double resistance.

The containment scenario ([Figure S2.4](#)) corresponds to a scenario in which some patients entering the hospital are infected with double-resistant bacteria to compare the ability of treatment strategies to contain the spread of pre-existing double resistance.

During the maximum-emergence scenario (Fig. 2.2) 50 % of the incoming patients are infected

with A-resistant bacteria, and the other 50 % are infected with B-resistant bacteria. These conditions maximally favour opportunities for horizontal gene transfer. The basic reproduction number was set to  $R_0 = 0.5$  ([Equation S2.1](#)) to ensure that double-resistant strains are flushed out, reducing the stochastic dependency on earlier emergence events while maintaining a high potential for new emergence.

**Instruction Sets.** Based on the parameter defined for each experiment (see Table 2.1), we generated instructions that were passed to the liquid handling platform. These instructions specify which patients are passaged or discharged and admitted, who infects whom, and the treatment for mixing therapy. Instructions are randomly generated prior to each transfer. We call the entirety of all instructions that come up during an experimental run an instruction set. Instruction sets are identical across all treatment arms and replicates.

**Computational Model.** We created a stochastic model ([SI Computational Model](#)) incorporating 94 in silico patients, each capable of adopting one of six resistance profiles  $\phi \in \{U, S, A_r, B_r, AB_r, (A_r \& B_r)\}$ . The model is structured analogue to the in vitro experiments ([Figure S2.1](#)) and alternates between modelling the transactions between wells and the effect of treatment during incubation. Admission and discharge (turnover) were simulated by replacing the resistance profile of the current patient with that of the incoming patient, as defined by the instruction set. Infections are simulated by combining the resistance profiles of the receiving well  $i$  and the infecting well  $j$ . The resulting resistance profile  $\phi_i + \phi_j$  is determined using the rules based on the dominance of resistance specified in [Table S2.9](#). Calculations involving more than two resistance profiles apply the associative law and are determined pairwise, e.g.  $(U + S) + A_r = S + A_r = A_r$ .

To model treatment effects, we use transition probabilities to assign the post-incubation resistance profile  $\phi(\hat{T})$  stochastically based on the treatment and the pre-incubation resistance profile  $\phi(T)$ . The transition probabilities ([Table S2.18 – S2.25](#)) were estimated based on experimental data across all experiments.

**In Silico Sensitivity Analysis.** To augment the experimental data, we conducted an in silico sensitivity analysis. We randomly generated 10,000 parameter sets with and 10,000 without pre-existing double resistance. Turnover and infection probabilities were uniformly sampled

[0.05, 0.95], allowing for  $R_0 \in [0.0526, 19]$ . The sampling proportions  $c_\phi$  for all incoming resistance profiles ( $\phi \in \{U, S, A_r, B_r, AB_r\}$ ) were randomised by sampling a number  $n_\phi \in [0, 1]$  from a uniform distribution and subsequently normalising by the sum:  $c_\phi = n_\phi / \sum_j n_j$ . We created ten randomised instruction sets for each parameter set and conducted one simulation per instruction set (Figure S2.3D) for 28 transfers.

For this analysis, the frequency of non-infected individuals during the last four transfers was used as a performance metric for treatment strategies, as it also indirectly reflects the frequency of both double- and single-resistant patients. We conducted an ANOVA test to assess if the effect of the treatment strategies significantly ( $p < 0.05$ ) influences the frequency of uninfecteds. For significant tests, we proceeded with Tukey's post hoc analysis ( $p < 0.05$ ), identifying significantly distinct pairs of strategies. Strategies not significantly inferior to others were classified as 'winners', while strategies not significantly superior to any were classified as 'losers'. Strategies that win or lose a parameter set alone are 'single winners' or 'single losers'.

**Data Availability** Experimental data and analysis scripts, as well as code for the computational model, have been deposited in Zenodo (<https://doi.org/10.5281/zenodo.14137410>).

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## 2.6 Supplementary Information Chapter 2

### SI Methods

**Strains** We chose two plasmid-carrying donors, ESBL9 and ESBL25, and two drugs, ceftazidime and tetracycline, based on the resistance conferred by the plasmids contained in the strains and the compatibility of the plasmids. ESBL9 and ESBL25 were collected as part of a clinical transmission study at the University Hospital Basel, Switzerland [1] and fully sequenced, including identification of the carried plasmids [2]. The strains were a generous gift from Adrian Egli, University Hospital Basel.

ESBL9 contains an IncI1 plasmid, referred to here as  $p_A$ , conferring, among others, resistance to ampicillin and ceftazidime but not tetracycline or chloramphenicol. ESBL25 contains an IncF1 plasmid, referred to here as  $p_B$ , conferring, among others, resistance to ampicillin and tetracycline but not ceftazidime or chloramphenicol.

The two plasmids were transferred by conjugation from the original clinical isolates to the chloramphenicol-resistant and ampicillin-sensitive Escherichia coli MDS42-YFP (recipient) [3], followed by selection for ampicillin and chloramphenicol resistance. This results in the ceftazidime-resistant (A-resistant) strain and the tetracycline-resistant (B-resistant) strain. The double-resistant (AB-resistant) strain was created by a further round of conjugation to receive both plasmids and subsequent selection for ceftazidime and tetracycline resistance. Strains are listed in Table 2.3. All transconjugants were verified by PCR replicon typing using primers specific for the respective replicon [4].

**Drugs.** We used ceftazidime, referred to as drug A, at a concentration of  $80 \frac{\mu\text{g}}{\text{mL}}$ .  $80 \frac{\mu\text{g}}{\text{mL}}$  is substantially lower than the MIC for A-resistant bacteria and more than 50 times the MIC for sensitive or B-resistant bacteria. Using the same reasoning, we used tetracycline, referred to as drug B, at a concentration  $40 \frac{\mu\text{g}}{\text{mL}}$ . The antibiotic concentrations in the liquid and the solid media were identical. To avoid contamination, we used  $25 \frac{\mu\text{g}}{\text{mL}}$  chloramphenicol for prevention scenario and  $5 \frac{\mu\text{g}}{\text{mL}}$  chloramphenicol for containment and maximum-emergence scenarios. We could not measure any significant growth effects of the chloramphenicol concentrations on the chloramphenicol-resistant strains (see Table 2.6).

**Conjugation Protocol.** We used ampicillin-resistant and chloramphenicol-sensitive original donors [1, 5] and the chloramphenicol-resistant, ampicillin-sensitive recipient [3]. Fresh overnight cultures of both donors and recipients were diluted 1:1000 and grown to mid-exponential phase. Following this, the donor and recipient cultures were combined in a culture tube and incubated for four hours at 37 °C with constant shaking at 180 rpm. We then spotted a 100  $\mu$ l drop of this mixture on an agar plate treated with 25  $\frac{\mu\text{g}}{\text{ml}}$  chloramphenicol and 100  $\frac{\mu\text{g}}{\text{ml}}$  ampicillin, allowing only the transconjugants to grow. Conjugation was verified by PCR replicon typing [4].

**Plasmid costs** To measure plasmid costs, we grew three replicates of overnight cultures of all strains in selective medium. The cultures were then diluted approximately 1:150 into LB with 5  $\mu\text{g}/\text{ml}$  chloramphenicol using the pintoool, following the same procedure as in the main experiments. Subsequently, we recorded OD growth curves using the same plate reader. The maximum growth rate was estimated by applying linear regression to a sliding window on the log-transformed data (window size: 1 hour, step size: approximately 5 minutes). Pairwise comparisons were performed between the maximum growth rates of the sensitive strain and the plasmid-carrying strains using the Mann-Whitney U test (scipy.stats [6]), followed by a Bonferroni correction to account for multiple testing. We observed no significant difference in the maximum growth rate between any of the pairs (Table 2.4).

**Segregational Loss** We estimated plasmid segregation loss over 24 hours ( $t_0 - t_1$ ) without treatment and with treatment as a control. For this, we grew overnight cultures in selective medium for three replicates  $k \in \{1, 2, 3\}$  of each plasmid-carrying strain. We diluted the cultures and plated each on drug-free agar plates, followed by replica plating onto selective plates to identify the presence or absence of resistance plasmids in each colony. This initial step represents time point  $t_0$ . The overnight cultures were then transferred to i) drug-free medium and ii) selective medium (control), using the same pintoool as in the main experiments. The cultures were incubated for 24 hours, after which we diluted and plated them again on drug-free plates and used replica plating on selective plates to assess plasmid presence for time point  $t_1$ . We compared the frequencies  $f_k(t)$  of plasmid presence between time points  $t_0$  and  $t_1$  using the Mann-Whitney U test (scipy.stats [6]). No significant loss of plasmids was observed in either the control or the experimental conditions (Table 2.5). We estimated the mean frequency of plasmid presence  $f(t)$  for

each strain and time point and the confidence intervals  $CI(t)$  for the frequency by bootstrapping the pooled colony presence-absence data.

**Growth rates and bacterial density.** We assessed the final bacterial density of overnight cultures following an 18-hour incubation period (Table 2.6) for each bacterial strain in its respective selective medium for two different chloramphenicol concentrations:  $5 \frac{\mu\text{g}}{\text{mL}}$  and  $25 \frac{\mu\text{g}}{\text{mL}}$ . To estimate the bacterial density, we plated  $200 \mu\text{L}$  of various dilutions of the cultures on agar plates using glass beads. The 95 % confidence intervals for the colony counts were calculated using the Poisson distribution.

In addition, we monitored the optical density (OD) in 384-well plates over an 18-hour period in the respective selective medium containing either  $5 \frac{\mu\text{g}}{\text{mL}}$  or  $25 \frac{\mu\text{g}}{\text{mL}}$  chloramphenicol. To determine the maximum growth rates, we employed a sliding window approach with a one-hour width, linearly fitting the growth rate to the log transformed values within this window for each replicate. The 95 % confidence intervals for the maximal growth rates were calculated using the Student's t-distribution.

**Basic Reproductive Number.**  $R_0$  represents the number of secondary infections generated by one patient in a fully susceptible population. Let  $\tau$  denote the probability that a patient leaves the hospital ward after one timestep,  $\tau' = 1 - \tau$  the probability that the patient stays and  $\beta$  the probability that a patient infects another during one timestep. Then we can write the total number of infections caused by one patient introduced into a susceptible population as a geometric series:

$$R_0 = (\tau')^0 \cdot \beta + (\tau')^1 \cdot \beta + (\tau')^2 \cdot \beta + \dots = \beta \sum_{n=0}^{\infty} (\tau')^n$$

This geometric series can then be rewritten as:

$$R_0 = \beta \cdot \frac{1}{1 - \tau'} = \frac{\beta}{\tau} \quad (\text{S1})$$

**Phenotyping -- Limitations.** The phenotyping procedure enables high throughput identification of resistance profiles. Although this method is generally reliable and effective for most wells, it is difficult to accurately determine the resistance profile for wells with very low bacterial densities

due to the potential for stochastic effects. We analysed 1784  $A_r$  turnover wells treated with antibiotic B during the maximum-emergence scenario. Here, we observed, alongside the expected  $A_r$  and  $U$  wells, 9% 'other' and 17%  $S$  wells, as detailed in Fig. 2.9a. It might be tempting to interpret the  $S$  wells as those in which all bacteria lost their plasmids and the 'other' wells as artefacts of measurement errors. Although these interpretations are not incorrect, a more critical factor influencing the measured resistance profile distribution is the inherent stochasticity of the method if applied to low-density wells.

As an example, we will analyse wells identified as  $A_r$ -wells during the previous transfer and subsequently treated with antibiotic B. To simplify the following analysis, we only consider agar plates treated with drug  $\vartheta \in \{N, A\}$  (no drug, drug A) and disregard plates treated with drug B (B-plates) and AB (AB-plates). Furthermore, we will use a prime ('') to indicate a counter probability ( $w' = 1 - w$ ). Drawing a drop with volume  $V_{\text{drop}}$  from a well with volume  $V_{\text{well}}$  leads to a probability  $p = \frac{V_{\text{drop}}}{V_{\text{well}}}$  of drawing a specific bacterium. The probability that the drawn drop contains no bacteria of phenotype  $\psi \in \{\emptyset, a\}$  (without resistance and A-resistance) is  $d'_\psi = (1 - p)^{Z_\psi}$ , with  $Z_\psi$  representing the number of bacteria with phenotype  $\psi$  inside the well. We denote the probability that a drop can grow on a plate treated with drug  $\vartheta$  as  $g_\vartheta$ . The probability  $g'_N$  of drawing a drop that will not form a colony on an N-plate can then be defined as  $g'_N = d'_\emptyset d'_a$ , and the probability  $g'_A$  of drawing a drop that will not grow on an A-plate is  $g'_A = d'_a$ .

Assuming that the drawn drop does not significantly alter the well volume and composition, we obtain the following probabilities for the possible resistance profiles (see also Fig. 2.9b):

$$\begin{aligned}\mathbb{P}(U) &= g'_N \cdot g'_A \\ \mathbb{P}(S) &= g_N \cdot g'_A \\ \mathbb{P}(A_r) &= g_N \cdot g_A \\ \mathbb{P}(\text{other}) &= g'_N \cdot g_A\end{aligned}$$

Assuming  $p = 0.006$  (value for our experiment), a well containing 25 A-resistant and 15 sensitive bacteria will result in 70%  $U$ , 26%  $S$ , 3%  $A_r$ , and 11% other. This result is similar to the experimentally measured distribution (compare Fig. 2.9a).

**Advanced Resistance Profiles** We introduced advanced phenotypes into our analysis to determine the approximate bacterial density inside the wells. Wells that retain their phenotype after one transfer, despite being sensitive to a particular antibiotic, are expected to exhibit a low bacterial density post-treatment and are labelled  $\phi^l$ . Conversely, wells that were either untreated or treated with an ineffective antibiotic are expected to contain a high bacterial density and are labelled  $\phi^h$ . In addition, wells that underwent a change in resistance profile or were mixed with other wells are denoted as  $\phi^?$  and are excluded from further analysis. We summarised the influence of the bacterial densities within the inoculating wells on the frequency of superinfections developing double resistance in Table 2.2.

**Statistical Analysis.** To compare the performance of different treatment strategies in vitro, we summarized the resistance profiles into groups and focused our analysis on three groups: uninfected, single-resistant and double-resistant. These labels stem from the properties within the wells. That means single resistant wells ( $A_r$ ,  $B_r$  or  $(A_r \& B_r)$ ) would be wells that contain only single resistant (or sensitive) bacteria, but no double resistant bacteria and therefore can be cured by combining drugs. In contrast, double-resistant wells cannot be cured using both antibiotics simultaneously since they contain AB-resistant bacteria. We then averaged the frequency of each group over the last four transfers for every replicate. Four transfers correspond to a complete cycle in the cycling strategy with a period of 2 (e.g., A-A-B-B). The effect of the treatment strategy on the average frequencies of uninfected, single-resistant, and double-resistant wells was then tested using a one-way ANOVA. In the case of a significant test ( $p < 0.05$ ), we conducted a pairwise Tukey post hoc analysis to relate the mean frequencies.

In addition, we analysed superinfections between patients and the emergence of double resistance across different strategies in the maximum-emergence scenario. We considered all measurement points from the fourth transfer onwards as at near-stationary level for the non-cycling strategies. Consequently, the initial conditions for each new transfer remain approximately the same or are repeated every fourth transfer in the case of cycling.

For this analysis, we counted the number of newly emerged double-resistant wells  $n_{\phi}$  and the number of superinfections  $n_{\mathcal{S}}$  across all replicates for each plate, with each plate representing one treatment arm for a single transfer. Newly emerged double-resistant wells are defined as those exhibiting double resistance but not having previously been passaged or infected by a

double-resistant well. Additionally, for each plate, we analysed all wells treated with treatment  $\vartheta$ , counting the number of superinfected wells  $n_{\mathcal{S}}^{\vartheta}$  and those among them that developed double resistance  $n_{\mathcal{E}}^{\vartheta}$ .

We then tested whether the treatment strategy significantly affects the emergence frequency  $f_{\mathcal{E}} = n_{\mathcal{E}}/n_p$  and the frequency of superinfection  $f_{\mathcal{S}} = n_{\mathcal{S}}/n_p$ , with  $n_p$  being the number of patients in a hospital ward across all replicates (376) using an ANOVA. Additionally, we used an ANOVA to assess if treatment  $\vartheta$  significantly influences the frequency of superinfected wells that develop double resistance  $n_{\mathcal{E}}^{\vartheta}/n_{\mathcal{S}}^{\vartheta}$ .

Subsequent to a significant ANOVA test, we conducted pairwise Tukey post hoc comparisons between the treatment arms ( $p < 0.05$ ).

All statistical analyses were performed in Python 3.8.5 using SciPy's `f_oneway()` [6] for ANOVA tests and Statsmodels' `pairwise_tukeyhsd()` [7] for conducting Tukey's honest significant difference post hoc analyses.

**Maximum-emergence scenario: Predicting the Emergence Probability.** We counted for each plate  $i$  the number of superinfected wells  $n_{\mathcal{S}}^i$  and normalized them by the number of patients per plate  $n_p$  to calculate the frequency of superinfection ( $f_{\mathcal{S}}^i = \frac{n_{\mathcal{S}}^i}{n_p}$ ). Then, we estimated the probability of superinfection  $\mathbb{P}(\mathcal{S})$  for each treatment arm by averaging  $f_{\mathcal{S}}^i$ . In addition, we approximated the probability of emergence for superinfected wells  $\mathbb{P}_{\vartheta}(\mathcal{E}|\mathcal{S})$  under treatment  $\vartheta$ , by normalizing the total number of newly emerged resistances across all plates  $N_{\mathcal{E}}^{\vartheta}$  by the total number of superinfected wells under treatment  $\vartheta$  ( $N_{\mathcal{S}}^{\vartheta}$ ):  $\frac{N_{\mathcal{E}}^{\vartheta}}{N_{\mathcal{S}}^{\vartheta}}$ . The estimates for  $\mathbb{P}_{\vartheta}(\mathcal{E}|\mathcal{S})$  were then utilized to approximate the average probability of superinfected wells developing double resistance for each treatment arm. The weighted average of all  $\mathbb{P}_{\vartheta}(\mathcal{E}|\mathcal{S})$  were computed using the proportion of patients receiving treatment  $\vartheta$  as weights. For example, in mixing, the average probability  $\bar{\mathbb{P}}(\mathcal{E}|\mathcal{S})$  is given by  $0.5\mathbb{P}_A(\mathcal{E}|\mathcal{S}) + 0.5\mathbb{P}_B(\mathcal{E}|\mathcal{S})$ . We then used Equation S2 to predict the average probability of emerging double resistance  $\mathbb{P}(\mathcal{E})$  for each strategy, as indicated in Figure 3A by black stars.

$$\mathbb{P}(\mathcal{E}) = \mathbb{P}(\mathcal{S})\bar{\mathbb{P}}(\mathcal{E}|\mathcal{S}) \quad (\text{S2})$$

## SI Results

**Impact of Treatment on the Emergence of Double Resistance.** As demonstrated in Figure 3B, our findings indicate that treatment substantially influences the frequency of emerging double resistance in superinfected wells. Population dynamics within wells can potentially explain these results. Here we approximate  $E_\theta$ , the expected number of conjugations during one treatment phase under treatment  $\vartheta$ , as  $E_\theta \propto \gamma_\vartheta \int_{t1}^{t2} {}^A X_\theta(t) {}^B X_\theta(t) dt$ .  ${}^i X$  represents the density of bacteria with resistance  $i$ , and  $\gamma_\vartheta$  is the treatment dependent conjugation rate. The experimentally generated data are insufficient for adequately estimating  $\gamma_\vartheta$ . However, if we assume identical initial bacterial populations, we can qualitatively rank the cumulative product of bacterial densities  $\int_{t1}^{t2} {}^A X_\theta(t) {}^B X_\theta(t) dt$ . The highest cumulative product is achieved when  ${}^A X$  and  ${}^B X$  grow without or with ineffective treatment. Additionally, we know that the clearance rate of ceftazidime (drug A) is substantially higher than that of tetracycline (drug B), as shown in Table 2.9, resulting in a larger cumulative product over time when treated with antibiotic B. Lastly, the lowest cumulative product is associated with treatment AB, where neither of the two strains can grow. Therefore, if we disregard  $\gamma_\vartheta$ , the above reasoning predicts the following ranking for the number of emergences per superinfection: None, B, A, AB. This predicted ranking aligns with the ranking observed in [Figure 3C](#).

Another potential explanation are potential differences in the plasmid-specific conjugation rates. If, for example,  $p_B$  had a higher conjugate rate than  $p_A$ , then drug A would have a stronger impact on the emergence of double resistance, even if we assumed identical clearance rates.

**Treatment strategies influence the number of bacteria inoculating superinfections.** We observed that the number of single-resistant bacteria that inoculate superinfections affects the emergence of double resistance (Table 2.2). At least one of the two superinfection-initiating inocula originates from infections between patients and is sourced from the previous assay plate. The cell densities and compositions within these source wells, which have already undergone treatment for one day, vary considerably depending on the resistance profile  $\phi$  and the treatment history. We used 'advanced phenotypes' (see SI Methods) to distinguish between high-density ( $\phi^h$ ) and low-density ( $\phi^l$ ) wells, assigning these based on the wells resistance profile ( $\phi$ ) and treatment history.

During the maximum-emergence scenario, which contained the highest number of superinfections, we made two noteworthy observations. First, superinfections between  $\phi^h$  and  $\phi^l$  ( $A_r^l + B_r^h$  (43 superinfections) and  $A_r^h + B_r^l$  (one superinfection)) occur significantly less frequently than superinfections between  $A_r^h + B_r^h$  (a total of 1176 superinfections, as outlined in Table 2.2). This discrepancy can be attributed to the high clearance rates of both drugs, resulting in a higher prevalence of  $\phi^h$  compared to  $\phi^l$ .

Second, none of the 44 superinfections involving  $A_r^l + B_r^h$  and  $A_r^h + B_r^l$  resulted in double resistance.

**Antagonism.** Adding the bacteriostatic antibiotic tetracycline (drug B) reduces the probability of clearing sensitive bacteria with the bactericidal antibiotic ceftazidime (drug A). The clearance probability drops from 0.97 to 0.86, as shown in Table 2.9. Antagonism between bactericidal and bacteriostatic antibiotics has been documented by various researchers since the 1950s, as exemplified by the works of Cates [8] and Jawetz [9], and also more recently by Ocampo [10]. The antagonistic effect may arise because the bacteriostatic drug (tetracycline) lowers the growth rate, resulting in a decreased kill rate of the bactericidal drug (ceftazidime) [11]. Accordingly, this antagonistic effect is anticipated to be less pronounced for a tetracycline-resistant strain, where the impact on the growth rate is diminished. This hypothesis is supported by the measured clearance rates for  $B_r$  wells, where the clearance probability remains at 0.98 for treatment with drugs A and AB Table 2.9.

### SI Computational Model

**Stochasticity** We conducted three experiments, each defined by one parameter set consisting of a turnover probability  $\tau$ , an infection probability  $\beta$  and the probability distribution for sampling patients  $c_\phi$  with different resistance profiles  $\phi \in \{U, S, A_r, B_r, AB_r\}$ . For each experiment, we randomly generated one instruction set based on the given parameter set. Due to the scale and complexity of the experiment, it was infeasible to conduct a unique instruction set for each replicate. Therefore, we opted to employ identical instruction sets for all replicates, which reduces the number of robot arm movements (and time) for infection and turnover by a factor of four.

As a consequence, our replicates may be interpreted as patients with identical histories regarding original infection, treatment and exchange with other patients. However, due to the accumulated biological stochasticity along the patient histories, their phenotypic properties may vary, as

reflected by the variance of the replicates.

Since we only tested one instruction set per replicate in vitro, we wondered whether the measured results depend on the randomisation of the instruction set and if we would expect a qualitatively different result if we reran the experiment 100 times. To answer this question, we created a computational model that, for a given parameter set, rerandomises the instruction set and conducts a stochastic simulation to mimic the biological variability. In Fig. 2.7, we visualised the different sources of experimental and computational variability.

**Transition Probabilities.** We used the experimental data to calculate the transition frequencies for all pre-treatment  $\phi_\vartheta^i(T)$  to post-treatment  $\phi_\vartheta^i(\hat{T})$  resistance profile combinations for each plate  $i$  and treatment  $\vartheta$ .  $\phi(\hat{T})$  is measured during the phenotyping procedure, while  $\phi(T)$  is estimated by employing one plate-handling simulation step to  $\phi(\hat{T} - 1)$  as described in the methods (e.g.  $A_r + S \rightarrow A_r$ ). Then, we estimated the transition probabilities as the weighted average of transition frequencies across all plates, with the count of  $\phi_\vartheta(T)$  on each plate as a weight.

For each treatment  $\vartheta$ , we created one transition matrix  $M^\vartheta$ , with the pre-treatment resistance profile  $\phi(T)$  as columns and the post-treatment profile  $\phi(\hat{T})$  in the rows (Table 2.19–2.22). To simulate the incubation phase, we use  $M^\vartheta$  to stochastically assign the post-treatment resistance profile  $\phi_\vartheta(\hat{T})$ , using the respective column of the transition matrix as a probability distribution.

**Transition Probabilities for Transfer 1.** All patients are untreated during transfer 0, leading to exceptionally high rates of superinfections and high emergence rates per superinfection during transfer 1. To account for this, we created four additional transition matrices for simulating the first transfer (see Table 2.23–2.26).

**Choice of Model.** We also considered using a continuous model. However, a typical population-based model would not match the experimental measurements for effectively treated patients. This is due to the discrete nature of our experimental setup. Here, the frequency of infected patients has a local maximum before treatment and a local minimum after treatment, creating sawtooth-shaped frequencies over time. We conducted the phenotyping at the end of the incubation period, at the local low point, diverging from the average frequencies predicted by a continuous model. Therefore, a continuous model would either use realistic clearance rates and

not fit through the experimental data points or use exaggerated clearance rates and fit through the data points. For example, treating an  $S$  well with a hypothetical drug  $C$  leads to a steady decline in bacterial density over time, resulting in a bacterial density below the detection limit after 24 hours (our first experimental measurement point). If initially, all wells are infected, and at the first measurement point, the infection drops to 0%, fitting a continuous compartmental model to these data would result in infinite clearance rates.

**Contamination of the Transition Matrix.** The computational simulations employ four transition matrices ( $M^{\text{none}}, M^A, M^B, M^{AB}$ ) derived from the observed transitions during the three experiments. During the experiments, we observed a low rate of contamination affecting neighbouring wells, likely due to pipette and plate movements by the liquid handling platform. Quantifying the exact contamination rate is challenging, though the observed mean transition probability from  $U$  to  $U$  is 0.99%.

These contaminations can be inconsequential; for example, an  $S$  well contaminating an  $A_r$  well will not cause a shift of the resistance profile in the contaminated well. However, they can also lead to artefactual transitions that are reflected in the transition matrices, such as  $U \rightarrow S$  (Table 2.19),  $U \rightarrow A_r$  (Table 2.20), or  $U \rightarrow B_r$  (Table 2.21). The impact of the recorded artefactual transitions in the transition matrices depends on the frequency and the transferred resistance profile. For instance, in Mono A, a high frequency of  $A_r$  contaminations is observed due to the predominant presence of  $A_r$  wells, creating the impossible transition  $U \rightarrow A_r$  described above, which now occurs in all simulations independent of the presence or frequency of  $A_r$  during the simulation. Similarly, in the containment experiment, the abundance of  $AB_r$  wells in all treatment arms led to a higher rate of double-resistant contamination reflected in the transitions:  $A_r \rightarrow AB_r$  (Table 2.19) and  $A_r \rightarrow AB_r$  &  $B_r \rightarrow AB_r$  (Table 2.21). Resistance mutations could also explain these transitions; however, because they occurred mainly during the containment scenario and the fact that they exclusively came up in wells neighbouring double-resistant wells, we believe that they are an artefact of unintended infections.

Artefactual transitions such as  $U \rightarrow S$ , or  $U \rightarrow A_r$  have a neglectable effect on the simulation of all scenarios, as their occurrence in the regular infection and admission processes outweighs the contribution through the artefactual transitions. Similarly, double-resistant contaminations minimally impact the simulations of both the containment scenario (where double resistance

is regularly admitted) and the maximum-emergence scenario (due to a low  $R_0$  and frequent emergence of double resistance). However, they pose a problem to the simulation of prevention scenario, where a low (untruthful) influx of  $AB_r$  can spread ( $R_0 > 1$ ).

**Filtered Transition Probabilities.** To mitigate the effect of contaminated transition matrices, we introduced filtered transition matrices. For this, we assumed no resistance mutations and forbidding impossible transitions (by setting the transition probability  $U \rightarrow U$  to 1; see Table 2.27 – 2.34).

Using these filtered probabilities to simulate the prevention scenario leads to better-matching results, almost removing the spread of double resistance in Mono A and the multidrug strategies and thereby matching the experimental data better (compare the green error bands between Fig. 2.6 & Fig. 2.11). In addition, we conducted a secondary sensitivity analysis with these filtered probabilities (see Fig. 2.12). Because the overall conclusions are consistent between the simulations using filtered and unfiltered transition probabilities, we opted to use the unfiltered transition probabilities in the main paper for a more direct representation of the experimental data.

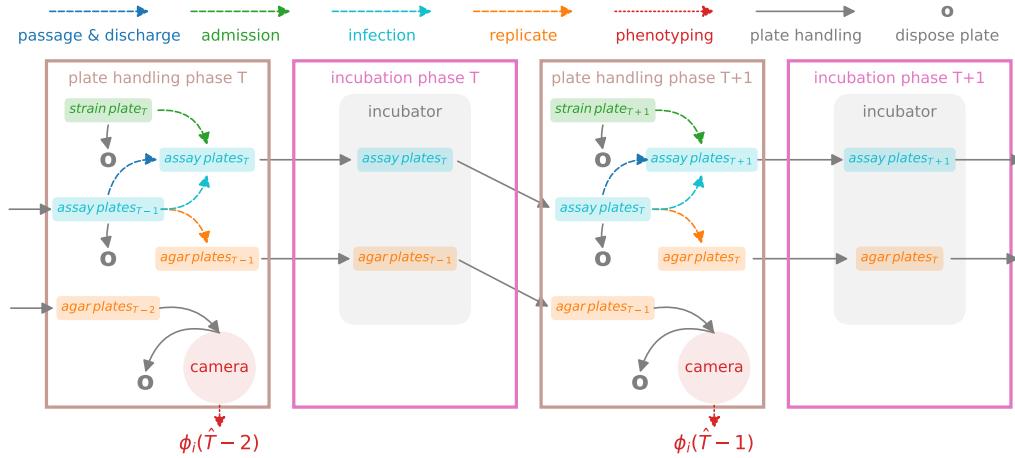


Fig. 2.5 Schematic illustrating the procedure used in the experiment for transfers  $T$  and  $T+1$  in the liquid handling platform after adding medium and drugs to the assay plates. Every transfer (day), we provide new assay and agar plates. Plates from the previous transfers are removed. To inoculate the new assay plates with newly admitted patients from the strain plate, along with staying patients and infection between patients from the previous assay plate, we use a pintoil with retractable pins (dashed lines). Discharged patients are not transferred (pins retracted) to the new assay plates. Plates are then automatically transferred (solid lines) to the incubator for overnight incubation. Subsequently, we replicate each assay plate onto four agar plates using the pintoil. These plates are treated with antibiotics A, B, and AB, and one remains untreated. Once the agar plates have been incubated overnight, we capture images (dotted lines) to determine the resistance profile  $\phi_i$  for each well  $i$ .

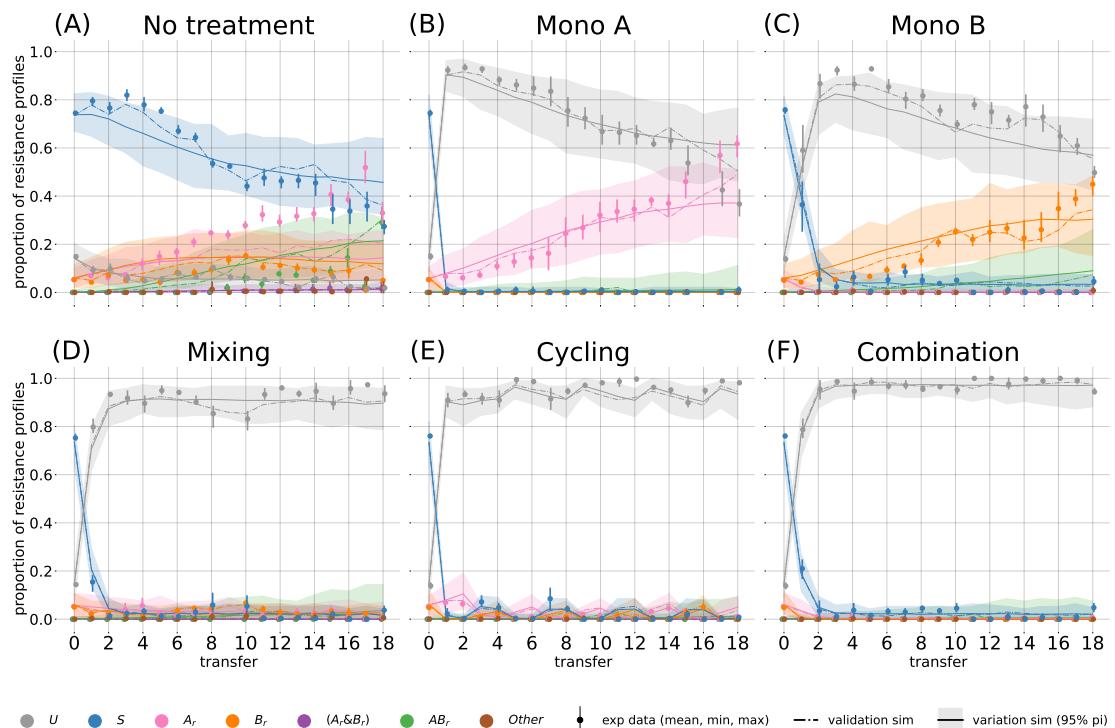


Fig. 2.6 Prevention scenario: Frequencies of resistance profiles (colours) over time during the prevention scenario. The dots show the experimental measurements, and the error bar indicates the min/max interval between the replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment. The solid line represents the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment. The shaded error band indicates the 95-percentile interval between the simulations.

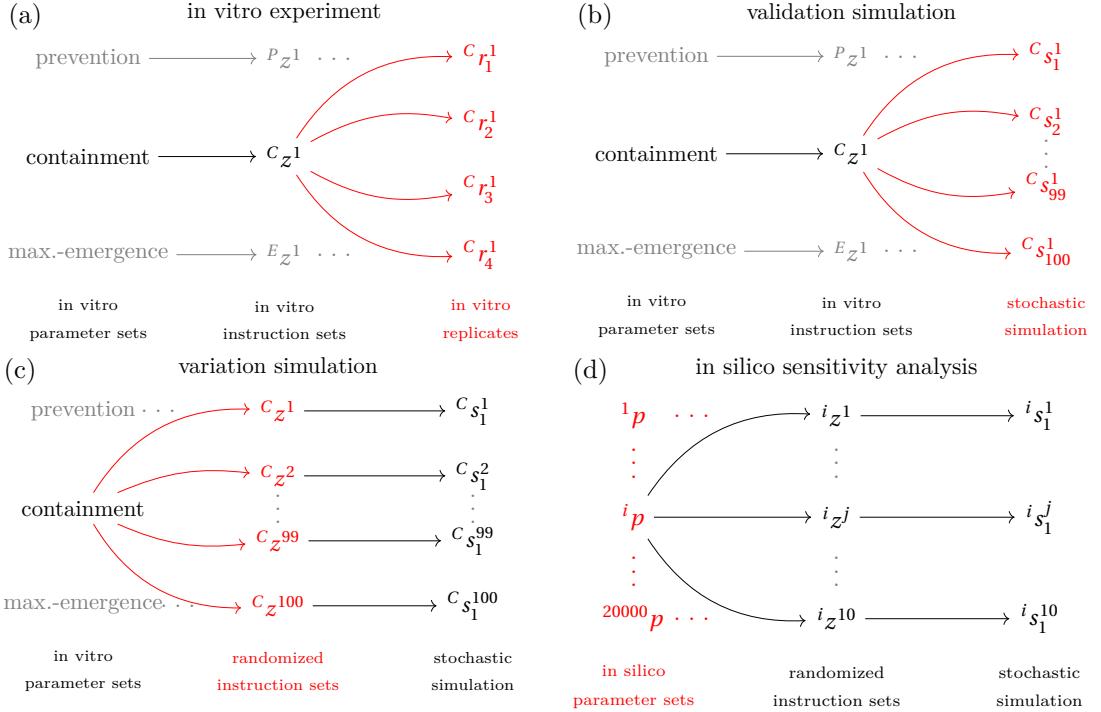


Fig. 2.7 Illustration depicting the various sources of stochasticity and variation in experiments and simulations. Our experiments and simulations investigate various sources of stochasticity and their contributions to the variability of the outcome. In panels A-D, we sketch the different sources of stochasticity for each experiment and simulation, highlighting our primary focus in red. (A) In vitro Experiment. Each experiment explores a scenario and is defined by a distinct parameter set (prevention ( $P$ )), containment ( $C$ ), maximum emergence ( $E$ )). We randomly generated one instruction set for each parameter set  $i$ :  $i_z^1$ . For each instruction set  $i_z^1$ , we replicated the cumulative in-well dynamics four times  $i_r_j^1$ . (B) Validation Simulation. To assess our computational model, we employed identical parameter sets and instruction sets  $i_z^1$ , as employed in the in vitro experiments. For each instruction set  $i_z^1$ , we conducted 100 stochastic simulations  $i_s_j^1$ . (C) Variation Simulation. For every in vitro parameter set, we randomly generated 100 alternative instruction sets  $i_z^k$  to quantify the influence of experimental decisions on the experiment's outcomes. For each instruction set, we performed one simulation  $i_s_j^k$ . (D) In Silico Sensitivity Analysis. We examined the sensitivity of our experimental findings to the input parameters by examining the effects of varying the input parameters on the resulting frequency of uninfected cases for different treatment strategies. To achieve this, we generated 20,000 alternative parameter sets  $i_p$ . We created ten randomised instruction sets  $i_z^k$  for each parameter set  $i_p$  and simulated each instruction set one time ( $i_s_j^k$ ).

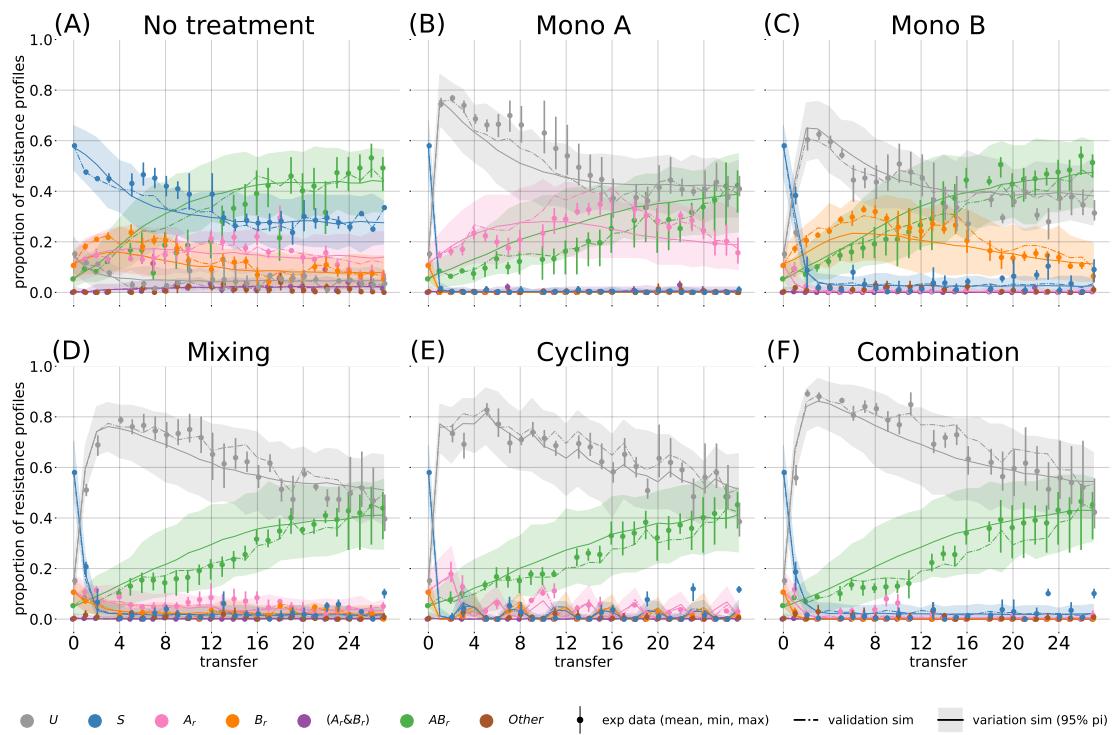


Fig. 2.8 Containment scenario: Frequencies of resistance profiles (colours) over time during the containment scenario. The dots show the experimental measurements, and the error bar indicates the min/max interval between the replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment. The solid line represents the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment. The shaded error band indicates the 95-percentile interval between the simulations.

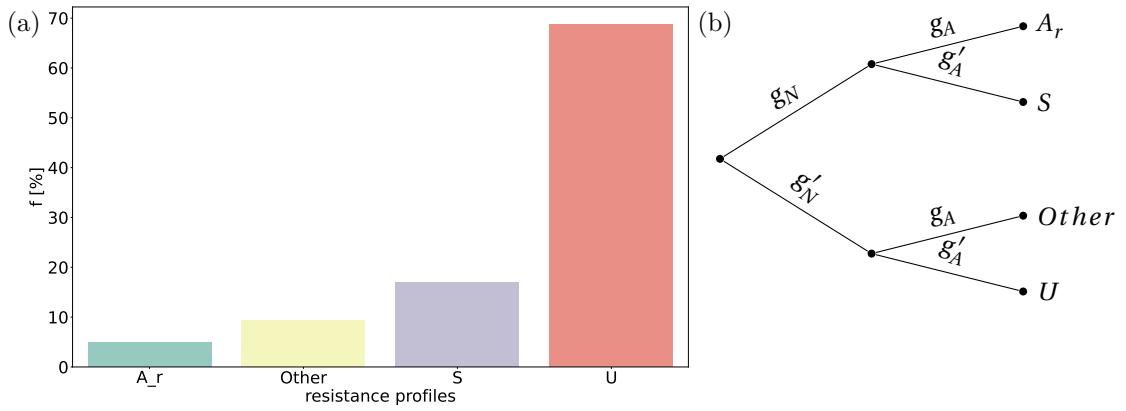


Fig. 2.9 (A) Experimentally measured resistance profiles for 1784 wells with the pre-treatment profile  $A_r$  and treatment with drug B during the maximum-emergence scenario. (B) Decision tree to calculate the distribution of measured phenotypes for a well that contains  $Z_\phi$  sensitive and  $Z_A$  A-resistant bacteria.  $g_\theta$  is the probability of drawing a drop that forms a colony on a plate treated with drug  $\theta$ , while  $g'_\theta$  is the probability that it does not form a colony.

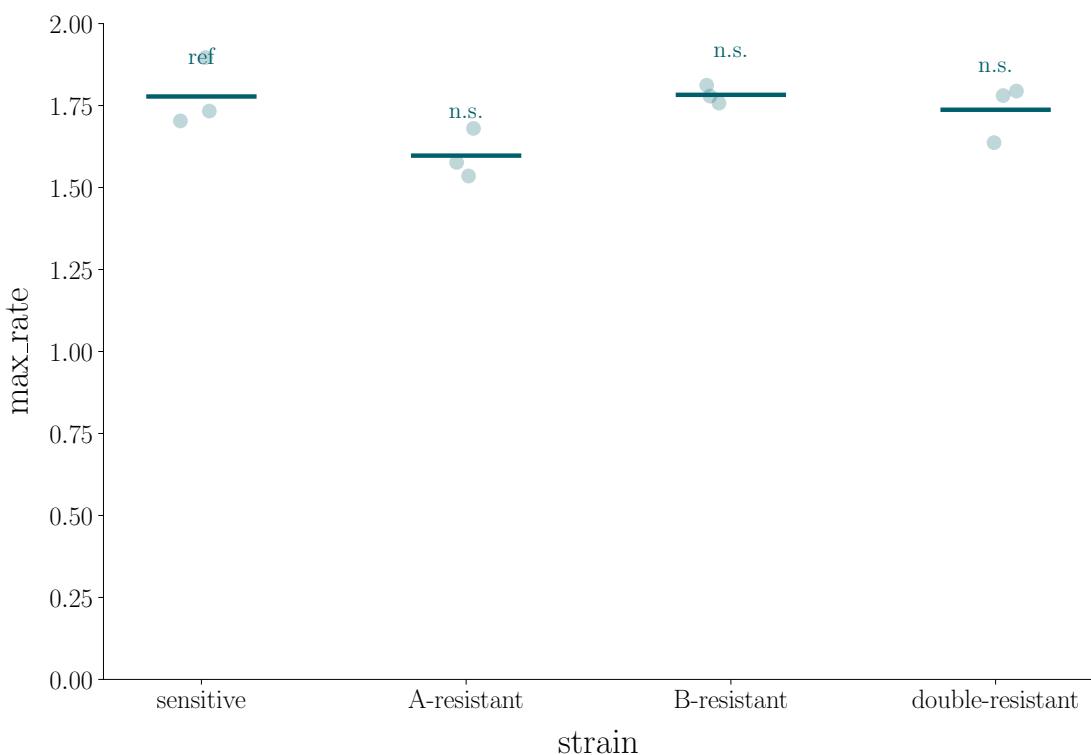


Fig. 2.10 Maximum growth rates of sensitive and plasmid-carrying strains, measured using OD-growth curves. Each dot represents an individual well, and vertical bars indicate the mean. The sensitive strain was used as the reference ("ref") for pairwise comparisons to the plasmid-carrying strains to identify potential plasmid costs. We used the Mann-Whitney U tests with the Bonferroni correction to identify significant differences in growth rates. All pairwise comparisons were not significant (n.s.).

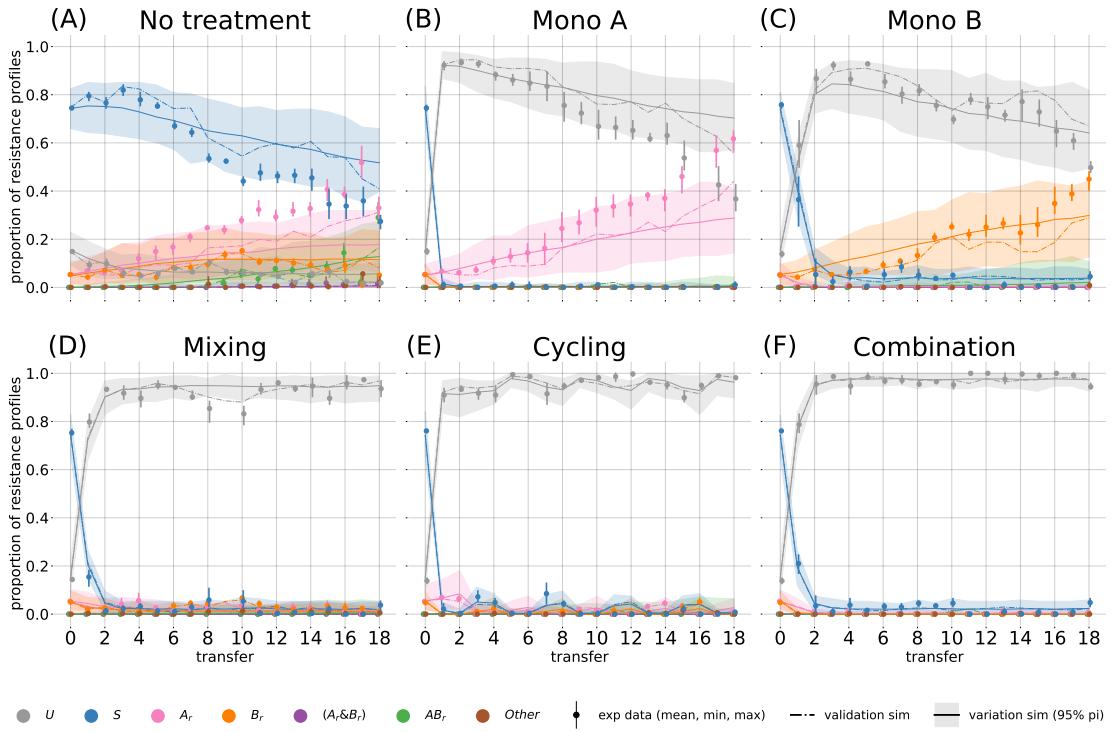


Fig. 2.11 Prevention scenario with filtered transition probabilities. Frequencies of resistance profiles (colours) over time during the prevention scenario. The dots show the experimental measurements, and the error bar indicates the min/max interval between the replicates. The dash-dotted line shows the mean value of 100 stochastic simulations based on the instruction set used in the in vitro experiment. The solid line represents the mean value of 100 simulations with randomly created instruction sets based on the parameter set used in the experiment. The shaded error band indicates the 95-percentile interval between the simulations.

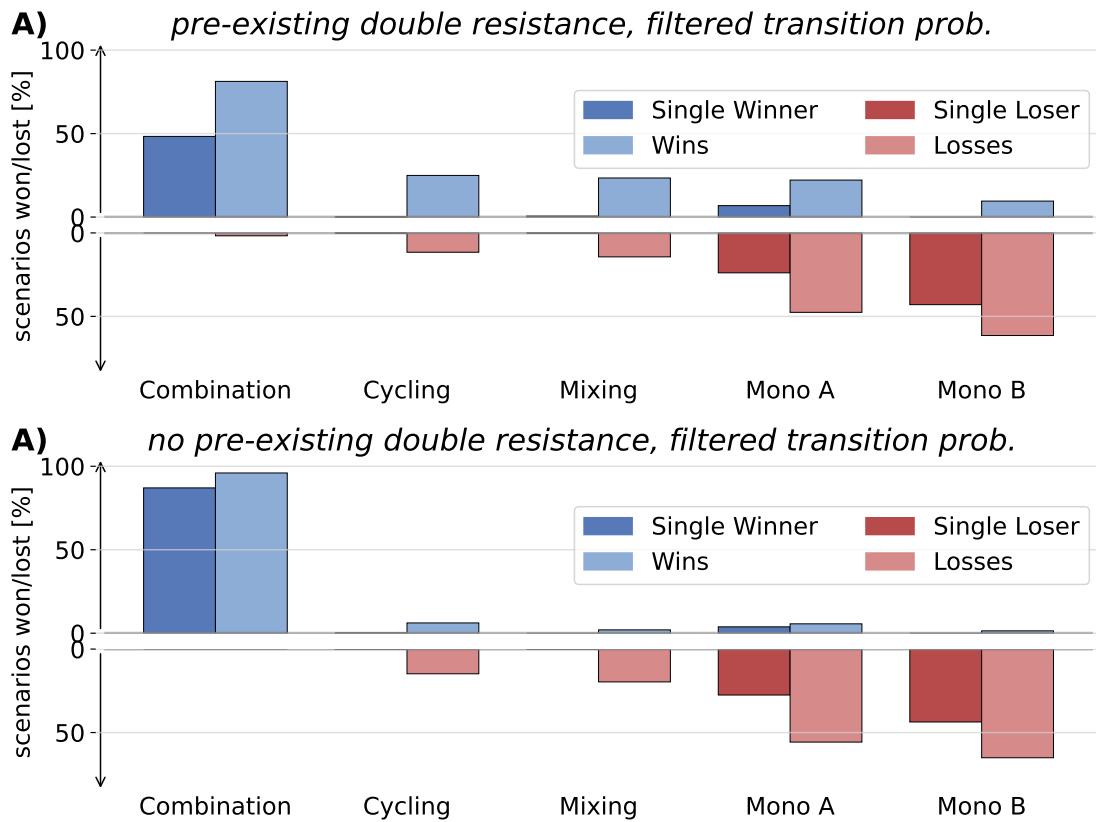


Fig. 2.12 Sensitivity analysis using filtered transition probabilities. We evaluated the effectiveness of the five treatment strategies in maximising the frequency of uninfected *in silico* patients across randomly generated parameter sets. Strategies not significantly better than any other are marked as losers (pastel red), with those significantly worse than all others being labelled as single losers (dark red). Conversely, strategies that are not significantly worse than any other are classified as winners (pastel blue), and those significantly better than all others as single winners (dark blue). (A) Evaluation of 10,000 parameter sets with preexisting double resistance. 659 out of 10,000 parameter sets yielded no significant difference between the strategies. (B) Evaluation of 10,000 parameter sets without preexisting double resistance. 8 out of 10,000 parameter sets yielded no significant difference between the strategies.

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Table 2.2 Number of superinfections ( $N_{\mathcal{S}}$ ) between high- and low-concentrated  $A_r$  and  $B_r$  wells and the number of double resistances that emerged ( $N_{\mathcal{E}}$ ) under treatment  $\vartheta$  across all three experiments.

Treatment $\vartheta$	$A_r^x$	$B_r^x$	$N_{\mathcal{E}}^\vartheta$	$N_{\mathcal{S}}^\vartheta$	$\frac{N_{\mathcal{E}}^\vartheta}{N_{\mathcal{S}}^\vartheta}$
A	$A_r^h$	$B_r^h$	55	399	0.14
AB	$A_r^h$	$B_r^h$	2	35	0.06
B	$A_r^h$	$B_r^h$	257	322	0.8
none	$A_r^h$	$B_r^h$	390	420	0.93
A	$A_r^h$	$B_r^l$	0	1	0.0
A	$A_r^l$	$B_r^h$	0	13	0.0
AB	$A_r^l$	$B_r^h$	0	15	0.0
B	$A_r^l$	$B_r^h$	0	15	0.0

Table 2.3 Strains used in this study and their relevant phenotypes. The phenotype in brackets is conferred by the respective plasmid. Cm<sup>R</sup>: Chloramphenicol resistance, Amp<sup>R</sup>: Ampicillin resistance, Caz<sup>R</sup>: Ceftazidime resistance, Tet<sup>R</sup>: Tetracycline resistance.

Name	Relevant phenotype	Reference
Escherichia coli MDS42-YFP;Cm <sup>R</sup> ;[3] A-resistant	Cm <sup>R</sup> pA (Amp <sup>R</sup> , Caz <sup>R</sup> )	this study, [2]
B-resistant	Cm <sup>R</sup> pB (Amp <sup>R</sup> , Tet <sup>R</sup> )	this study, [2]
AB-resistant	Cm <sup>R</sup> pA (Amp <sup>R</sup> , Caz <sup>R</sup> ) pB (Amp <sup>R</sup> , Tet <sup>R</sup> )	this study, [2]

Table 2.4 Statistical comparison of maximum growth rates between the sensitive and plasmid-carrying strains. We used a Mann-Whitney U test for pairwise comparisons, and the p-values were adjusted using the Bonferroni correction.

Comparison	U-statistic	P-value	Corrected P-value	Significant after Bonferroni
sensitive vs A-resistant	9.000000	0.100000	0.300000	False
sensitive vs B-resistant	3.000000	0.700000	1.000000	False
sensitive vs double-resistant	5.000000	1.000000	1.000000	False

Table 2.5 Plasmid segregation loss was estimated over 24 hours without treatment and with selective treatment as a control. Frequencies of plasmid retention were compared between  $t_0 - t_1$  using the Mann-Whitney U test. Confidence intervals and the mean frequencies were estimated by bootstrapping the binary data (plasmid retained or lost) pooled across replicates. No significant plasmid loss was observed in either the main data or the control.

plasmids	$f(t_0)$	$CI(t_0)$	$f(t_1)$	$CI(t_1)$	$p$ -value	$f(t_1)$ control	$CI(t_1)$ control	$p$ -value control
$p_A$	1.00	(1.0, 1.0)	1.00	(1.0, 1.0)	1.00	1.00	(1.0, 1.0)	1.00
$p_B$	1.00	(1.0, 1.0)	0.97	(0.93, 1.0)	0.20	1.00	(1.0, 1.0)	1.00
$p_A \& p_B$	0.99	(0.97, 1.0)	1.00	(1.0, 1.0)	0.50	1.00	(1.0, 1.0)	0.50

Table 2.6 95 % confidence intervals for the final bacterial density measured by colony plating and the maximal growth rates measured by evaluating OD-growth curves.

strain	antibiotic	cmp [ $\mu\text{g}/\text{ml}$ ]	cfu [ $1/\mu\text{l}$ ]	growthrate [ $1/\text{h}$ ]
double-resistant	AB	5	$(2.14 - 3.33) \times 10^5$	(0.43 – 0.86)
double-resistant	AB	25	$(2.68 - 3.99) \times 10^5$	(0.4 – 0.85)
A-resistant	A	5	$(1.58 - 2.62) \times 10^5$	(0.51 – 0.7)
A-resistant	A	25	$(2.05 - 3.21) \times 10^5$	(0.46 – 0.71)
sensitive	None	5	$(1.03 - 1.27) \times 10^6$	(0.68 – 0.74)
sensitive	None	25	$(1.15 - 1.4) \times 10^6$	(0.55 – 0.96)
B-resistant	B	5	$(4.05 - 5.62) \times 10^5$	(0.45 – 0.74)
B-resistant	B	25	$(3.53 - 5.01) \times 10^5$	(0.4 – 0.87)

Table 2.7 Definition of resistance profiles (rows) by growth patterns on differently treated agar plates (columns). X indicates colony formation, whereas o indicates no growth.

	None	A	B	AB
$U$	o	o	o	o
$S$	X	o	o	o
$A_r$	X	X	o	o
$B_r$	X	o	X	o
$(A_r \& B_r)$	X	X	X	o
$AB_r$	X	X	X	X

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Table 2.8 Association between bacterial phenotypes (rows) and resistance profiles  $\phi$  (columns). An 'X' denotes that a phenotype is obligatory for a given profile, while a ' $\checkmark$ ' indicates that it is optional.

	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
sensitive	X	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$
A-resistant		X		X	$\checkmark$	
B-resistant			X	X	$\checkmark$	
double-resistant					X	

Table 2.9 Clearance probability of well phenotypes across the three experiments.

	$S$	$A_r$	$B_r$
A	0.97	0.02	0.98
B	0.73	0.62	0.01
AB	0.86	0.4	0.98

Table 2.10 Mixing rules. During the plate-handling phase, we mix wells due to infections. The resulting phenotype of the two mixed wells can be calculated using this table. More than two phenotypes can be combined by applying associative logic.

$\phi_1(\hat{T}) \backslash \phi_2(\hat{T})$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
S	$S$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$A_r$	$A_r$	$A_r$	$A_r$	$(A_r \& B_r)$	$(A_r \& B_r)$	$AB_r$
$B_r$	$B_r$	$B_r$	$(A_r \& B_r)$	$B_r$	$(A_r \& B_r)$	$AB_r$
$(A_r \& B_r)$	$(A_r \& B_r)$	$(A_r \& B_r)$	$(A_r \& B_r)$	$(A_r \& B_r)$	$(A_r \& B_r)$	$AB_r$
$AB_r$	$AB_r$	$AB_r$	$AB_r$	$AB_r$	$AB_r$	$AB_r$

Table 2.11 Mean parameter leading to  $n$  single wins during the sensitivity analysis without preexisting double resistance. Strategies that did not yield at least one single win were excluded.

	turnover	infection	U	S	A_r	B_r	AB_r	n
Combination	0.50	0.50	0.26	0.24	0.24	0.25	0.00	9311.00
Mono A	0.80	0.27	0.26	0.29	0.37	0.08	0.00	56.00
Cycling	0.10	0.39	0.33	0.42	0.14	0.10	0.00	4.00
Mixing	0.11	0.33	0.12	0.49	0.38	0.01	0.00	1.00

Table 2.12 Mean parameter leading to  $n$  single losses during the sensitivity analysis without preexisting double resistance. Strategies that did not yield at least one single loss were excluded.

	turnover	infection	U	S	A_r	B_r	AB_r	n
Mono B	0.60	0.43	0.26	0.25	0.16	0.33	0.00	4132.00
Mono A	0.55	0.43	0.27	0.25	0.36	0.12	0.00	2648.00
Cycling	0.07	0.87	0.28	0.34	0.27	0.11	0.00	8.00
Mixing	0.07	0.90	0.69	0.25	0.01	0.05	0.00	1.00

Table 2.13 Mean parameter leading to  $n$  single wins during the sensitivity analysis with preexisting double resistance. Strategies that did not yield at least one single win were excluded.

	turnover	infection	U	S	A_r	B_r	AB_r	n
Combination	0.61	0.47	0.21	0.18	0.22	0.22	0.17	5487.00
Mono A	0.52	0.56	0.23	0.20	0.16	0.15	0.26	365.00
Mixing	0.13	0.30	0.23	0.21	0.18	0.15	0.22	40.00
Cycling	0.14	0.20	0.23	0.20	0.21	0.20	0.16	9.00

Table 2.14 Mean parameter leading to  $n$  single losses during the sensitivity analysis with preexisting double resistance. Strategies that did not yield at least one single loss were excluded.

	turnover	infection	U	S	A_r	B_r	AB_r	n
Mono B	0.57	0.46	0.21	0.19	0.13	0.28	0.18	4250.00
Mono A	0.51	0.43	0.23	0.19	0.30	0.10	0.18	2359.00
Cycling	0.42	0.72	0.23	0.21	0.14	0.14	0.28	6.00
Mixing	0.34	0.71	0.23	0.20	0.18	0.09	0.30	2.00

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Table 2.15 Wins and losses during the sensitivity analysis. With preexisting double resistance. 606 parameter sets yielded an insignificant result.

strategy	single winner [%]	single loser [%]	loser [%]	winner [%]	single winner	single loser	loser	winner
Combination	54.87	0.00	0.95	86.76	5487	0	95	8676
Cycling	0.09	0.06	12.10	23.32	9	6	1210	2332
Mixing	0.40	0.02	14.78	20.54	40	2	1478	2054
Mono A	3.65	23.59	48.57	17.49	365	2359	4857	1749
Mono B	0.00	42.50	62.29	8.11	0	4250	6229	811

Table 2.16 Wins and losses during the sensitivity analysis without preexisting double resistance. 100 parameter sets yielded an insignificant result.

strategy	single winner [%]	single loser [%]	loser [%]	winner [%]	single winner	single loser	loser	winner
Combination	93.11	0.00	0.00	98.35	9311	0	0	9835
Cycling	0.04	0.08	18.89	3.49	4	8	1889	349
Mixing	0.01	0.01	22.05	1.32	1	1	2205	132
Mono A	0.56	26.48	57.03	1.65	56	2648	5703	165
Mono B	0.00	41.32	63.44	1.11	0	4132	6344	111

Table 2.17 Wins and losses during the sensitivity analysis, using filtered transition probabilities and preexisting double resistance. 659 parameter sets yielded an insignificant result.

strategy	single winner [%]	single loser [%]	loser [%]	winner [%]	single winner	single loser	loser	winner
Combination	48.31	0.00	1.72	81.27	4831	0	172	8127
Cycling	0.07	0.10	11.57	24.94	7	10	1157	2494
Mixing	0.59	0.03	14.34	23.40	59	3	1434	2340
Mono A	6.85	23.91	47.57	22.15	685	2391	4757	2215
Mono B	0.00	42.97	61.45	9.57	0	4297	6145	957

Table 2.18 Wins and losses during the sensitivity analysis with filtered transition matrices and no preexisting double resistance. 8 parameter sets yielded an insignificant result.

strategy	single winner [%]	single loser [%]	loser [%]	winner [%]	single winner	single loser	loser	winner
Combination	87.04	0.00	0.00	95.98	8704	0	0	9598
Cycling	0.08	0.06	14.80	6.16	8	6	1480	616
Mixing	0.00	0.04	19.66	2.00	0	4	1966	200
Mono A	3.82	27.53	55.71	5.62	382	2753	5571	562
Mono B	0.00	43.67	65.10	1.41	0	4367	6510	141

Table 2.19  $M^{\text{none}}$ . Unfiltered transition matrix for untreated wells.

$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$\phi(\hat{T})$	U	0.97	0.0	0.0	0.0	0.0
	S	0.03	0.98	0.01	0.02	0.01
	$A_r$	0.0	0.01	0.96	0.0	0.07
	$B_r$	0.0	0.01	0.0	0.97	0.02
	$(A_r \& B_r)$	0.0	0.0	0.01	0.01	0.08
	$AB_r$	0.0	0.0	0.02	0.0	0.82

Table 2.20  $M^A$ . Unfiltered transition matrix for wells treated with antibiotic A.

$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$	
$\phi(\hat{T})$	U	0.99	0.96	0.02	0.97	0.03	0.02
	S	0.0	0.02	0.0	0.01	0.0	0.0
	$A_r$	0.01	0.02	0.98	0.01	0.59	0.01
	$B_r$	0.0	0.0	0.0	0.01	0.0	0.0
	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.18	0.01
	$AB_r$	0.0	0.0	0.0	0.0	0.2	0.96

Table 2.21  $M^B$ . Unfiltered transition matrix for wells treated with antibiotic B.

$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$	
$\phi(\hat{T})$	U	0.99	0.8	0.63	0.01	0.03	0.02
	S	0.0	0.19	0.18	0.0	0.0	0.0
	$A_r$	0.0	0.0	0.18	0.0	0.0	0.0
	$B_r$	0.01	0.01	0.0	0.98	0.21	0.0
	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.03	0.0
	$AB_r$	0.0	0.0	0.01	0.01	0.73	0.98

Table 2.22  $M^{AB}$ . Unfiltered transition matrix for wells treated with antibiotic AB.

$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$	
$\phi(\hat{T})$	U	1.0	0.9	0.49	0.98	1.0	0.02
	S	0.0	0.1	0.32	0.02	0.0	0.0
	$A_r$	0.0	0.0	0.19	0.0	0.0	0.0
	$B_r$	0.0	0.0	0.0	0.0	0.0	0.0
	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.0	0.0
	$AB_r$	0.0	0.0	0.0	0.0	0.0	0.98

Table 2.23  $M_1^{\text{none}}$ . Unfiltered transition matrix for the first time point in untreated wells.

$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$\phi(\hat{T})$	U	0.96	0.0	0.0	0.0	0.0
	S	0.03	0.99	0.0	0.0	0.0
	$A_r$	0.01	0.01	1.0	0.0	0.0
	$B_r$	0.0	0.0	0.0	1.0	0.17
	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.02
	$AB_r$	0.0	0.0	0.0	0.0	0.81

Table 2.24  $M_1^A$ . Unfiltered transition matrix for the first time point in wells treated with antibiotic A.

$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$	
$\phi(\hat{T})$	U	0.99	0.97	0.0	0.99	0.23	0.0
	S	0.0	0.02	0.0	0.01	0.0	0.0
	$A_r$	0.01	0.01	1.0	0.0	0.54	0.0
	$B_r$	0.0	0.0	0.0	0.0	0.0	0.0
	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.11	0.0
	$AB_r$	0.0	0.0	0.0	0.0	0.12	1.0

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Table 2.25  $M_1^B$ . Unfiltered transition matrix for the first time point in wells treated with antibiotic B.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	U	1.0	0.48	0.51	0.02	0.01	0.0
S	S	0.0	0.52	0.21	0.01	0.0	0.0
$A_r$	$A_r$	0.0	0.0	0.28	0.0	0.0	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.97	0.31	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.1	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.58	1.0

Table 2.26  $M_1^{AB}$ . Unfiltered transition matrix for the first time point in wells treated with antibiotic AB.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	U	0.98	0.79	0.32	0.67	0.24	0.0
S	S	0.02	0.21	0.21	0.24	0.21	0.0
$A_r$	$A_r$	0.0	0.0	0.47	0.0	0.41	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.09	0.02	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.07	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.05	1.0

Table 2.27  $M^{\text{none}}$ . Filtered transition matrix for untreated wells.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	U	1.0	0.0	0.0	0.0	0.0	0.0
S	S	0.0	1.0	0.01	0.02	0.01	0.01
$A_r$	$A_r$	0.0	0.0	0.99	0.0	0.07	0.03
$B_r$	$B_r$	0.0	0.0	0.0	0.98	0.02	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.08	0.04
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.82	0.92

Table 2.28  $M^A$ . Filtered transition matrix for wells treated with antibiotic A.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	U	1.0	0.98	0.02	0.98	0.03	0.02
S	S	0.0	0.02	0.0	0.01	0.0	0.0
$A_r$	$A_r$	0.0	0.0	0.98	0.0	0.59	0.01
$B_r$	$B_r$	0.0	0.0	0.0	0.01	0.0	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.18	0.01
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.2	0.96

Table 2.29  $M^B$ . Filtered transition matrix for wells treated with antibiotic B.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	U	1.0	0.8	0.64	0.01	0.03	0.02
S	S	0.0	0.2	0.18	0.0	0.0	0.0
$A_r$	$A_r$	0.0	0.0	0.18	0.0	0.0	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.99	0.21	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.03	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.73	0.98

Table 2.30  $M^{AB}$ . Filtered transition matrix for wells treated with antibiotic AB.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
U	U	1.0	0.9	0.49	0.98	1.0	0.02
S	S	0.0	0.1	0.32	0.02	0.0	0.0
$A_r$	$A_r$	0.0	0.0	0.19	0.0	0.0	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.0	0.0	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.0	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.0	0.98

Table 2.31  $M_1^{\text{none}}$ . Filtered transition matrix for the first time point in untreated wells.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$U$	$U$	1.0	0.0	0.0	0.0	0.0	0.0
$S$	$S$	0.0	1.0	0.0	0.0	0.0	0.0
$A_r$	$A_r$	0.0	0.0	1.0	0.0	0.0	0.0
$B_r$	$B_r$	0.0	0.0	0.0	1.0	0.17	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.02	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.81	1.0

Table 2.32  $M_1^A$ . Filtered transition matrix for the first time point in wells treated with antibiotic A.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$U$	$U$	1.0	0.98	0.0	0.99	0.23	0.0
$S$	$S$	0.0	0.02	0.0	0.01	0.0	0.0
$A_r$	$A_r$	0.0	0.0	1.0	0.0	0.54	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.0	0.0	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.11	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.12	1.0

Table 2.33  $M_1^B$ . Filtered transition matrix for the first time point in wells treated with antibiotic B.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$U$	$U$	1.0	0.48	0.51	0.02	0.01	0.0
$S$	$S$	0.0	0.52	0.21	0.01	0.0	0.0
$A_r$	$A_r$	0.0	0.0	0.28	0.0	0.0	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.97	0.31	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.1	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.58	1.0

Table 2.34  $M_1^{AB}$ . Filtered transition matrix for the first time point in wells treated with antibiotic AB.

$\phi(\hat{T})$	$\phi(T)$	$U$	$S$	$A_r$	$B_r$	$(A_r \& B_r)$	$AB_r$
$U$	$U$	1.0	0.79	0.32	0.67	0.24	0.0
$S$	$S$	0.0	0.21	0.21	0.24	0.21	0.0
$A_r$	$A_r$	0.0	0.0	0.47	0.0	0.41	0.0
$B_r$	$B_r$	0.0	0.0	0.0	0.09	0.02	0.0
$(A_r \& B_r)$	$(A_r \& B_r)$	0.0	0.0	0.0	0.0	0.07	0.0
$AB_r$	$AB_r$	0.0	0.0	0.0	0.0	0.05	1.0

Table 2.35 Prevention scenario: Effect of treatment strategies on the frequency of uninfecteds (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.833	5	0.567	779.436	< 0.001
Within Groups	0.013	18	< 0.001		
Total	2.846	23			

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Table 2.36 Prevention scenario: Multiple comparison between the effects of treatment strategies on the frequencies of uninfecteds using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	-0.027	0.729	-0.087	0.034	False
Combination	Mixing	-0.041	0.317	-0.101	0.020	False
Combination	Mono A	-0.525	0.000	-0.586	-0.465	True
Combination	Mono B	-0.360	0.000	-0.421	-0.300	True
Combination	No treatment	-0.951	0.000	-1.011	-0.890	True
Cycling	Mixing	-0.014	0.975	-0.074	0.047	False
Cycling	Mono A	-0.499	0.000	-0.559	-0.438	True
Cycling	Mono B	-0.334	0.000	-0.394	-0.273	True
Cycling	No treatment	-0.924	0.000	-0.985	-0.864	True
Mixing	Mono A	-0.485	0.000	-0.545	-0.424	True
Mixing	Mono B	-0.320	0.000	-0.380	-0.259	True
Mixing	No treatment	-0.910	0.000	-0.971	-0.850	True
Mono A	Mono B	0.165	0.000	0.104	0.226	True
Mono A	No treatment	-0.425	0.000	-0.486	-0.365	True
Mono B	No treatment	-0.590	0.000	-0.651	-0.530	True

Table 2.37 Prevention scenario: Effect of treatment strategies on the frequency of single resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.133	5	0.227	290.494	< 0.001
Within Groups	0.014	18	< 0.001		
Total	1.147	23			

Table 2.38 Prevention scenario: Multiple comparison between the effects of treatment strategies on the frequencies of single resistance using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.030	0.659	-0.033	0.093	False
Combination	Mixing	0.035	0.518	-0.028	0.097	False
Combination	Mono A	0.501	0.000	0.438	0.563	True
Combination	Mono B	0.359	0.000	0.296	0.422	True
Combination	No treatment	0.479	0.000	0.417	0.542	True
Cycling	Mixing	0.005	1.000	-0.058	0.067	False
Cycling	Mono A	0.471	0.000	0.408	0.533	True
Cycling	Mono B	0.329	0.000	0.266	0.392	True
Cycling	No treatment	0.450	0.000	0.387	0.512	True
Mixing	Mono A	0.466	0.000	0.403	0.529	True
Mixing	Mono B	0.325	0.000	0.262	0.387	True
Mixing	No treatment	0.445	0.000	0.382	0.508	True
Mono A	Mono B	-0.142	0.000	-0.204	-0.079	True
Mono A	No treatment	-0.021	0.884	-0.084	0.042	False
Mono B	No treatment	0.120	0.000	0.058	0.183	True

Table 2.39 Prevention scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.061	5	0.012	157.486	< 0.001
Within Groups	0.001	18	< 0.001		
Total	0.063	23			

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Table 2.40 Prevention scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.000	1.000	-0.020	0.020	False
Combination	Mixing	0.003	0.998	-0.017	0.022	False
Combination	Mono A	0.002	1.000	-0.018	0.022	False
Combination	Mono B	0.000	1.000	-0.020	0.020	False
Combination	No treatment	0.136	0.000	0.117	0.156	True
Cycling	Mixing	0.003	0.998	-0.017	0.022	False
Cycling	Mono A	0.002	1.000	-0.018	0.022	False
Cycling	Mono B	0.000	1.000	-0.020	0.020	False
Cycling	No treatment	0.136	0.000	0.117	0.156	True
Mixing	Mono A	-0.001	1.000	-0.021	0.019	False
Mixing	Mono B	-0.003	0.998	-0.022	0.017	False
Mixing	No treatment	0.134	0.000	0.114	0.153	True
Mono A	Mono B	-0.002	1.000	-0.022	0.018	False
Mono A	No treatment	0.134	0.000	0.115	0.154	True
Mono B	No treatment	0.136	0.000	0.117	0.156	True

Table 2.41 Containment scenario: Effect of treatment strategies on the frequency of uninfecteds (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.639	5	0.128	28.906	< 0.001
Within Groups	0.080	18	0.004		
Total	0.718	23			

Table 2.42 Containment scenario: Multiple comparison between the effects of treatment strategies on the frequencies of uninfecteds using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	-0.008	1.000	-0.157	0.141	False
Combination	Mixing	-0.041	0.951	-0.190	0.109	False
Combination	Mono A	-0.104	0.282	-0.253	0.046	False
Combination	Mono B	-0.158	0.034	-0.308	-0.009	True
Combination	No treatment	-0.474	0.000	-0.623	-0.325	True
Cycling	Mixing	-0.033	0.980	-0.182	0.117	False
Cycling	Mono A	-0.096	0.361	-0.245	0.054	False
Cycling	Mono B	-0.150	0.048	-0.300	-0.001	True
Cycling	No treatment	-0.466	0.000	-0.616	-0.317	True
Mixing	Mono A	-0.063	0.758	-0.212	0.086	False
Mixing	Mono B	-0.118	0.175	-0.267	0.032	False
Mixing	No treatment	-0.433	0.000	-0.583	-0.284	True
Mono A	Mono B	-0.054	0.850	-0.204	0.095	False
Mono A	No treatment	-0.370	0.000	-0.520	-0.221	True
Mono B	No treatment	-0.316	0.000	-0.465	-0.166	True

Table 2.43 Containment scenario: Effect of treatment strategies on the frequency of single resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.129	5	0.026	40.881	< 0.001
Within Groups	0.011	18	< 0.001		
Total	0.140	23			

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Table 2.44 Containment scenario: Multiple comparison between the effects of treatment strategies on the frequencies of single resistance using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.019	0.895	-0.038	0.075	False
Combination	Mixing	0.054	0.067	-0.003	0.110	False
Combination	Mono A	0.197	0.000	0.140	0.253	True
Combination	Mono B	0.102	0.000	0.045	0.158	True
Combination	No treatment	0.169	0.000	0.112	0.225	True
Cycling	Mixing	0.035	0.389	-0.021	0.092	False
Cycling	Mono A	0.178	0.000	0.122	0.235	True
Cycling	Mono B	0.083	0.002	0.027	0.140	True
Cycling	No treatment	0.150	0.000	0.094	0.207	True
Mixing	Mono A	0.143	0.000	0.086	0.199	True
Mixing	Mono B	0.048	0.125	-0.009	0.104	False
Mixing	No treatment	0.115	0.000	0.059	0.172	True
Mono A	Mono B	-0.095	0.001	-0.152	-0.039	True
Mono A	No treatment	-0.028	0.626	-0.084	0.029	False
Mono B	No treatment	0.067	0.015	0.011	0.124	True

Table 2.45 Containment scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.038	5	0.008	1.169	0.362
Within Groups	0.116	18	0.006		
Total	0.154	23			

Table 2.46 Maximum-emergence scenario: Effect of treatment strategies on the frequency of uninfecteds (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.432	5	0.286	383.054	< 0.001
Within Groups	0.013	18	< 0.001		
Total	1.445	23			

Table 2.47 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of uninfecteds using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	-0.306	0.000	-0.368	-0.245	True
Combination	Mixing	-0.386	0.000	-0.447	-0.324	True
Combination	Mono A	-0.414	0.000	-0.476	-0.353	True
Combination	Mono B	-0.499	0.000	-0.561	-0.438	True
Combination	No treatment	-0.823	0.000	-0.885	-0.762	True
Cycling	Mixing	-0.079	0.008	-0.141	-0.018	True
Cycling	Mono A	-0.108	0.000	-0.169	-0.046	True
Cycling	Mono B	-0.193	0.000	-0.254	-0.131	True
Cycling	No treatment	-0.517	0.000	-0.578	-0.455	True
Mixing	Mono A	-0.029	0.681	-0.090	0.033	False
Mixing	Mono B	-0.114	0.000	-0.175	-0.052	True
Mixing	No treatment	-0.438	0.000	-0.499	-0.376	True
Mono A	Mono B	-0.085	0.004	-0.146	-0.024	True
Mono A	No treatment	-0.409	0.000	-0.470	-0.347	True
Mono B	No treatment	-0.324	0.000	-0.385	-0.262	True

Table 2.48 Maximum-emergence scenario: Effect of treatment strategies on the frequency of single resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.311	5	0.262	524.241	< 0.001
Within Groups	0.009	18	< 0.001		
Total	1.320	23			

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Table 2.49 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of single resistance using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.342	0.000	0.292	0.393	True
Combination	Mixing	0.408	0.000	0.358	0.459	True
Combination	Mono A	0.549	0.000	0.499	0.600	True
Combination	Mono B	0.536	0.000	0.486	0.586	True
Combination	No treatment	0.761	0.000	0.710	0.811	True
Cycling	Mixing	0.066	0.006	0.016	0.116	True
Cycling	Mono A	0.207	0.000	0.157	0.257	True
Cycling	Mono B	0.194	0.000	0.143	0.244	True
Cycling	No treatment	0.418	0.000	0.368	0.469	True
Mixing	Mono A	0.141	0.000	0.091	0.191	True
Mixing	Mono B	0.128	0.000	0.077	0.178	True
Mixing	No treatment	0.352	0.000	0.302	0.403	True
Mono A	Mono B	-0.013	0.956	-0.064	0.037	False
Mono A	No treatment	0.211	0.000	0.161	0.262	True
Mono B	No treatment	0.225	0.000	0.174	0.275	True

Table 2.50 Maximum-emergence scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.109	5	0.022	71.779	< 0.001
Within Groups	0.005	18	< 0.001		
Total	0.115	23			

Table 2.51 Maximum-emergence scenario: Effect of treatment strategies on the frequency of double resistance (ANOVA).

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.058	0.002	0.019	0.097	True
Combination	Mixing	0.071	0.000	0.032	0.110	True
Combination	Mono A	0.009	0.980	-0.031	0.048	False
Combination	Mono B	0.069	0.000	0.030	0.108	True
Combination	No treatment	0.206	0.000	0.167	0.245	True
Cycling	Mixing	0.013	0.884	-0.026	0.052	False
Cycling	Mono A	-0.049	0.009	-0.088	-0.010	True
Cycling	Mono B	0.011	0.937	-0.028	0.051	False
Cycling	No treatment	0.148	0.000	0.109	0.188	True
Mixing	Mono A	-0.062	0.001	-0.102	-0.023	True
Mixing	Mono B	-0.002	1.000	-0.041	0.037	False
Mixing	No treatment	0.135	0.000	0.096	0.174	True
Mono A	Mono B	0.060	0.001	0.021	0.100	True
Mono A	No treatment	0.198	0.000	0.158	0.237	True
Mono B	No treatment	0.137	0.000	0.098	0.176	True

Table 2.52 Maximum-emergence scenario: Effect of treatment strategies on the frequency of newly emerging double resistance (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.035	5	0.007	41.272	< 0.001
Within Groups	0.010	60	< 0.001		
Total	0.045	65			

2. THE IMPACT OF TREATMENT STRATEGIES ON THE EPIDEMIOLOGICAL DYNAMICS OF  
PLASMID-CONFERRED ANTIBIOTIC RESISTANCE

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Table 2.53 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of newly emerging double resistance using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.013	0.227	-0.004	0.029	False
Combination	Mixing	0.021	0.004	0.005	0.038	True
Combination	Mono A	0.003	0.990	-0.013	0.020	False
Combination	Mono B	0.024	0.001	0.008	0.041	True
Combination	No treatment	0.070	0.000	0.053	0.086	True
Cycling	Mixing	0.009	0.597	-0.007	0.025	False
Cycling	Mono A	-0.009	0.569	-0.026	0.007	False
Cycling	Mono B	0.012	0.308	-0.005	0.028	False
Cycling	No treatment	0.057	0.000	0.041	0.073	True
Mixing	Mono A	-0.018	0.022	-0.035	-0.002	True
Mixing	Mono B	0.003	0.997	-0.014	0.019	False
Mixing	No treatment	0.048	0.000	0.032	0.065	True
Mono A	Mono B	0.021	0.005	0.004	0.037	True
Mono A	No treatment	0.066	0.000	0.050	0.083	True
Mono B	No treatment	0.045	0.000	0.029	0.062	True

Table 2.54 Maximum-emergence scenario: Effect of treatment strategies on the frequency of super-infections (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.019	5	0.004	11.731	< 0.001
Within Groups	0.017	52	< 0.001		
Total	0.036	57			

Table 2.55 Maximum-emergence scenario: Multiple comparison between the effects of treatment strategies on the frequencies of superinfections using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
Combination	Cycling	0.024	0.202	-0.007	0.055	False
Combination	Mixing	0.036	0.015	0.005	0.067	True
Combination	Mono A	0.035	0.021	0.004	0.066	True
Combination	Mono B	0.026	0.160	-0.005	0.057	False
Combination	No treatment	0.068	0.000	0.037	0.099	True
Cycling	Mixing	0.011	0.674	-0.011	0.034	False
Cycling	Mono A	0.010	0.768	-0.013	0.033	False
Cycling	Mono B	0.002	1.000	-0.022	0.025	False
Cycling	No treatment	0.043	0.000	0.021	0.066	True
Mixing	Mono A	-0.001	1.000	-0.024	0.021	False
Mixing	Mono B	-0.010	0.810	-0.033	0.013	False
Mixing	No treatment	0.032	0.001	0.009	0.055	True
Mono A	Mono B	-0.009	0.881	-0.032	0.015	False
Mono A	No treatment	0.033	0.001	0.011	0.056	True
Mono B	No treatment	0.042	0.000	0.019	0.065	True

Table 2.56 Maximum-emergence scenario: Effect of drug  $\vartheta$  on the frequency of emergence per superinfection (ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.194	3	3.398	143.661	< 0.001
Within Groups	1.443	61	0.024		
Total	11.637	64			

Table 2.57 Maximum-emergence scenario: Multiple comparison between the effects of drug  $\vartheta$  on the frequencies of emergence per superinfection using Tukey's post-hoc analysis.

group1	group2	meandiff	p-adj	lower	upper	reject
A	AB	-0.082	0.752	-0.300	0.136	False
A	B	0.740	0.000	0.624	0.855	True
A	none	0.857	0.000	0.711	1.002	True
AB	B	0.822	0.000	0.602	1.042	True
AB	none	0.939	0.000	0.701	1.176	True
B	none	0.117	0.174	-0.032	0.266	False

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# 3

## High-Throughput Quantification of Population Dynamics using Luminescence

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### 3.1 abstract

The dynamics of bacterial population decline at antibiotic concentrations above the minimum inhibitory concentration (MIC) remain poorly characterized. This is because measuring colony-forming units (CFU), the standard assay to quantify inhibition, is slow, labour-intensive, costly, and can be unreliable at high drug concentrations. Luminescence assays are widely used to quantify population dynamics at subinhibitory concentrations, yet their limitations and reliability at super-MIC concentrations remain underexplored. To fill this gap, we compared luminescence- and CFU-based rates across 20 antimicrobials. In our experiments luminescence- and CFU-based rates did not differ significantly for half of them. For the other half, CFU-based estimates of rates of decline were consistently higher. The estimates differed for two main reasons: First, because light intensity tracks biomass more closely than population size, luminescence declined more slowly than the population when bacteria filamented. Second, CFU-based estimates indicated a steeper decline when antimicrobial treatment reduced the number of colonies formed per plated bacterium. This effect can result from changes in clustering behaviour, physiological changes that impair culturability, or antimicrobial carry-over. Thus, the suitability of luminescence to quantify bacterial decline depends on the physiological effects of the antimicrobial used (e.g. filamentation) and whether the quantity of interest is cell number or biomass. Within these limitations, luminescence can serve as an efficient, high-throughput alternative for quantifying bacterial dynamics at super-MIC concentrations.

### 3.2 Introduction

Accurate characterization of changes in population size under treatment is essential for understanding the evolution of antibiotic resistance. Commonly, the effect of treatment on bacterial

populations is quantified by pharmacodynamic (PD) curves. PD curves quantify the relationship between drug concentration and the rate of population change (net growth) [1]. These range from no antibiotic, through sub-MIC concentrations that only reduce population growth, to super-MIC concentrations that kill bacteria and lead to a population decline.

The growth parameter most often used in PD curves is the exponential rate of change in living bacteria,  $\psi_B$ , reflecting both division and death. However, other population properties, such as changes in the number of culturable bacteria or total biomass, may also be relevant, depending on the specific biological question.

In practice, PD-curves are fitted to the rate of change of a measured proxy signal such as optical density (OD), colony-forming units (CFU) or bioluminescent light intensity.

OD is a cost-effective approach for real-time, high-throughput monitoring of culture turbidity without sacrificing the population. OD is positively correlated (within a certain range) to cell density. However, since OD cannot distinguish between living and dead cells, this estimate of cell density is only reliable for increasing or stable population sizes, making it unsuitable for quantifying negative rates (kill rates).

CFU assays estimate bacterial density by counting the colonies that grow on permissive agar media from plated samples. They remain the gold standard for measuring population size under both sub-MIC and super-MIC conditions and are widely used to quantify pharmacodynamic curves (e.g. [1, 2]).

Luminescence assays measure the light emitted by bioluminescent bacterial cultures and can be used as a proxy to estimate changes in population size. Two main approaches for biological assays are: eukaryotic luc systems and the prokaryotic lux systems. The luc system, derived from eukaryotes such as fireflies, uses an ATP-dependent luciferase that oxidises luciferin to emit light [3]. It was adapted for bacterial reporters by chromosomal integration in *Mycobacterium tuberculosis* [4] and later tested for quantifying antibiotic killing in *Streptococcus gordonii* [5]. However, luc-based assays are limited by sensitivity to intracellular ATP, the need for addition of a costly substrate, and luciferin degradation, making continuous measurement in the same culture impractical.

By contrast, the lux operon of prokaryotes such as *Photobacterium luminescens* encodes all components required to sustain the bioluminescence reaction [6, 7]. No external substrate is needed,

so light production can be recorded continuously in the same culture, making the lux system better suited for high-throughput applications than the luc system. Accordingly, it has been widely used to record growth curves and quantify sub-MIC treatment effects [8–13].

While high-throughput OD and luminescence measurements at sub-MIC concentrations provide valuable insights into drug effects on growth rates, the super-MIC range is clinically more relevant. A comprehensive investigation of super-MIC population dynamics (for example, pharmacodynamics of drug combinations or resistance mutations) using CFU remains impractical, as it is labor-intensive and inherently low-throughput.

Whether lux luminescence can be extended to super-MIC ranges remains unclear, as direct comparisons between CFU- and luminescence-based measurements are scarce and so far limited to only a few drugs [14–16]. Here we evaluate the limitations of lux luminescence assays and assess whether they can reliably quantify growth rates at super-MIC concentrations. For this, we compare changes in light intensity with changes in CFU counts across 20 antimicrobials spanning 11 classes, including penicillins, cephalosporins, carbapenems, polymyxins, quinolones, rifamycins, tetracyclines, amphenicols, folate antagonists, fosfomycin, and antimicrobial peptides. Luminescence- and CFU-based rates aligned for some antimicrobials (e.g., colistin, amoxicillin) but diverged for others (e.g., ciprofloxacin). Here, we explore the potential and limitations of both methods, identify the conditions under which they align with the rate of change of population size, and discuss their implications for studying antimicrobial effectiveness across sub-MIC and super-MIC ranges.

### 3.3 Results

To investigate the validity of luminescence assays as a high-throughput measure for bacterial population size and its change across the entire range of antimicrobial concentrations, we compared this measure to the number of colony-forming units (CFU). Specifically, we tested whether the rates of change in light intensity,  $\psi_I$ , and CFU,  $\psi_{\text{CFU}}$ , agree for various drugs, and if not, we explored the reasons for any discrepancies.

We used a modified luminescence operon luxCDABE from *P. luminescens*. To minimize plasmid copy-number effects on the light emitted by a single cell (cell-specific luminosity), we excised the operon from the pCS- $\lambda$  plasmid ([8]) and inserted it into the *Escherichia coli* chromosome.

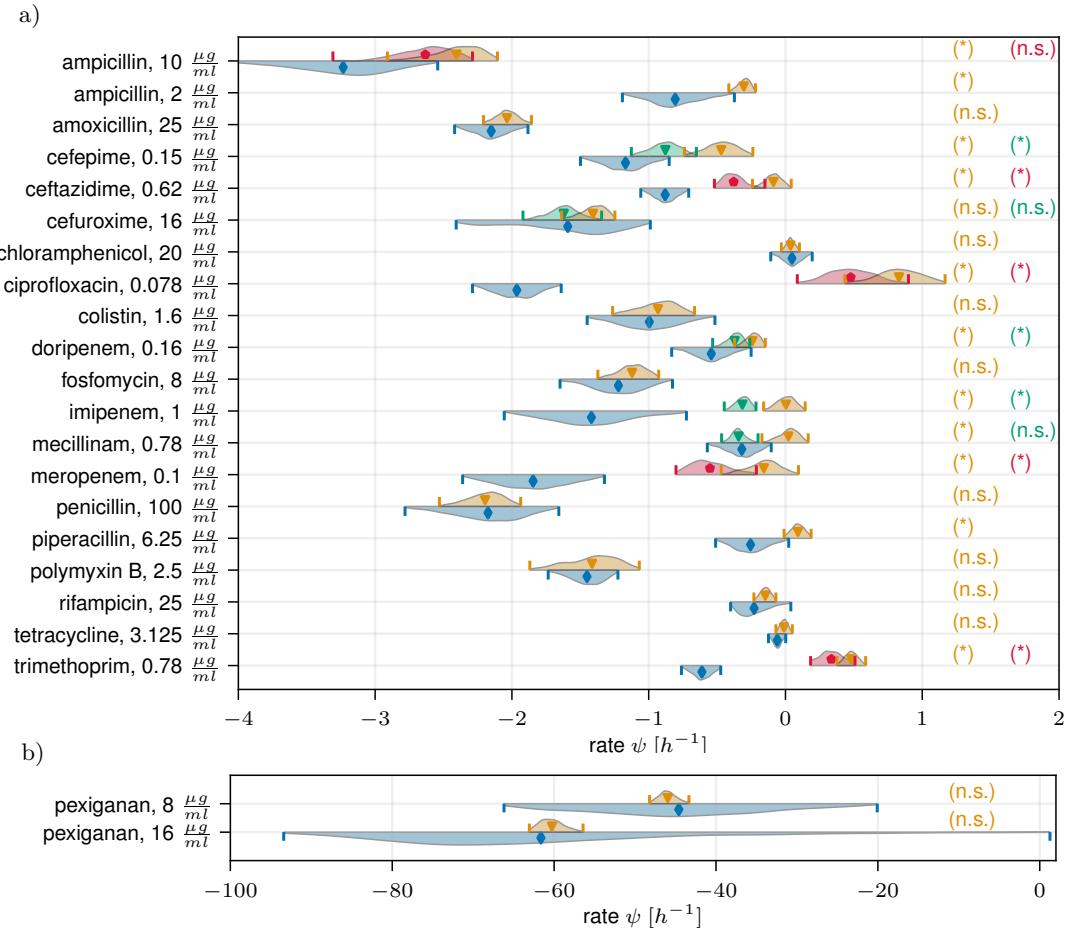


Fig. 3.1 Comparison of CFU-based and luminescence-based rates of change. For each drug, we generated 2000 bootstrapped datasets by resampling time-series CFU and light-intensity data with replacement and fitted an exponential function to each bootstrap replicate to obtain distributions of rates. Panel (a) shows these distributions for 20 drug-concentration assays across 19 antibiotics; panel (b) shows the antimicrobial peptide pexiganan at 8  $\mu\text{g}/\text{mL}$  and 16  $\mu\text{g}/\text{mL}$ . The distribution of  $\psi_{CFU}$  is shown in blue (lower half of each violin, diamond). The distribution of  $\psi_I$  is shown in orange (upper half, triangle). Green distributions ( $\psi^*$ ; triangle) represent luminescence-based rates calculated from data starting at the first peak onward. Red distributions show volume-adjusted luminescence rates ( $\psi_J$ ; pentagon). Vertical lines mark the 95 % confidence intervals. Asterisk (\*) or letters (n.s.) indicate whether CFU-based rates differ significantly from the corresponding luminescence-based rate or not (see Methods), with color coding matching the respective luminescence-based distribution. Wide confidence intervals for pexiganan reflect biphasic killing, steep curves, and noisy CFU data.

**Light intensity is proportional to bacterial density.** We first evaluated how the observed bioluminescent light intensity,  $I$ , which represents a fraction  $\kappa$  of the total light emitted by the culture, correlates with bacterial density. To this end, we prepared three replicate overnight cultures of bioluminescent *E. coli*, serially diluted them tenfold, and measured the light intensity for each dilution. We found that light intensity increased linearly with bacterial density for signals above approximately 20 rlu (Figure S3.1;  $R^2 = 0.987$ ,  $n = 20$ ,  $p < 10^{-14}$ ), with a proportionality constant  $m = 0.006 \frac{\text{rlu ml}}{\text{CFU}}$ . From this observation, we conclude that  $\kappa$  remains independent of bacterial density, indicating that, up to one-tenth of the stationary-phase density, high cell densities do not attenuate emitted light. Thus, provided we maintain the same luminescence plate-reader setup, we can assume  $\kappa$  to be constant for all subsequent analyses.

#### Luminescence-based rates agree with CFU-based kill rates in 11 out of 22 antimicrobial assays.

Given the linearity between light intensity and bacterial density shown above, we tested whether the rate of change of light intensity  $\psi_I$  aligns with the rate of change of bacterial population size ( $\psi_B$ ) under super-MIC antimicrobial concentrations. Since we cannot measure  $\psi_B$  directly, we first compared  $\psi_I$  to the CFU-based rate,  $\psi_{\text{CFU}}$ , and then discussed their relation to  $\psi_B$ . We measured CFU and light intensity over time for 20 drugs (Table S3.1) using an automated liquid handler (Methods) and estimated the distributions of the rates of change of CFU ( $\psi_{\text{CFU}}$ ) and light intensity ( $\psi_I$ ) by bootstrap (Fig. 3.1, Table S3.2). We classified the luminescence and CFU-based rate distributions as “not significantly different” (n.s.) if each mean fell within the other’s 95% percentile and otherwise significantly different (\*). All time-series data are presented in Figures S3.2–S3.20. For amoxicillin, cefuroxime, chloramphenicol, colistin, fosfomycin, penicillin, pexiganan, polymyxin B, rifampicin, and tetracycline,  $\psi_I$  and  $\psi_{\text{CFU}}$  did not differ significantly. However, we observed significant discrepancies for ampicillin, cefepime, ceftazidime, ciprofloxacin, doripenem, imipenem, mecillinam, meropenem, piperacillin, and trimethoprim.

For all cases where significant discrepancies were observed, the light intensity declined more slowly than the CFU signal. In the following we investigate potential reasons why the light signal may decline more slowly and the CFU signal more rapidly than the “true”  $\psi_B$ .

**No support for SOS-driven increase in luminescence promoter activity.** To explain the observed discrepancies between CFU- and luminescence-based rates, we tested whether the SOS response

might upregulate lux expression, increasing the cell-specific luminosity. To this end, we exposed the strains to UV light to induce the SOS response. Specifically, we alternated between measuring light intensity and optical density, and exposing cultures to UV ([SI Appendix 3.4](#)).

UV treatment impaired bacterial growth significantly, yet the OD-normalized light intensity ( $\propto$  cell-specific luminosity) of UV-treated cells was lower than that of untreated controls ([Figure S3.21d](#); t-test,  $p = 3 \cdot 10^{-5}$  for the last time point). This implies that SOS induction did not upregulate lux expression, as cells under SOS had lower light output per cell than controls. While we cannot exclude the possibility that a non-UV-induced SOS response enhances cell-specific luminosity, our findings suggest that promoter upregulation is unlikely to explain why luminescence-based rates exceed CFU-based rates.

**Filamentation aligns with divergence between CFU- and luminescence-based rates.** Antibiotic pressure is known to impair septation and induce bacterial filamentation. Our second hypothesis was that larger, filamented cells might emit more light per cell, thereby driving the divergence between CFU- and luminescence-based rates. We explored this for a subset of the antibiotics tested by imaging bacteria before and after two hours of antibiotic treatment ([Figure S3.22–S3.34](#); see Methods). From these images, we measured bacterial length and width ([Figure S3.35](#)) and calculated the cell volumes (Fig. 3.2, [SI Appendix 3.4](#)).

For antibiotics where microscopy showed no significant filamentation (amoxicillin, colistin, fosfomycin, rifampicin, and tetracycline, [Table S3.3](#)), luminescence-based and CFU-based rates did not differ significantly (Fig. 3.1, [Table S3.2](#)). In contrast, for those drugs where microscopy data indicated significant filamentation (ampicillin, ceftazidime, ciprofloxacin, meropenem, and trimethoprim), luminescence-based rates were significantly higher than CFU-based rates.

**Filamentation model predicts divergence between luminescence- and CFU-based rates.** To further investigate the link between filamentation and the recorded light signal, we developed a simplified population dynamical model which incorporates bacterial filamentation ([SI Appendix 3.3](#)). In this model, cells elongate linearly [17], causing the population to converge to an equilibrium cell volume, provided the division rate remains above zero ([Figure S3.36](#)). We assume that cell-specific luminosity scales with cell volume — i.e., the volume-specific luminosity remains constant.

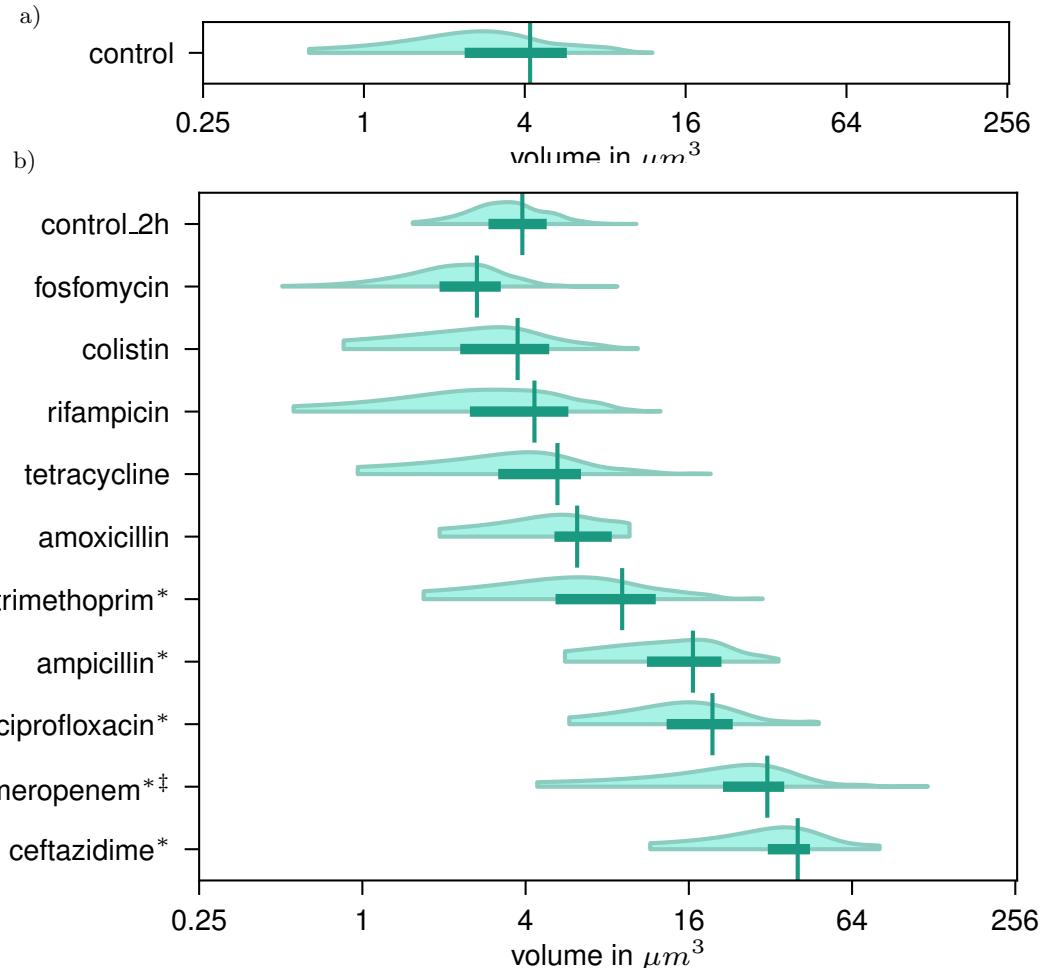


Fig. 3.2 Density distributions (2.5–97.5% percentile range) of pooled cell volumes acquired by microscopy imaging, shown (a) before and (b) after 2 h of antibiotic treatment. Boxes indicate the 25–75 % interquartile range, and vertical bars mark the mean. Significance (\*) was assessed by bootstrapping cell volumes 200 times with replacement for each replicate (image) and treatment, pooling the bootstrapped volumes by treatment, and comparing the resulting 95% confidence interval of the mean to that of the untreated control (control\_2h). ‡ Carbapenems tend to deform cells into a lemon-like shape (Fig. 3.33), resulting in poor fitting quality since our algorithm assumes a cylindrical geometry.

We simulate filament-inducing treatment by reducing the division rate by  $\Delta\lambda$ , which causes an initial rapid increase in average cell size. As the mean cell volume stabilizes and cell-specific luminosity reaches equilibrium, the luminescence-based rate converges to the rate of change of population size  $\psi_B$  (Figure S3.36). Depending on the division and death rates, this dynamic can result in an initial peak in light intensity followed by a decline (Figure S3.36b).

This initial peak in light intensity, as predicted by the model, was experimentally observed for several drugs associated with filamentation (Figures S3.3, S3.5, S3.9, S3.11, S3.12, and S3.13).

To investigate the dependence of  $\psi_B$  and  $\psi_I$  on treatment-induced changes in division rate and death rate ( $\delta$ ), we simulated four hours of treatment (details and parameters in SI Appendix 3.3). Our model shows that a reduction in  $\lambda$  leads to higher luminescence-based rates  $\psi_I$  relative to the rate of change of population size  $\psi_B$ , with particularly large discrepancies when the death rate is low (Fig. 3.3). The results from this model suggest that excluding early data points, where the mean cell volume changes rapidly, improves agreement between the estimated rates  $\psi_I$  and  $\psi_B$ . This is evident in Figure S3.36b, where the slopes inferred from luminescence and from population size differ initially but are nearly identical at later times.

We tested this approach by refitting all experimentally acquired luminescence-based rates that exhibited an initial peak, excluding data points recorded before the light signal reached its maximum. The resulting distributions of  $\psi_I^*$  (green) are shown in Fig. 3.1. An exception was made for meropenem, for which we know the change of cell volume and thus applied an alternative correction as described below. This adjustment substantially reduced the difference between CFU- and luminescence-based estimates for all tested drugs and fully eliminated the discrepancy for mecillinam.

**Adjusting luminescence intensities by changes in volume narrows the gap between CFU- and luminescence-based rates.** Given the model-predicted differences between  $\psi_I$  and  $\psi_B$  in filamenting populations, we next tested whether combining morphological data with measured light intensities can help infer  $\psi_B$ . This approach only works if the volume-specific luminosity is constant (SI Appendix 3.2, Equation S3.12). We used the mean cell volume data acquired by microscopy imaging before ( $v_{\text{obs},0}$ , Fig. 3.2a), and after treatment ( $v_{\text{obs},2h}$ , Fig. 3.2b), for all drugs that caused significant filamentation (ampicillin, ceftazidime, ciprofloxacin, meropenem,

and trimethoprim). The light intensities were then volume-corrected as  $J(t) = I(t) \nu_{\text{obs},0} / \nu(t)$ , where  $\nu(t)$  is derived from the filamentation model (Equation S3.43, SI Appendix 3.3). The free parameters were determined by minimizing Equation S3.51. All adjusted light signals  $J$  are shown in Figures S3.2a, S3.4, S3.7, and S3.13.

For ampicillin, the CFU-based and volume-corrected luminescence-based rates ( $\psi_J$ ) did not differ significantly (Table S3.2). For ceftazidime, ciprofloxacin, and meropenem, volume correction reduced the discrepancy, but  $\psi_J$  remained significantly above  $\psi_{\text{CFU}}$ . We observed in all experiments that volume correction narrowed but never reversed the discrepancy ( $\psi_{\text{CFU}} \leq \psi_J \leq \psi_I$ , Equation S3.23). From this observation and the derivation in the SI (SI Appendix 3.2), we conclude that  $\psi_I$  is closer to  $\psi_V$  (rate of total cell volume change) than to  $\psi_B$  (rate of bacterial number change).

Three factors may explain these residual differences: (i) the assumption of constant volume-specific luminosity may not hold; (ii) the approximation of  $\nu(t)$  may be inaccurate, and excluding entangled or overlapping cells from the analysis introduces a bias that underestimates the volume of heavily filamented cells; (iii) the CFU-based method may underestimate  $\psi_B$ .

For ciprofloxacin, the large disparity between  $\psi_{\text{CFU}}$  and  $\psi_I$  is unlikely to be explained by factors (i) and (ii) alone. Bringing the two rates into agreement solely by adjusting cell-specific luminosity would require an almost four-order-of-magnitude increase, which appears implausible. We therefore conclude that, in this case, CFU-based measurements likely overestimate the rate of population decline, corresponding to an underestimation of  $\psi_B$  (iii).

**CFU-based estimates can overestimate the rate of population decline.** After exploring why luminescence assays can underestimate the rate of population decline, we now explore why CFU assays may overestimate it. By definition, the rate of change of the CFU signal,  $\psi_{\text{CFU}}$ , only matches  $\psi_B$  if the number of colonies emerging per plated bacterium,  $\eta \in [0, 1]$  (Equation S3.4), is constant over time (SI Appendix 3.2, Equation S3.6). This assumption can be problematic for three main reasons:

- a) Loss of culturability: The number of colonies emerging per plated bacterium,  $\eta$ , depends on the division rate  $\lambda$ , which can be affected temporarily or permanently by treatment ([18]), e.g. due to DNA damage. In extreme cases, viable and culturable cells can be converted into

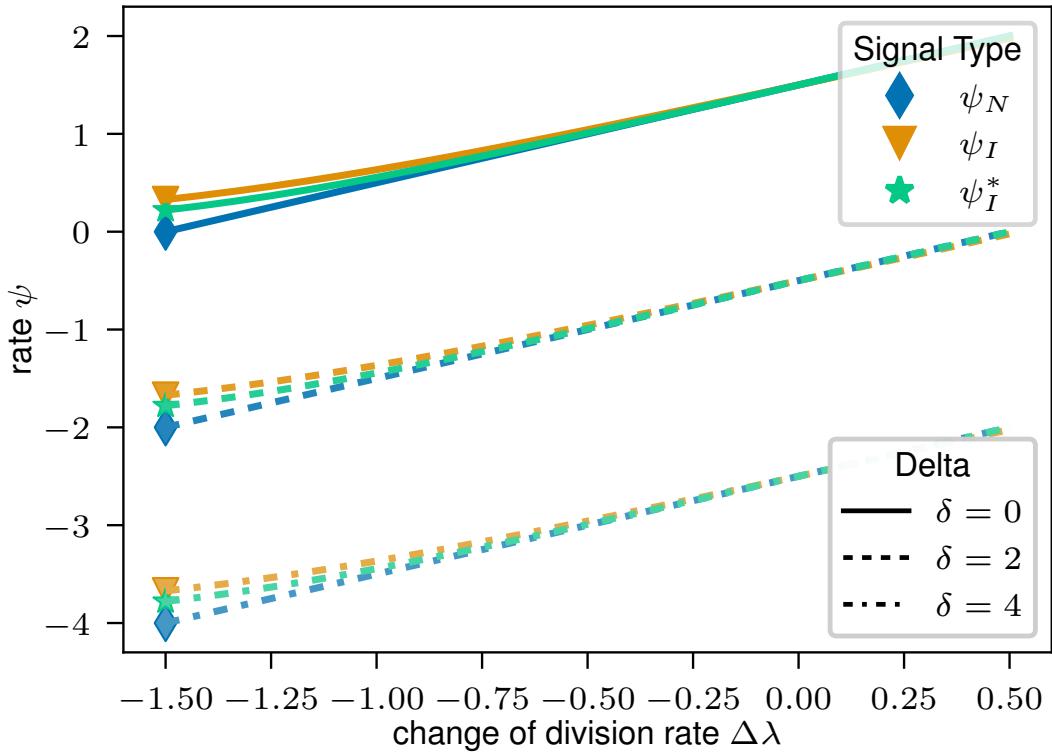


Fig. 3.3 Simulations based on the filamentation model quantifying how changes in division rate due to treatment ( $\Delta\lambda$ ) and death rate ( $\delta$ ) influence the rate of change of population size ( $\psi_B$ , blue), the rate of change of light intensity ( $\psi_I$ , orange), and the rate of change of light intensity when the first 2 hours of data are excluded ( $\psi_I^*$ , green). These illustrative simulations were conducted using an initial division rate  $\lambda_0 = 1.5\text{h}^{-1}$ . More details and all parameter values can be found in [SI Appendix 3.3](#).

viable but non-culturable (VBNC) cells ([19–21]), meaning they continue to be metabolically active but cease to divide ( $\lambda = 0$ ) and therefore no longer form colonies on agar. Typically, cells can reproduce at the start of a time-kill assay but may, depending on the drug, partially or completely lose this ability as the assay progresses, causing an underestimation of  $\psi_B$ .

- b) Antimicrobial carryover: Antibiotics transferred onto agar by plating a diluted culture can experience a residual treatment effect; either on the division rate  $\lambda$  or the death rate  $\delta$ , depending on the mode of action of the drug and thus reduce the probability of colony formation. This phenomenon, known as antimicrobial carryover, has been described in previous studies [22–24]. Its effect is usually minimal at the start of a time-kill assay, when bacterial density is high and plated samples are highly diluted. However, as the assay pro-

gresses and bacterial density declines, less dilution is needed, increasing the concentration of the transferred antibiotic. As a consequence, CFU-based rates would underestimate  $\psi_B$  between two time points  $t_0$  and  $t_1$  by  $\frac{\ln(\eta_1) - \ln(\eta_0)}{\Delta t}$ , where  $\eta_i$  denotes the mean number of colonies formed per plated bacterium at time  $t_i$ .

- c) Aggregation: Filamentation or altered cell adhesiveness can change the size distribution of colony-initiating clusters on agar after plating ([Equation S3.3](#)). Changes in cluster size in turn, affect the average number of clusters per plated bacterium, thereby biasing estimates of  $\psi_B$ .

#### **Partial loss of culturability causes CFU to underestimate $\psi_B$ for ciprofloxacin and trimethoprim treatment.**

During ciprofloxacin treatment, CFU counts fell steeply while light intensity continued to rise ([Figure S3.7](#)). This discrepancy is consistent with previous reports comparing CFU and luminescence during fluoroquinolone killing [14, 25]. To investigate the cause of this discrepancy, we plated treated cultures on phosphate-buffered saline (PBS) agar containing propidium iodide (PI), a red fluorescent dye that binds to nucleic acids but cannot penetrate intact cell membranes. PI is a red fluorescent dye that binds to nucleic acids and is widely used to stain permeable cells due to its inability to penetrate intact cell membranes. Microscopic imaging ([Figure S3.27](#)) revealed almost no red fluorescence, indicating that the cells remained impermeable. Although impermeability alone does not confirm viability, additional observations support the conclusion that most cells were still alive: the absence of bacterial debris (as has been observed for drugs with similar decline in CFU such as amoxicillin), visible growth indicated by increased cell size compared to two hours earlier, and continued (and even increased) light emission. These findings suggest that most cells remain alive but are unable to form colonies under the provided conditions, possibly due to DNA damage induced by ciprofloxacin [26]. This observation aligns well with previous studies on ciprofloxacin, which found that CFU can underestimate viability relative to non-culture-based methods [19, 27, 28].

Trimethoprim treatment showed similar, though less pronounced, results ([Figure S3.19](#)). Trimethoprim, which impairs DNA replication ([29]), likewise caused an increase in light intensity and a decline in CFU counts, while microscopy revealed intact, mostly impermeable, filamented cells ([Figure S3.34](#)).

**Antimicrobial carryover causes underestimation of  $\psi_B$  for pexiganan using CFU.** Building on our understanding of when luminescence assays accurately estimate  $\psi_B$ , we hypothesized that antimicrobial peptides (AMPs) would be an ideal application for this method. We expected that, during the AMP's short killing phase, changes in the cell-specific luminosity would remain negligible compared to the high kill rates AMPs can achieve.

Initially, however, we failed to recover almost any colonies on agar, despite the light intensity indicating a high enough bacterial density. Moreover, colony counts were inconsistent across dilutions: wells diluted 100-fold and 1000-fold from the same time point yielded similar colony numbers, instead of reflecting the tenfold difference. We suspected that AMPs from the liquid culture, including those attached to the bacterial surface, were carried over into the PBS dilution medium, causing continued cell death during dilution and after plating.

To test this, cultures treated with pexiganan for 1 min were diluted 1:100 in PBS and subsequently sampled and plated at four time points, approximately 45 minutes apart. As dilution medium, we tested PBS supplemented with various concentrations of  $\text{CaCl}_2$  and  $\text{MgCl}_2$ . These compounds were selected based on prior evidence that they inhibit the activity of other AMPs ([30]).

Our results show that supplementing the dilution medium increased the measured CFU substantially ([Figure S3.38](#), [SI Appendix 3.4](#)). This indicates that bacterial survival probability increases when diluted in the supplemented medium. Conversely, diluting in unsupplemented PBS does not stop bacteria from dying. Supplementing 100 mM  $\text{MgCl}_2$  yielded the highest CFU count for the first time point ([Table S3.4](#)). Since CFU cannot systematically overestimate bacterial density, this count represents the best estimate of the bacterial density. Consequently, we supplemented the PBS with 100 mM  $\text{MgCl}_2$  in subsequent pexiganan experiments.

Given this insight into the residual killing effect of pexiganan and how to mitigate it, we repeated the CFU time-kill experiment using two different dilution media ([SI Appendix 3.4](#)). We recorded three replicates for each of the two time-kill curves. We diluted the cultures at each time point in either pure PBS or PBS supplemented with 100 mM  $\text{MgCl}_2$  and counted colonies on all agar plates from three dilution steps. To lower the detection limit by one order of magnitude, we increased the plated volume from 10  $\mu\text{L}$  to 100  $\mu\text{L}$ . Since this volume exceeds the capacity of the automated high-throughput setup, we used the standard manual CFU plating method instead.

We observed a much steeper initial decline in CFU for the cultures diluted in pure PBS compared

to the supplemented ones (Figure S3.39). In pure PBS, more highly diluted samples consistently yielded higher CFU estimates (Figure S3.39a), supporting the antimicrobial carryover hypothesis. This pattern diminished over time, suggesting a reduction in the residual killing effect of pexiganan, which we discuss below.

#### **Luminescence and CFU show identical decline rates for pexiganan time-kill curves if residual killing is prevented.**

To confirm that eliminating residual pexiganan killing aligns CFU and luminescence, we supplemented PBS with 100 mM MgCl<sub>2</sub> and measured both signals at pexiganan concentrations of 8  $\frac{\mu\text{g}}{\text{mL}}$  and 16  $\frac{\mu\text{g}}{\text{mL}}$  using the “rapid luminescence-CFU assay setup” (Methods). We observed no significant difference between the CFU- and luminescence-based rates for either of the tested pexiganan concentrations (Fig. 3.1b, Table S3.2). However, examining the time series (Figure S3.20) revealed that while CFU and luminescence signals declined in parallel for the 8  $\frac{\mu\text{g}}{\text{mL}}$  treatment, they diverged for the 16  $\frac{\mu\text{g}}{\text{mL}}$  kill curve. In this case, the CFU signal initially declined much faster (rates below  $-200\text{h}^{-1}$ ), and subsequently declined more slowly than the corresponding luminescence signal, ultimately resulting in a similar average rate (approximately  $-60\text{h}^{-1}$ ).

This pattern suggests that luminescence assays may not be able to capture extremely rapid kill rates, potentially due to short delays between irreversible cell damage, actual cell death, and the subsequent cessation of luminescence. This limitation should not affect measurements for most conventional antibiotics, for which rates rarely exceed  $-10\text{ h}^{-1}$ .

**Pexiganan rapidly loses killing capacity.** During the pexiganan experiments, we observed an initial steep decline in bacterial density, predicted by both CFU and luminescence, followed by almost constant signals (Figures Figure S3.20, Figure S3.39). Two possible, non-exclusive explanations for this observation are: first, pexiganan is deactivated or sequestered from the medium by attaching to targets on the bacteria over time; and second, the remaining bacteria are unaffected by the AMP because they are resistant or persisters. To investigate the first explanation, we exposed bacteria to pexiganan for 5 min at 16  $\frac{\mu\text{g}}{\text{mL}}$ , after which the supernatant was collected and tested for its ability to kill bacteria (SI Appendix 3.4). While the initial treatment showed rapid bacterial killing ( $\psi_{\text{CFU}} \approx -46\text{h}^{-1}$  between  $t_0 = 0$  and  $t_1 = 5\text{ min}$ ), bacteria exposed to the supernatant alone showed no significant reduction in viability (Figure S3.40,

[Table S3.5](#)). These results show that the supernatant has no residual killing effect. This makes deactivation or sequestration through attachment of pexiganan the likely explanation for the flattening CFU signal, even though we did not assess whether the surviving cells are resistant or persisters.

### 3.4 Discussion

We evaluated whether luminescence can serve as a high-throughput proxy for population dynamics by comparing it with CFU assays. We found no significant difference between CFU- and luminescence-based rates for treatments that neither induce substantial changes in culturability nor provoke strong morphological changes, such as filamentation. However, for drugs that induce filamentation and/or loss of culturability, the two methods can yield significantly different results. The divergence between the two rates does not imply that either method is incorrect; rather, each captures a different aspect of the population.

The CFU method counts bacteria capable of forming colonies on permissive media (i.e., culturable cells). When inferring growth rates from CFU, the observed rate of change  $\psi_{\text{CFU}}$  reflects both the rate of change of population size  $\psi_B$  and changes in the probability that a plated bacterium forms a colony,  $\eta$  ([Equation S3.4](#)). The CFU-based rate equals  $\psi_B$  only if  $\eta$  remains constant over time.

However,  $\eta$  can change for three main reasons. First, altered clustering behavior can change how many bacteria seed a single colony. This directly shifts the observed colony count. Second, physiological changes to the bacteria may lead to a temporary or permanent change in culturability ([18–21, 27, 31]), which may increase the fraction of viable cells that fail to form colonies, e.g. due to a reduced division rate. Third, residual drug activity carried over to the agar can alter on-plate conditions, thereby reducing division or increasing the death rate ([22–24]).

Preventing antibiotic carryover when handling low-density cultures treated with highly concentrated antimicrobials is challenging and, in some cases, infeasible. Centrifugation-based washing (pelleting bacteria and replacing the supernatant) can remove residual drug, but only if the processing delay is negligible relative to the antibiotic’s killing kinetics — a condition unlikely to hold for fast-acting agents such as pexiganan. Moreover, although bacteria generally tolerate high centrifugal forces ([32]), the impact on compromised cells, such as those with destabilized

walls, is unknown. As an alternative, we found that supplementing the dilution medium with  $\text{MgCl}_2$  effectively neutralizes residual AMP activity, preventing residual killing effects in CFU assays for pexiganan. However, this strategy is not generalizable, as for many antimicrobials the corresponding deactivating agents are unknown — or may not even exist. For these cases, it may be impossible to accurately derive  $\psi_B$  from CFU counts at high drug concentrations.

In contrast to CFU assays, luminescence assays become more reliable at high, fast-killing concentrations. This is because  $\psi_I$  reflects both the rate of population size change,  $\psi_B$ , and changes in cell-specific luminosity (Equation S3.9); when population declines rapidly,  $\psi_I$  converges to  $\psi_B$ . One exception was observed for extremely rapid kill rates (exceeding  $-60\text{h}^{-1}$ ), as seen for highly concentrated pexiganan, likely due to short delays between cell death and cessation of luminescence.

For lower kill rates, we observed that the absence of filamentation was a good indicator of stable cell-specific luminosity. In cases where drugs induced filamentation, CFU- and luminescence-based rates diverged. We further demonstrated that correcting for the increased cell size partly compensates for the difference between CFU- and luminescence-based estimates, and we found that the rate of change in light intensity is closer to the change in total cell volume than to the change in total cell number (SI Appendix 3.2). This makes a constant volume-specific (or mass-specific) luminosity a better assumption than a constant cell-specific luminosity.

Changes in both CFU and luminescence are used as proxy signals for population net growth rates ([1, 2, 8–10, 12, 13]). Whether discrepancies between the changes in these proxy signals and the changes in living bacteria pose a problem depends on the underlying biological question. The rate of change of living bacteria,  $\psi_B$ , is most commonly applied in theoretical modeling to create predictions, making its estimation important. If, instead, the aim is to assess a population’s reproductive potential, for example, in studies focusing on evolutionary dynamics, examining changes in the number of culturable cells (as approximated by CFU) may be more relevant than  $\psi_B$ , as only culturable cells contribute to subsequent generations and thus to evolution. Additionally, tracking changes in total biomass (closer to  $\psi_I$ ) can be more relevant than the number of living bacteria, as biomass accounts for a potential “catch-up” effect, whereby filamented cells fragment into multiple viable units once antibiotic pressure is removed ([31, 33]).

A notable challenge of using luminescence assays is the absence of a fundamental biological prin-

ciple linking cell-specific luminosity uniquely to a single biological property, such as biomass, cell number, or gene copy number. We therefore recommend applying luminescence-based methods primarily over short to intermediate timescales, during which abiotic conditions remain relatively stable. When the impact of treatment on cell size is unknown, one should assume that  $\psi_I$  primarily reflects the rate of change of biomass rather than cell number. Thus, to reliably estimate  $\psi_B$ , it is essential to ensure either that cells do not filament, correct for changes in mean cell volume, or restrict analyses to intervals during which volume changes are minimal relative to population decline. Finally, because most drugs in this study were tested at only one concentration, the generalizability of our drug-specific findings remains to be shown.

Our results show that neither CFU nor luminescence is optimal for every experimental scenario. Instead, CFU and luminescence work best under different conditions, measure different population properties and complement each other well. At low and intermediate drug concentrations, changes in CFU accurately reflect changes in bacterial density, but CFU becomes unreliable at high drug concentrations.

Luminescence, by contrast, becomes more reliable at high concentrations, where CFU becomes unreliable. In practice, the luminescence method significantly reduces labor, consumables, and costs: eight PD curves with twelve concentrations and four replicates each can be fit on a single 384-well plate, whereas measuring CFU would require more than 8,000 agar plates, hundreds of dilution plates, and substantial manual labor. Given their scalability and cost-effectiveness, luminescence assays offer a valuable alternative for high-throughput analysis, particularly at high antimicrobial concentrations, where traditional methods become unreliable or even unusable.

### 3.5 Methods

**Strains.** We generated a bioluminescent strain by integrating a modified *P. luminescens* luxCD-ABE operon, driven by the constitutive  $\lambda$ -Pr promoter, together with a kanamycin resistance cassette (as a marker) into the chromosome of *Escherichia coli* MG1655. This integration replaced the galK gene and was achieved using  $\lambda$ -Red recombination ([34]), following a protocol by Hughes [35, pp. 19–26]. The integrated elements were derived from the pCS- $\lambda$  plasmid ([8, 36]). Primers are listed in [Table S3.6](#). For all time-course experiments, we prepared three replicate exponential cultures by diluting overnight cultures (grown for approximately 18 hours) 1:100 and

growing them to exponential phase for 1–1.5 hours.

**Media.** We used LB (Sigma L3022) as a liquid medium and, as a solid medium for CFU plating, LB with 1.5 % agar. Cultures were treated by diluting the tenfold working concentration of one of 20 antimicrobials 1:10. All MICs, determined by broth microdilution ([37]), and the concentrations used are listed in [Table S3.1](#). Working concentrations were centered around the MIC, with some variation due to rounding convenience and variability in repeated MIC tests. For colistin and polymyxin B, we used lower concentrations, as higher concentrations in our setup consistently yielded too few colonies for meaningful analysis.

Phosphate-buffered saline (PBS, Sigma 79383) was used as the diluent for CFU assays. If cultures were treated with pexiganan, 100 mM MgCl<sub>2</sub> was added. For microscopy, we added 1  $\frac{\mu\text{g}}{\text{mL}}$  propidium iodide (PI) to the liquid medium and used PBS/PI agar plates (containing PBS with 1.5 % agar and 1  $\frac{\mu\text{g}}{\text{mL}}$  PI) as solid medium.

**Automated CFU plating.** We automated the high-throughput colony-count method described by [38] using an Evo 200 liquid-handling platform (Tecan) integrated with a Liconic STX100 incubator. The platform handles liquids and automatically moves, images, and incubates plates. We produced six colony streaks by spotting six 10  $\mu\text{L}$  drops of diluted bacterial culture onto a one-well agar plate. Plates were automatically tilted for 7 s on a custom-built tiltar integrated into the handling platform, to spread the drops and distribute the bacteria. After incubation, plate images were captured using the Pickolo camera (SciRobotics).

The platform is controlled by custom-generated worklists executed in the native software “Evoware”. These worklists were generated using the Python package `pypetting` (version 1.0.1). We analyzed the captured images of the agar plates using a custom colony-recognition script that automatically identifies colonies and allowed the manual addition of unidentified colonies and the removal of mismatched ones. All Python classes for generating the worklists and analyzing colonies are available at Zenodo (DOI: 10.5281/zenodo.15261184).

**Luminescence measurements.** To record the luminescent light intensity, we used an Infinite F200 spectrophotometer plate reader (Tecan), which is also integrated into the liquid-handling platform, with an exposure time of one second. We set 20 rlu as the lower detection limit and

excluded all data points below.

**Luminescence-CFU assay setup.** To measure the CFU and light intensity at seven time points, we treated the exponential cultures and then distributed them onto seven (one for each time point) white 384-well plates (Greiner, 781073), with each culture well containing 54  $\mu$ L medium and 6  $\mu$ L 10x stock solution. We adjusted the duration of the experiments between two and five hours, depending on the anticipated kill rate. For each time point, an assay plate was transferred from the incubator to the plate reader for luminescence measurement. Subsequently, a dilution series was conducted directly in the white plate and plated using the automated plating method, after which the plate was discarded.

**Rapid luminescence-CFU assay setup.** This experiment is a variation of the Luminescence-CFU assay setup, adjusted to measure rapid kill curves for the antimicrobial peptide pexiganan. In this setup, we captured four time points within 5 min. Cultures were treated in a 96-deep-well plate (Greiner, 780285) by adding 100  $\mu$ L of the 10x stock solution to 900  $\mu$ L exponential phase culture. 60  $\mu$ L of the treated culture was then transferred to a 384-well white plate (Greiner, 781073) and placed in the plate reader for continuous luminescence recording. For the four CFU time points, samples were taken directly from the deep-well plate, automatically diluted in PBS supplemented with 100mM MgCl<sub>2</sub> in a 96-well plate (Greiner, 655101) to halt the antimicrobial activity and then plated.

**Morphology experiments.** To assess treatment-induced morphological changes, we imaged treated (for 2 hours) and untreated bacteria by spotting 2  $\mu$ L droplets onto PBS/PI-agar plates. The spots were cut out and flipped onto Ibidi  $\mu$ -dishes (Ibidi, 80136) for imaging. We used an Eclipse Ti2 microscope (Nikon) with a 100x objective connected to a DS-Qi2 Nikon Scientific CMOS (sCMOS) camera to image the bacterial cells. The microscope setup included an additional 1.5x zoom, which was used only for some images due to unintentional variation. We estimated the width and length of the bacterial cells using a custom Python script, as described in [SI Appendix 3.4](#).

**Fitting rates of change  $\psi$ .** To compare the rates of change of two signals, we first excluded all data below the detection limits (empty plates or light intensity below 20 rlu). We then truncated

both signals at the latest time point where both remained above the detection limit, ensuring the same time frame was used for comparison. Next, we bootstrapped 200 datasets with replacement per signal, while ensuring that each dataset contained more than one time point. For each dataset, we applied a simple regression to fit an exponential function to all time points of each time-kill curve resulting in distributions with 200 rate estimates each.

**Significance.** We classify two distributions of rates as not significantly different (n.s.) if the mean of each distribution falls within the 95% confidence interval of the other. Otherwise, we classify them as significantly different (\*).

### Data, Materials, and Software Availability

Experimental datasets are available at Zenodo (DOI: 10.5281/zenodo.15261454). Analysis scripts, plate-handling worklists, colony-recognition code, and model code are available at Zenodo (DOI: 10.5281/zenodo.15261184).

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### 3.6 Supplementary Information Chapter 3

#### 3.7 Mathematical descriptions

##### Light-related terminology.

In this manuscript, total luminosity ( $L$ ) refers to the total light output produced by  $B$  bacteria of a bioluminescent bacterial culture in a well with volume  $V_w$ . During the luminescence assays, we capture a fraction  $\kappa$  of the total luminosity ( $\phi B$ ) as light intensity  $I(t) = \kappa\phi B$ . We call the exponential decline rates based on these intensities luminescence-based rates,  $\psi_I$ . We use cell-specific luminosity ( $\phi$ ) for light output per cell and volume-specific luminosity ( $\theta$ ) for light output per unit cell volume. When  $I(t)$  is normalized by the measured optical density, we obtain  $\omega$ , the OD-normalized light intensity.  $J(t)$  is the light intensity  $I(t)$  adjusted by the relative change in cell volume  $\frac{v_0}{v(t)}$ .

Testing for linearity between bacterial density and luminescent light intensity.

To test whether bacterial density and bacterial luminescent light intensity are linearly related, we grew three replicate overnight cultures. To replenish nutrients, we diluted each culture 1:10

in fresh LB medium and incubated for 60 minutes. We subsequently performed a 10-fold dilution series in a 384-well white plate (Greiner, 781073) and immediately measured the light intensity (see Fig. 1 in the main text). Each plate included wells containing only medium to determine a blank (median  $\approx 37.33$  rlu), which we subtracted from all measurements.

We assessed linearity between light intensity ( $I$ ) and bacterial density ( $b$ ) by fitting a linear model without intercept on the original scale,

$$I_i = m \cdot b_i,$$

which corresponds to a log–log regression with intercept:

$$\ln(I_i) = \ln(m) + \ln(b_i).$$

Summing over all  $n$  observations gives

$$n \ln(m) = \sum_{i=1}^n \ln(I_i) - \sum_{i=1}^n \ln(b_i),$$

The optimal conversion factor  $m$  is then

$$m = \frac{\left(\prod_{i=1}^n I_i\right)^{\frac{1}{n}}}{\left(\prod_{i=1}^n b_i\right)^{\frac{1}{n}}},$$

yielding  $m = 0.006 \frac{\text{rlu}}{\text{CFU}}$ .

We observed a linear relationship ( $R^2 = 0.987$ ,  $F = 1164.14$ ,  $p < 10^{-14}$ ) between bacterial density and light intensity for intensities above 20 rlu ( $\sim 3 \times 10^2$  CFU/ml). Consequently, we use 20 rlu as the lower detection limit for luminescence. Additionally, we conclude that  $\kappa$  is independent of the bacterial density (no overshadowing effects), within the relevant range of densities. Since the plate reader setup remains constant within an experiment, we assume  $\kappa$  to be constant for following analyses.

Rate of change of CFU count.

To infer the rate of change of CFU, we assume bacteria spend only a short time in the low-nutrient dilution medium, so replication and death are negligible during that phase.

Most colonies originate from clusters only comprising a single cell, but some from founding clusters of multiple cells. Let  $f(n)$  be the probability that a randomly chosen cluster contains  $n$

bacteria. A cluster of size  $n$  forms a colony with probability

$$1 - (p_E)^n, \quad (3.1)$$

where  $p_E$  is the probability that a lineage originating from a single bacterium goes extinct (extinction probability). The mean probability that a plated cluster forms a colony is:

$$\bar{g} = \sum_{n=1}^N f(n) (1 - (p_E)^n). \quad (3.2)$$

The mean cluster size is

$$\bar{n} = \sum_{n=1}^N n f(n). \quad (3.3)$$

The mean number of colonies emerging per plated bacterium can be approximated by:

$$\eta = \frac{\bar{g}}{\bar{n}}, \quad \eta \in [0, 1]. \quad (3.4)$$

The predicted CFU per ml given a bacterial density  $b = \frac{B}{V_w}$ , with  $B$  the number of bacteria per well and  $V_w$  the constant well volume, is:

$$\text{CFU} = \eta \frac{B}{V_w}. \quad (3.5)$$

Taking the logarithmic derivative yields:

$$\psi_{\text{CFU}} = \frac{d}{dt} \ln(\text{CFU}) = \frac{d}{dt} \ln(\eta) + \frac{d}{dt} \ln(B). \quad (3.6)$$

In practice  $\eta$  is often unknown. We can only estimate  $\psi_B$  from CFU data if we assume that  $\eta$  is constant over time.

$$\psi_{\text{CFU}} = \frac{d}{dt} \ln(B) = \psi_B. \quad (3.7)$$

To justify a constant  $\eta$  we must assume that the cluster-size distribution  $f(n)$  and the extinction probability  $p_E$  do not change over time. Both assumptions may fail, for example if cells filament or if the division or death rate change. We discuss the behaviour of  $p_E$  below.

Rate of change of luminescence.

The observed light intensity  $I$  is a fraction  $\kappa$  of the total luminosity ( $\phi B$ ) of a bioluminescent culture, where  $\phi$  is the cell-specific luminosity (the amount of light emitted by one bacterium).

The light intensity can thus be written as:

$$I = \kappa \phi B. \quad (3.8)$$

The rate of change of light intensity is therefore:

$$\psi_I = \frac{d}{dt} \ln(I) = \frac{d(\ln(\kappa) + \ln(\phi) + \ln(B))}{dt}. \quad (3.9)$$

We assume that  $\kappa$  remains constant over time, as we explained above (“Testing for linearity between bacterial density and luminescent light intensity”). If we assume that the cell-specific luminosity  $\phi$  is also constant,  $\psi_I$  equals the rate of bacterial count change  $\psi_B$ , as Equation 3.9 simplifies to:

$$\psi_I = \frac{d}{dt} \ln(I) = \frac{d}{dt} \ln(B) = \psi_B. \quad (3.10)$$

Rate of change of volume-corrected luminescence.

Alternatively, we can link the measured light intensity to the number of bacteria using the mean cell-specific volume ( $v$ ) and the volume-specific luminosity ( $\theta$ ):

$$I = \kappa \theta v B. \quad (3.11)$$

Defining the volume-corrected luminescence as  $J(t) = I(t)v_0/v(t)$ , we can compute its rate of change as:

$$\psi_J = \frac{d(\ln(I \cdot v_0/v))}{dt} = \frac{d(\ln(\kappa) + \ln(v_0) + \ln(\theta) + \ln(B))}{dt}. \quad (3.12)$$

If we assume that the volume-specific luminosity  $\theta$  is constant, this estimate equals the rate of change of the number of living bacteria ( $B$ ):

$$\psi_J = \frac{d}{dt} \ln(I \cdot v_0/v) = \frac{d}{dt} \ln(B) = \psi_B. \quad (3.13)$$

Change of light intensity is closer to the rate of change of total cell-volume than to the rate of change of number of bacteria.

We made two empirical observations under all tested drug conditions:

- (i) For the subset of drugs imaged using microscopy, the mean specific cell volume never significantly decreased between the first and second time point:

$$\frac{d \ln v}{dt} \geq 0 \quad (3.14)$$

The rate of change of total cell volume is given by:

$$\psi_V = \frac{d \ln(vB)}{dt} = \frac{d \ln v}{dt} + \psi_B. \quad (3.15)$$

From this relation and observation (i), we directly obtain:

$$\boxed{\psi_V \geq \psi_B} \quad (3.16)$$

- (ii) The rate of change of volume-corrected light intensity was never significantly lower than the corresponding rate of change of CFU:

$$\psi_I = \frac{d \ln J}{dt} \geq \psi_{CFU} \quad (3.17)$$

Since we can express the light intensity as  $I = \kappa\theta vB$ , the volume-corrected luminescence rate becomes:

$$\psi_I = \frac{d}{dt} \ln(\kappa\theta v_0 B) = \psi_B + \frac{d \ln \theta}{dt}. \quad (3.18)$$

Observation (ii) thus implies:

$$\psi_B + \frac{d \ln \theta}{dt} \geq \psi_{CFU} \quad (3.19)$$

If we make the assumption that the rate of change of CFU equals that of bacterial count ( $\psi_{CFU} = \psi_B$ ) we obtain:

$$\frac{d \ln \theta}{dt} \geq 0 \quad (3.20)$$

Using the rate of change of light intensity we get:

$$\psi_I = \frac{d \ln(\kappa\theta vB)}{dt} = \psi_V + \frac{d \ln \theta}{dt}. \quad (3.21)$$

From this relation and Equation 3.20, we follow:

$$\boxed{\psi_I \geq \psi_V} \quad (3.22)$$

Combining Equation 3.16 and Equation 3.22 yields:

$$\boxed{\psi_I \geq \psi_V \geq \psi_B} \quad (3.23)$$

allowing us to conclude that, during our experiments, the luminescence-based rate is closer to the rate of change of total cell volume (likely identical to the rate of change of biomass) than to the rate of change of bacterial count.

#### Colony formation -- birth-death Markov Model.

We use a basic birth-death Markov model, as described by [1], [2], [3], and [4], to model the probability that a single plated bacterium creates a colony.

In this model, as in [1],  $P_0(t)$  describes the probability that the population originating from this single bacterium goes extinct by the time  $t$ :

$$P_0(t) = \frac{\delta}{\lambda} \cdot \frac{E(t) - 1}{E(t) - \frac{\delta}{\lambda}}, \quad (3.24)$$

where  $\delta$  is the death rate,  $\lambda$  the division rate, and  $E(t) = e^{(\lambda-\delta)t}$ . For  $t \rightarrow \infty$ ,  $P_0(t)$  converges to the extinction probability  $p_E$  for a lineage originating from a single cell.

$$p_E = \begin{cases} 1, & \text{if } \lambda \leq \delta, \\ \frac{\delta}{\lambda}, & \text{if } \lambda > \delta. \end{cases} \quad (3.25)$$

#### Colony formation for bacteriostatic and bactericidal drugs.

In the following, we call the death and division rate in the absence of treatment  $\delta_0$  and  $\lambda_0$ , respectively, and the treatment-induced increase in death and reduction in division rate  $\delta_T$  and  $\lambda_T$ , respectively. We then rewrite the net growth rate as:

$$\psi = \lambda_0 - \lambda_T - \delta_0 - \delta_T \quad (3.26)$$

Furthermore, we define the combined treatment effect:

$$\tau = \psi_0 - \psi = \delta_T + \lambda_T \quad (3.27)$$

We write the probability of colony formation (from a single cell) as:

$$p_E = \begin{cases} 1, & \text{if } \lambda_0 - \lambda_T \leq \delta_0 + \delta_T, \\ \frac{\delta_0 + \delta_T}{\lambda_0 - \lambda_T}, & \text{if } \lambda_0 - \lambda_T > \delta_0 + \delta_T. \end{cases} \quad (3.28)$$

We then define the extinction probability for purely bacteriostatic drugs ( $\delta_T = 0$  and  $\lambda_T = \tau$ ) as:

$$p_{E,\text{stat}} = \begin{cases} 1, & \text{if } \lambda_0 - \tau \leq \delta_0, \\ \frac{\delta_0}{\lambda_0 - \tau}, & \text{if } \lambda_0 - \tau > \delta_0. \end{cases} \quad (3.29)$$

We define the extinction probability for purely bactericidal drugs ( $\delta_T = \tau$  and  $\lambda_T = 0$ ) as:

$$p_{E,\text{cidal}} = \begin{cases} 1, & \text{if } \lambda_0 \leq \delta_0 + \tau, \\ \frac{\delta_0 + \tau}{\lambda_0}, & \text{if } \lambda_0 > \delta_0 + \tau. \end{cases} \quad (3.30)$$

In Fig. 3.40, we plot the colony formation probability for a single bacterium plated on agar ( $p_C = 1 - p_E$ ) as a function of the treatment effect  $\tau$ , showing purely bacteriostatic (red) and purely bactericidal (blue) drugs.

### 3.8 Filamentation Model

To model bacterial filamentation we discretize the cell volumes into  $K$  classes indexed by  $i \in \{1, 2, \dots, K\}$ . Each class contains the bacterial density  $b_i(t)$  of cells with volume

$$v_i = i \epsilon, \quad (3.31)$$

where  $\epsilon$  is a unit volume increment. We define the following rules for growth, division, and death of cells

- cells in class  $i = 1$  cannot divide
- cells in class  $i = K$  cannot grow
- cells in class  $i = 1, \dots, K - 1$  shift from class  $i$  to  $i + 1$  at rate  $\gamma$
- cells in class  $i = 2, \dots, K$  divide at rate  $\lambda$ , resulting in a redistribution cells from class  $i$  into smaller classes (e.g. if  $i$  is even, two cells appear in class  $i/2$ ; if  $i$  is odd, one cell each appears in classes  $\frac{i-1}{2}$  and  $\frac{i+1}{2}$ ).

- cells in all classes die at rate  $\delta$ .

We collect the populations into a vector

$$\vec{b}(t) = (b_1(t), b_2(t), \dots, b_K(t))^T,$$

and write the dynamics as

$$\frac{d\vec{b}}{dt} = \lambda \Lambda \vec{b} + \gamma \Gamma \vec{b} - \delta \vec{b}, \quad (3.32)$$

where  $\Lambda$  and  $\Gamma$  are transition matrices for division and growth, respectively. An example form for  $\Gamma$  (volume acquisition) is

$$\Gamma = \begin{pmatrix} -1 & 0 & 0 & 0 & \cdots & 0 & 0 \\ 1 & -1 & 0 & 0 & \cdots & 0 & 0 \\ 0 & 1 & -1 & 0 & \cdots & 0 & 0 \\ \vdots & \vdots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & 0 & 0 & 0 & \cdots & 1 & 0 \end{pmatrix},$$

which shifts cells from class  $i$  to  $i+1$ . An example  $\Lambda$  (division) might be

$$\Lambda = \begin{pmatrix} 0 & 2 & 1 & 0 & \cdots & 0 \\ 0 & -1 & 1 & 0 & \cdots & 0 \\ 0 & 0 & -1 & 1 & \cdots & 0 \\ \vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & 0 & 0 & \cdots & -1 \end{pmatrix}.$$

We assume that the volume of the two new cells after division is identical to the original volume of the parent cell before division:

$$\sum_{i=1}^K i (\Lambda \vec{b})_i = 0. \quad (3.33)$$

Furthermore the volume acquisition does not impact the number of cells:

$$\sum_{i=1}^K (\Gamma \vec{b})_i = 0. \quad (3.34)$$

Population-level quantities.

We define the total bacterial density across all volume classes as

$$b(t) = \sum_{i=1}^K b_i(t), \quad (3.35)$$

and total biovolume density:

$$V(t) = \epsilon \sum_{i=1}^K i b_i(t) = \sum_{i=1}^K v_i b_i(t). \quad (3.36)$$

Then the mean cell volume is

$$v(t) = \frac{V(t)}{b(t)}. \quad (3.37)$$

In the finite model, boundary effects arise because the smallest cells cannot divide and the largest cannot grow. In the continuum limit  $\epsilon \rightarrow 0$ ,  $K \rightarrow \infty$ , these effects vanish and all cells experience uniform rates, so the following equalities hold:

$$\frac{db}{dt} = (\lambda - \delta) b, \quad (3.38)$$

$$\frac{dV}{dt} = \epsilon \gamma b - \delta V, \quad (3.39)$$

$$\frac{dv}{dt} = \epsilon \gamma - \lambda v. \quad (3.40)$$

Setting  $\frac{dv}{dt} = 0$  yields the equilibrium mean volume

$$v_{\text{eq}} = \frac{\epsilon \gamma}{\lambda}. \quad (3.41)$$

Integrating Equation 3.38 gives

$$b(t) = b_0 \exp((\lambda - \delta)t). \quad (3.42)$$

Substituting this result into Equation 3.39 and integrating with the integrating factor  $\exp(\delta t)$  yields the analytic solution for the total biovolume density accross all size classes:

$$V(t) = v_{\text{eq}} b(t) + (V_0 - v_{\text{eq}} b_0) \exp(-\delta t). \quad (3.43)$$

Finally, writing  $v_0 = V_0/b_0$ , we obtain the analytical solution for the mean cell volume:

$$v(t) = v_{\text{eq}} + (v_0 - v_{\text{eq}}) \exp(-\lambda t). \quad (3.44)$$

Parameter sensitivity.

We evaluated the impact of changes in the division rate,  $\Delta\lambda \in [-1.5, 0.5]$ , and the death rate,  $\delta \in \{0, 2, 4\}$ , on the difference between the luminescence-based rate and the true net growth rate. To this end, we set the treatment-free division rate to  $\lambda_0 = 1.5$ , the treatment-free death rate to  $\delta_0 = 0$ , the biovolume acquisition rate in the presence and absence of treatment to  $\gamma = 150h^{-1}$ , the size of a volume increment to  $\epsilon = 0.04\mu m^3$ , and volume-specific luminosity to  $\theta = 0.015$ . We simulated each parameter set for four hours using  $K = 1000$ . Furthermore we added the estimate based on luminescence if the first two hours of data are excluded.

#### Volume correction and parameter estimation

To correct the light signal for dynamic changes in biovolume, we combine two sources of information: (i) two morphology snapshots before ( $v_{\text{obs},0}$ ) and after 2 hours of treatment ( $v_{\text{obs},2h}$ ), and (ii) the luminescence time series  $I_{\text{obs}}(t_i)$ .

We first rewrite Equation 3.44 using the equilibrium-to-initial volume ratio  $\alpha = v_{\text{eq}}/v_0$  as:

$$v(t) = \alpha v_0 + (1 - \alpha) v_0 e^{-\lambda t}. \quad (3.45)$$

To avoid fitting  $\alpha$  as a free parameter, we insert  $v_{\text{obs},0}$  and  $v_{\text{obs},2h}$  into Equation 3.45 to express  $\alpha$  as a function of  $\lambda$ :

$$\alpha(\lambda) = \frac{v_{\text{obs},2h} - v_{\text{obs},0} e^{-2\lambda}}{v_{\text{obs},0}(1 - e^{-2\lambda})}, \quad \alpha > 0. \quad (3.46)$$

Assuming constant volume-specific luminosity, we insert this constrained  $\alpha$  into Equation 3.11 to obtain:

$$I(t) = I_0 e^{(\lambda-\delta)t} \left[ \alpha + (1 - \alpha) e^{-\lambda t} \right], \quad (3.47)$$

with scale parameter  $I_0 = \kappa\theta v_{\text{obs},0} b_0 V_w$ .

To estimate the division rate  $\lambda$  and death rate  $\delta$ , we eliminate the nuisance parameter  $I_0$  by defining

$$F_i := e^{(\lambda-\delta)t_i} \left[ \alpha + (1 - \alpha) e^{-\lambda t_i} \right], \quad (3.48)$$

so that

$$\ln I(t_i) = \ln I_0 + \ln F_i. \quad (3.49)$$

Minimizing the residual sum of squares over  $\ln I_0$  yields the optimal

$$\ln I_0^* = \overline{\ln I_{\text{obs}}} - \overline{\ln F}, \quad (3.50)$$

where the overline denotes the sample mean across all  $i$ .

Minimizing the residual  $\ln I(t_i) - \ln I_{\text{obs}}(t_i)$  by substituting (3.49) and (3.50) yields the final loss function:

$$\mathcal{L}(\lambda, \delta) = \sum_{i=1}^n \left[ (\ln F_i - \overline{\ln F}) - (\ln I_{\text{obs}}(t_i) - \overline{\ln I_{\text{obs}}}) \right]^2. \quad (3.51)$$

To balance the dataset of observed specific volumes  $v$ , we sample 200 values per replicate and pool them. We then use the same bootstrapped light intensity datasets as in the main method for estimating  $\psi_I$ . For each bootstrap sample, we randomly pair one  $v_{\text{obs},0}$  and one  $v_{\text{obs},2h}$  with one luminescence trajectory and minimize (3.51) over the biologically plausible region:

$$0.01 \leq \lambda \leq 1.75, \quad \delta \geq 0.$$

The remaining quantities  $\psi = \lambda - \delta$ ,  $\alpha(\lambda)$ , and  $I_0^*$  are computed algebraically.

### 3.9 Experiments

#### SOS experiment.

We conducted this experiment to test whether activating the SOS response by UV light would increase the specific luminosity and thereby explain the shallower decline of light intensity compared to the decline in CFU counts. This hypothesis rests on the possibility that the phage promoter driving the lux cassette up-regulates when the cell experiences stress.

To test this, we diluted three replicate overnight cultures 1:100 and grew them for approximately 1.5 h to mid-exponential phase. Each of the three exponential-phase cultures was split into two aliquots: one was assigned to UV treatment and placed in the upper half of a white 96-well plate (rows B–D), while the other served as an untreated control in the lower half (rows E–G) (Greiner, 655098).

We started the experiment by measuring luminescence and OD in the plate reader. Then we alternated between exposing the strains for repeated intervals to UV light in a cross-linker (Hoefer, UVC 500 crosslinker, at  $10 \frac{\mu\text{J}}{\text{cm}^2}$ ) in a temperature-regulated environment ( $36.5^\circ\text{C}$ ); followed by luminescence and OD measurements. During UV exposure, we shielded the control samples by

covering the lower half of the plate with a metal lid. The durations of UV treatment were 30 s, 1 min, 2 min, 4 min and 8 min.

OD increased in both UV-treated and control cultures; however, UV exposure visibly impaired OD growth compared to the controls (Fig. 3.24a). We normalized the luminescence signals (Fig. 3.24b) by dividing through the OD signal, resulting in the OD-normalized light intensity  $\omega$  (Fig. 3.24c). We observed that the OD-normalized light intensity of UV-treated cultures falls with the duration of treatment compared to the OD-normalized light intensity of the controls ( $\omega_{UV} - \omega_{ctrl}$ ; see Fig. 3.24d; t-test,  $p = 3 \cdot 10^{-5}$  for the last timepoint).

Based on these results, we find it unlikely that upregulation of the promoter explains the shallower decline of light intensity compared to that of CFU count. However, we cannot exclude the possibility that this result does not hold if the SOS response is triggered by another mechanism.

#### Morphology evaluation.

We analyzed the microscopy images in several steps. First, we manually applied lower and upper thresholds to the red and green channel to enhance the visual contrast (Fig. 3.25–Fig. 3.37). Next, we used “Ilastik-1.4.0” to infer the probability that each pixel in the thresholded green channel image belonged to a bacterium. Ilastik employs a neural network trained directly on the microscopy images.

Subsequently, we used a Python script ([5]) to convert these probabilities into markers representing individual bacteria. Misidentified markers were manually excluded, e.g., if they only partially covered a bacterium or covered multiple overlapping bacteria. For each marker, we fitted a spline through its center, providing the spline length  $l_s$ . We then optimized the radius  $r_s$  by maximizing the marker area within a distance  $r_s$  from the spline while minimizing the area within  $r_s$  that did not belong to the marker.

Using these parameters, we calculated for each bacterium the length  $l_b = l_s + 2 \cdot r_s$ , width  $w_b = 2 \cdot r_s$ , and volume  $v = 2 \cdot \pi \cdot r_s^2 \cdot l_s + \frac{4}{3} \cdot \pi \cdot r_s^3$ . To create a volume distribution for each treatment, we resampled the fitted volume estimates from each image (replicate) 200 times with replacement, preserving the original sample size, and aggregated the resulting datasets. Based on these distributions, we determined whether cells were significantly filamented using the significance criterion described in the methods section of the main paper.

#### Antimicrobial peptide deactivation experiment.

To assess whether pexiganan-treated bacteria continued to die in a 1:100 diluted PBS environment, we exposed exponential-phase cultures to 16  $\frac{\mu\text{g}}{\text{mL}}$  pexiganan for 1 min. Following this treatment, 10  $\mu\text{L}$  of each culture was diluted in 990  $\mu\text{L}$  of PBS supplemented with 0, 1, 10, or 100 mM CaCl<sub>2</sub> or MgCl<sub>2</sub>. Every 45 min, we sampled from each diluted culture and plated 10  $\mu\text{L}$  aliquots using the automated plating method described in the methods section of the main paper. We observed a substantial effect of both supplements on the measured bacterial density (Fig. 3.41a, b). Increasing the supplement concentration consistently resulted in higher bacterial densities, indicating that the supplemented ions reduce bacterial killing. Most data points for the unplemented medium resulted in empty agar plates. The highest CFU count was observed for strains diluted in 100 mM MgCl<sub>2</sub> (Table 3.4).

#### Manual pexiganan time-kill curve experiment.

In this setup, we captured four time points within 5 min using CFU plus the pre-treatment bacterial density. The experiment was performed manually using the traditional CFU plating method. Round agar plates (Sarstedt, 82.1473.001) containing 25 mL of agar were used, and 100  $\mu\text{L}$  of each dilution was plated. This approach increases sensitivity by using 100  $\mu\text{L}$ , rather than 10  $\mu\text{L}$ , for plating.

Cultures were treated in a 96-deepwell plate (Greiner, 780285) by adding 100  $\mu\text{L}$  of a 10x stock to 900  $\mu\text{L}$  of exponential-phase culture. Samples were taken directly from the deepwell plate, and two simultaneous dilution series were prepared in a 96-well plate (Greiner, 655101) at each time point to halt the killing. One dilution series was prepared in pure PBS, and the other in PBS supplemented with 100 mM MgCl<sub>2</sub>.

For this experiment, we plated three dilutions (factors of 100, 1,000, and 10,000) and counted the colonies on all plates. We observed that when PBS without MgCl<sub>2</sub> was used, higher dilution factors led to higher CFU estimates (Fig. 3.42). This discrepancy diminished over time, in parallel with a weakening of the observed kill rate. In contrast, when the dilution medium was supplemented with MgCl<sub>2</sub>, we did not observe this effect.

### Supernatant experiment.

During the measured pexiganan kill curve described above, we observed a steep decline in CFU counts, followed by a nearly constant plateau. Two non-exclusive explanations may account for this observed decrease in killing: (i) the surviving bacteria are persisters or resistant to the AMP, or (ii) the AMP molecules become deactivated, leaving the supernatant without killing activity. To investigate the supernatant's remaining bactericidal effects, we conducted a multi-step experiment:

Step 1: preparation. Three overnight (O/N) cultures were diluted 1:100 in LB and incubated at 37 °C with shaking for 2 h. From each culture, we took three samples: one to measure the bacterial density before treatment, the second (1 ml) to accumulate pure bacteria for supernatant exposure, and the third 1.35 mL was reserved for supernatant production and measuring the initial kill rate.

Step 2: purification of bacteria. To purify bacteria we pelleted the previously collected 1 mL bacterial aliquots at 3000 ×g for 5 min, discarded the supernatant and stored them in the fridge.

Step 3: initial killing and supernatant production. To generate the supernatant, each 1.35 mL sample was treated with a 160  $\frac{\text{ng}}{\text{mL}}$  pexiganan stock at a 1:10 ratio, yielding a final concentration of 16  $\frac{\text{ng}}{\text{mL}}$ . After 5 min, we plated samples for CFU counts and centrifuged the remaining culture at maximum speed for 2 min to remove cellular debris. Plate counts of these treated samples revealed rapid bacterial killing (rate of CFU count change  $\psi_{\text{CFU}} \approx -46$  per hour) (Fig. 3.43, Table 3.5). We collected 1 mL of the clarified supernatant for the subsequent exposure experiment.

Step 4: supernatant killing. In that final step we dissolved the pelleted bacteria (from step 2) in the supernatant collected during step 3. After another 5 min incubation, we plated samples (dilutions 1:100, 1:1,000, and 1:10,000) to estimate bacterial density. No significant killing was observed, indicating that the supernatant alone no longer exhibited bactericidal activity—supporting explanation (ii), without rejecting (i).

Our current hypothesis is that AMP molecules bind to the surface of intact bacteria and to newly exposed targets from lysed bacteria, thereby becoming deactivated. Thus, cells that survive the initial kill phase may have an increased chance of continued survival. Possible explanations for why specific bacteria survive this phase include reduced surface area due to clumping or adhesion

to well walls, smaller cell size, and other factors that might confer protection, potentially related to the cell cycle.

Table 3.1 Drugs used in this study, their MICs, working concentrations, and stock solvents. In the MIC column, we report the highest concentration of the dilution series (numerator) and the maximum inhibiting dilution (denominator). Kanamycin ( $50 \frac{\mu\text{g}}{\text{mL}}$ ) was used as the selection marker for the lux operon.

drug	MIC [ $\frac{\mu\text{g}}{\text{mL}}$ ]	cwork [ $\frac{\mu\text{g}}{\text{mL}}$ ]	cwork [MIC]	solvent	supplier
Amoxicillin	$\frac{10}{4} = 2.50$	25	10	DMSO	Sigma, A8523
Ampicillin	$\frac{100}{128} = 0.78$	10, 2	12.8, 2.56	water	Sigma, A9518
Cefepime	$\frac{4}{256} = 0.02$	0.15	9.6	DMSO	ThermoFisher, J66237
Ceftazidime	$\frac{4}{64} = 0.06$	0.62	10	DMSO	Sigma, PHR1847
Cefuroxime	$\frac{4}{2} = 2.00$	16	8	water	Sigma, C4417
Chloramphenic	$\frac{128}{64} = 2.00$	20	10	DMSO	Sigma, C0378
Ciprofloxacin	$\frac{1}{128} = 0.01$	0.08	10	water	Sigma, 17850
Colistin	$\frac{50}{64} = 0.78$	1.6	2.05	water	Sigma, C4461
Doripenem	$\frac{4}{256} = 0.02$	0.16	10.24	water	VWR, ACRO463870010
Fosfomycin	$\frac{4}{4} = 1.00$	8	8	water	VWR, APOS-BIM0107
Imipenem	$\frac{5}{64} = 0.08$	1	12.8	water	Sigma, PHR1796
Mecilinam	$\frac{10}{128} = 0.08$	0.78	10	DMSO	Sigma, 33447
Meropenem	$\frac{5}{512} = 0.01$	0.1	10.24	water	Sigma, PHR1772
Penicillin	$\frac{500}{32} = 15.62$	100	6.4	water	Roth, HP48.2
Pexiganan	$\frac{64}{32} = 2.00$	8, 16	4, 8	water	Sigma, SML3787
Piperacillin	$\frac{10}{16} = 0.62$	6.25	10	DMSO	Sigma, J66419
Polymyxin B	$\frac{50}{32} = 1.56$	2.5	1.6	water	Roth, 0235.1
Rifampicin	$\frac{40}{16} = 2.50$	25	10	DMSO	Sigma, R3501
Tetracycline	$\frac{10}{32} = 0.31$	3.12	10	DMSO	Sigma, T3383
Trimethoprim	$\frac{10}{128} = 0.08$	0.78	10	DMSO	Sigma, T7883

Table 3.2 Point estimates and 95% percentile intervals of  $\psi_{CFU}$ ,  $\psi_I$ ,  $\psi_I^*$ , and  $\psi_J$  for different treatments.  $Sig_x$  indicates whether the rate of change of signal  $X \in \{I, I^*, J\}$  differs significantly (\*) from the distribution of  $\psi_{CFU}$ , or not (n.s.), based on the significance criterion defined in the Methods section. Estimates are based on data from the CFU-luminescence assays (see Methods).

	$\psi_{CFU} [\frac{1}{h}]$	$\psi_I [\frac{1}{h}]$	$sig_I$	$\psi_I^* [\frac{1}{h}]$	$sig_I^*$	$\psi_J [\frac{1}{h}]$	$sig_J$
ampicillin 10 $\frac{\mu g}{ml}$	-3.23 [-4.11, -2.54]	-2.41 [-2.91, -2.11]	*			-2.63 [-3.31, -2.29]	n.s.
ampicillin 2 $\frac{\mu g}{ml}$	-0.81 [-1.19, -0.37]	-0.30 [-0.41, -0.22]	*				
amoxicillin 25 $\frac{\mu g}{ml}$	-2.15 [-2.42, -1.88]	-2.04 [-2.21, -1.86]	n.s.				
cefepime 0.15 $\frac{\mu g}{ml}$	-1.17 [-1.50, -0.85]	-0.47 [-0.74, -0.24]	*	-0.88 [-1.13, -0.65]	*		
ceftazidime 0.62 $\frac{\mu g}{ml}$	-0.88 [-1.06, -0.71]	-0.09 [-0.24, 0.04]	*			-0.38 [-0.52, -0.15]	
cefuroxime 16 $\frac{\mu g}{ml}$	-1.59 [-2.41, -0.99]	-1.41 [-1.64, -1.25]	n.s.	-1.62 [-1.92, -1.34]	n.s.		
chloramphenicol 20 $\frac{\mu g}{ml}$	0.05 [-0.11, 0.20]	0.04 [-0.03, 0.10]	n.s.				
ciprofloxacin 0.078 $\frac{\mu g}{ml}$	-1.96 [-2.29, -1.64]	0.83 [0.44, 1.17]	*			0.48 [0.09, 0.90]	
colistin 1.6 $\frac{\mu g}{ml}$	-1.00 [-1.45, -0.52]	-0.93 [-1.26, -0.66]	n.s.				
doripenem 0.16 $\frac{\mu g}{ml}$	-0.54 [-0.83, -0.25]	-0.24 [-0.37, -0.15]	*	-0.37 [-0.53, -0.26]	*		
fosfomycin 8 $\frac{\mu g}{ml}$	-1.22 [-1.65, -0.83]	-1.12 [-1.37, -0.93]	n.s.				
imipenem 1 $\frac{\mu g}{ml}$	-1.42 [-2.06, -0.72]	0.00 [-0.16, 0.14]	*	-0.32 [-0.45, -0.22]	*		
mecillinam 0.78 $\frac{\mu g}{ml}$	-0.32 [-0.57, -0.10]	0.02 [-0.17, 0.17]	*	-0.34 [-0.47, -0.20]	n.s.		
meropenem 0.1 $\frac{\mu g}{ml}$	-1.85 [-2.36, -1.32]	-0.16 [-0.47, 0.10]	*			-0.55 [-0.80, -0.21]	
penicillin 100 $\frac{\mu g}{ml}$	-2.17 [-2.78, -1.66]	-2.20 [-2.53, -1.94]	n.s.				
piperacillin 6.25 $\frac{\mu g}{ml}$	-0.26 [-0.51, 0.02]	0.09 [-0.01, 0.19]	*				
polymyxinB 2.5 $\frac{\mu g}{ml}$	-1.45 [-1.73, -1.22]	-1.41 [-1.87, -1.07]	n.s.				
rifampicin 25 $\frac{\mu g}{ml}$	-0.23 [-0.40, 0.04]	-0.15 [-0.23, -0.07]	n.s.				
tetracycline 3.125 $\frac{\mu g}{ml}$	-0.06 [-0.12, 0.00]	-0.01 [-0.07, 0.05]	n.s.				
trimethoprim 0.78 $\frac{\mu g}{ml}$	-0.61 [-0.76, -0.47]	0.48 [0.38, 0.59]	*			0.34 [0.18, 0.51]	
pexiganan 8 $\frac{\mu g}{ml}$	-44.59 [-66.19, -20.08]	-45.95 [-48.21, -43.34]	n.s.				
pexiganan 16 $\frac{\mu g}{ml}$	-61.63 [-93.40, 1.26]	-60.27 [-63.06, -56.42]	n.s.				

Table 3.3 Bootstrapped 95% confidence intervals and point estimates for the length, width, and volume of cells after 2 h of treatment, estimated from microscopy images. Significance was assessed by comparing the confidence intervals of cell volumes for each antibiotic treatment to the untreated control (**control\_2h**), as described in the Methods section of the main paper.

	L_lower	L_upper	L_mean	W_lower	W_upper	W_mean	V_lower	V_upper	V_mean
control_2h	2.28	4.92	3.41	0.77	1.4	1.09	2	6.51	3.89
amoxicillin	2.64	10.77	5.09	0.47	1.37	1.17	1.93	9.67	6.21
ampicillin	3.87	27.14	12.36	0.93	1.67	1.27	5.7	30.39	16.67
ceftazidime	32.05	68.69	54.54	0.67	1.22	0.95	11.52	80.86	40.42
ciprofloxacin	10.88	36.82	21.77	0.7	1.27	1.03	6.35	45.7	19.55
colistin	2.19	4.53	3.14	0.6	1.59	1.07	1	8.32	3.71
fosfomycin	1.77	5.32	3.18	0.69	1.15	0.93	1.09	4.75	2.65
meropenem	3.44	10.61	5.9	1.13	3.71	2.26	5.33	71.01	31.11
rifampicin	2.41	8.38	4.77	0.59	1.4	0.98	1.15	9.44	4.32
tetracycline	2.43	7.7	4.71	0.65	1.71	1.08	1.29	13.4	5.25
trimethoprim	4.67	30.74	12.19	0.65	1.29	0.93	2.64	19.79	9.05

Table 3.4 Estimated group means and 95% confidence intervals from an ordinary least squares (OLS) model fitted to log-transformed CFU data, collected from the first sampled time point after diluting pexiganan-treated strains in supplemented PBS. Grouping is based on the supplement (CaCl<sub>2</sub> or MgCl<sub>2</sub>) and concentration (0 mM to 100 mM). Confidence intervals were computed using heteroscedasticity-consistent standard errors (HC3). The compact letter display (cld) indicates groups that are not significantly different by sharing a common letter, based on mutual inclusion of their 95% confidence intervals.

group	mean	ci_lower	ci_upper	cld
CaCl <sub>2</sub> (0 mM)	-0.00	-0.00	0.00	a
CaCl <sub>2</sub> (1 mM)	2.77	-0.56	6.09	b
CaCl <sub>2</sub> (10 mM)	5.06	4.92	5.20	c
CaCl <sub>2</sub> (100 mM)	5.85	5.38	6.31	d
MgCl <sub>2</sub> (0 mM)	-0.00	-0.00	0.00	a
MgCl <sub>2</sub> (1 mM)	2.77	-0.56	6.09	b
MgCl <sub>2</sub> (10 mM)	5.31	5.20	5.42	e
MgCl <sub>2</sub> (100 mM)	6.36	6.27	6.46	f

Table 3.5 Comparison of kill rates [ $h^{-1}$ ] between cultures treated with pexiganan ( $16 \frac{\mu\text{g}}{\text{mL}}$ ) for 5 minutes and cultures exposed to the supernatant collected after the kill assay. The two rates differ significantly; the confidence interval of the rate of change of CFU in the supernatant includes zero.

experiment	mean	lower	upper
pexiganan	-46.980603	-63.153906	-35.797836
supernatant	1.281551	-2.187859	5.111196

Table 3.6 Primer sequences used for  $\lambda$ -red mediated integration of the luxCDABE operon into E. coli. Lowercase letters indicate homology regions binding to the lux operon on the plasmid; uppercase letters indicate chromosomal homology regions at the integration site.

Primer name	Sequence (5' → 3')
forward primer	CGGTACGGCTGACCATCGGGTGCAGTGCAGGAGTTTCGTAccaggtaaggcagcggtatc
reverse primer	AGTCAGCGATATCCATTTCGCGAATCCGGAGTGTAAGAAtaggtctagggccggcggaa

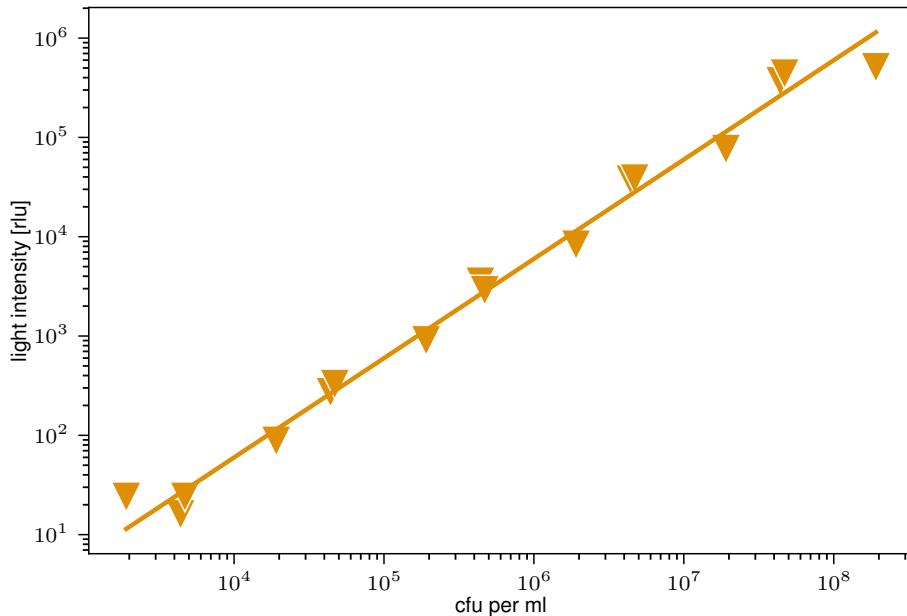


Fig. 3.4 Light intensity scales linearly with bacterial density. Serial tenfold dilutions of bacterial cultures were prepared in a 384-well white microplate, and luminescence was measured immediately. Linear regression of the luminescence signal against bacterial density (CFU) yielded a conversion factor of  $m_{\text{fit}} = 0.006 \text{ rlu} \cdot \text{ml} \cdot \text{CFU}^{-1}$ . The high correlation ( $R^2 = 0.987$  in log-log space) confirms a linear relationship between luminescence and bacterial density.

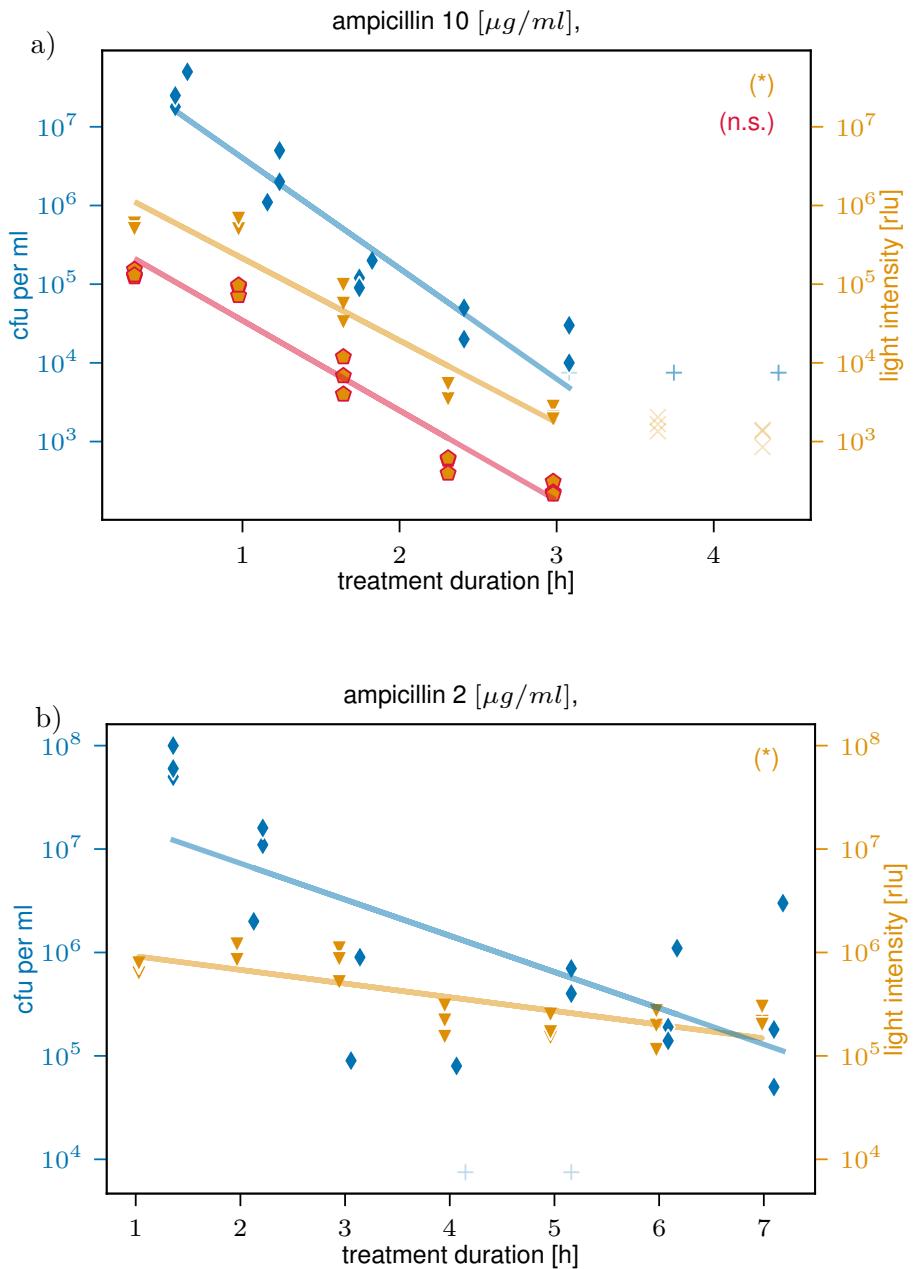


Fig. 3.5 Ampicillin. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. The morphology-corrected luminescence signal is shown as orange pentagons with red frames, and the corresponding rate fit indicated by a red line. ‘+’ indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data), and If a signal ends early (due to dropping below its detection limit), the corresponding data point of the other signal was cut to the same endpoint for a consistent comparison. These excluded data points are marked as ‘X’.

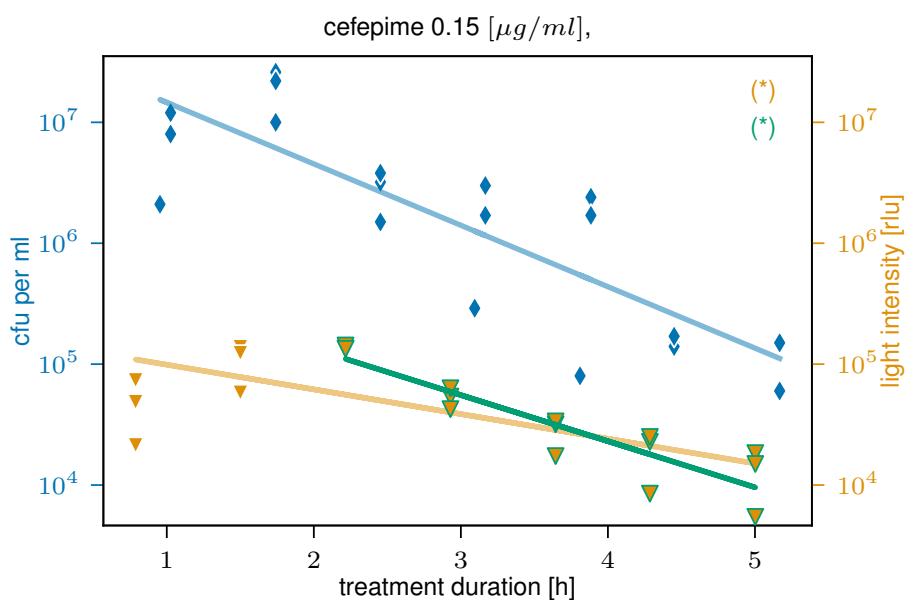


Fig. 3.6 Cefepime. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. Green-framed data points and corresponding green fit lines indicate analyses excluding early data points until the first peak.

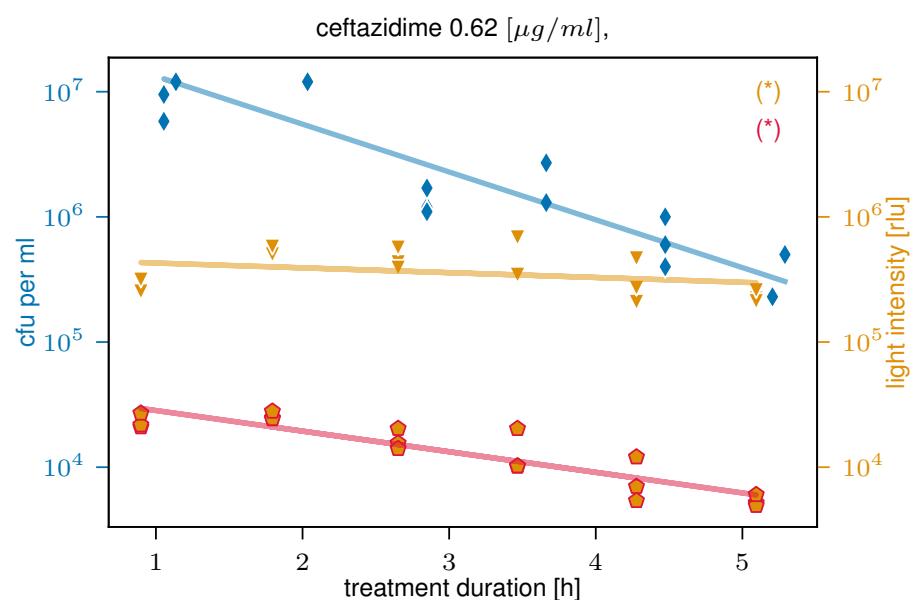


Fig. 3.7 Ceftazidime. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. The morphology-corrected luminescence signal is shown as orange pentagons with red frames, and the corresponding rate fit indicated by a red line.

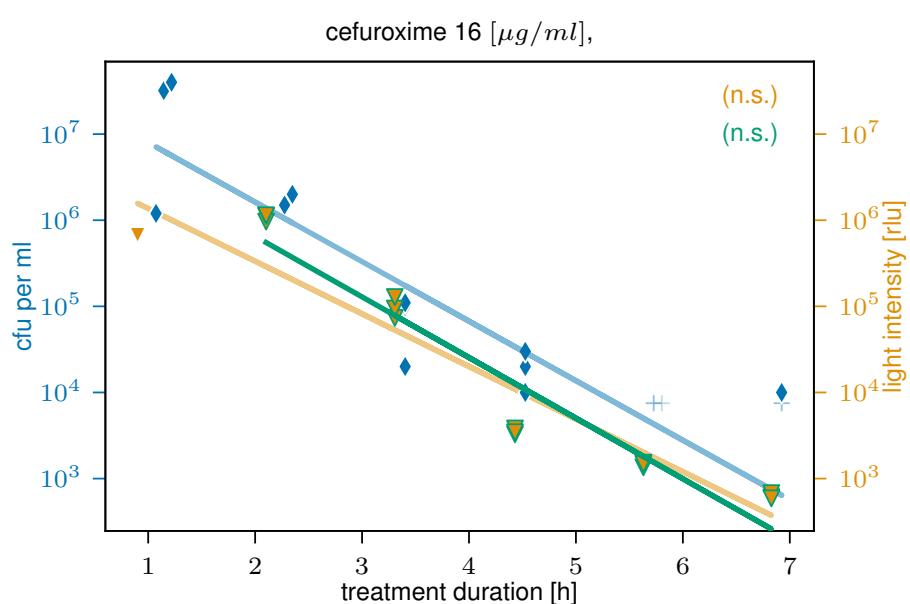


Fig. 3.8 Cefuroxime. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. ‘+’ indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data).

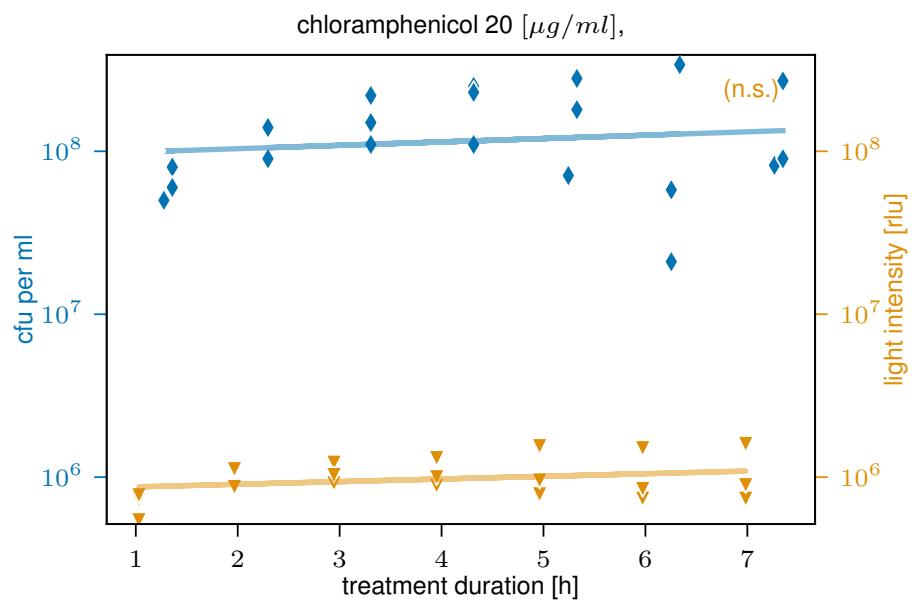


Fig. 3.9 Chloramphenicol. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals.

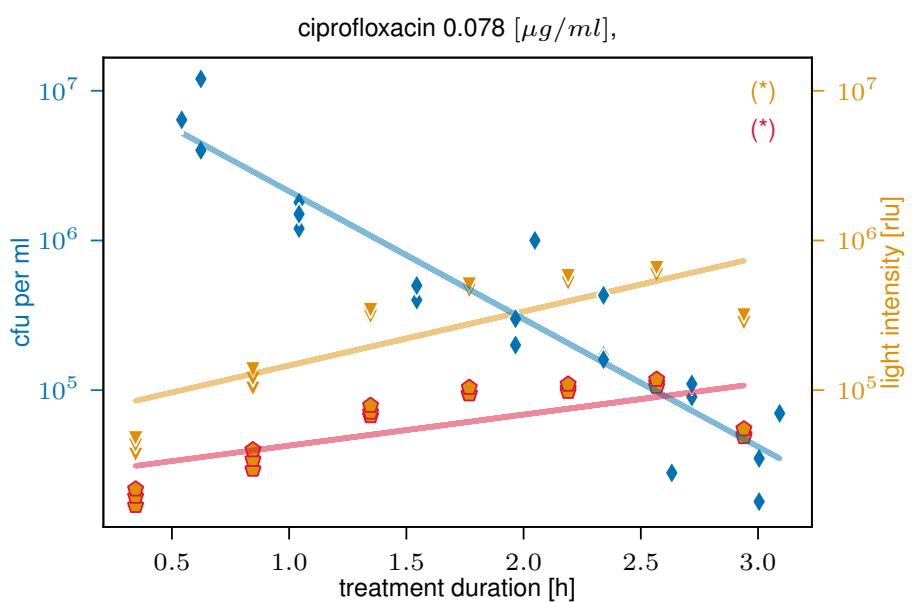


Fig. 3.10 Ciprofloxacin. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. The morphology-corrected luminescence signal is shown as orange pentagons with red frames, and the corresponding rate fit indicated by a red line.

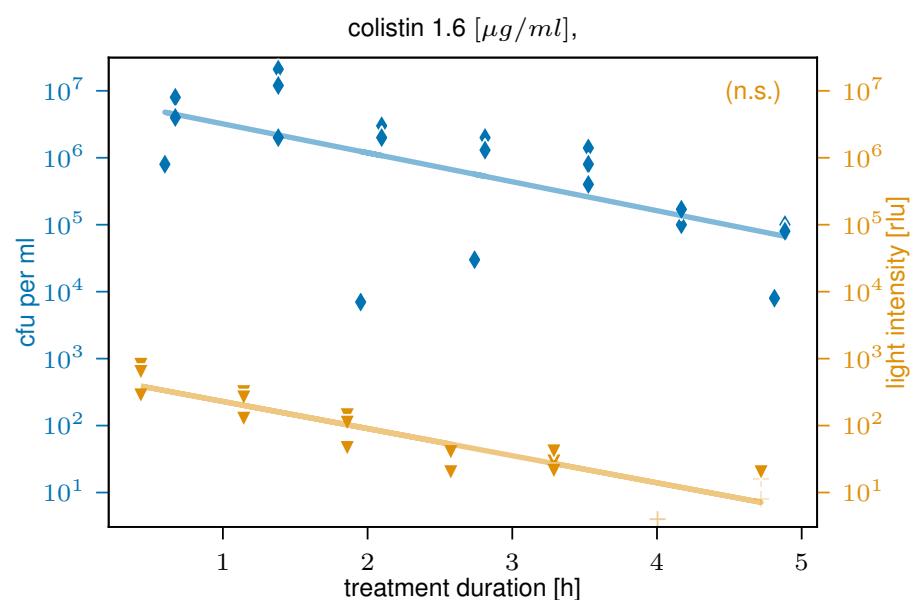


Fig. 3.11 Colistin. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. ‘+’ indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data)

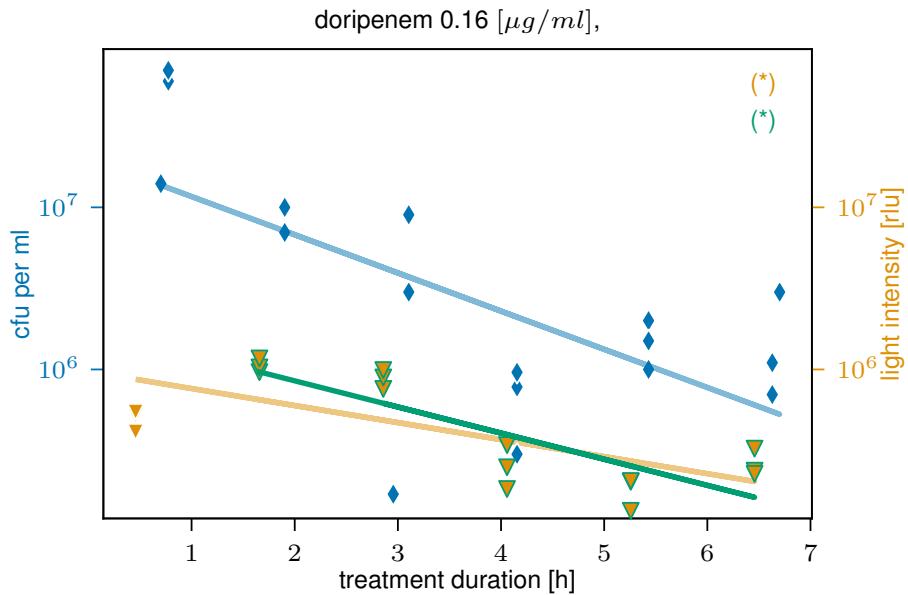


Fig. 3.12 Doripenem. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. Green-framed data points and corresponding green fit lines indicate analyses excluding early data points until the first peak.

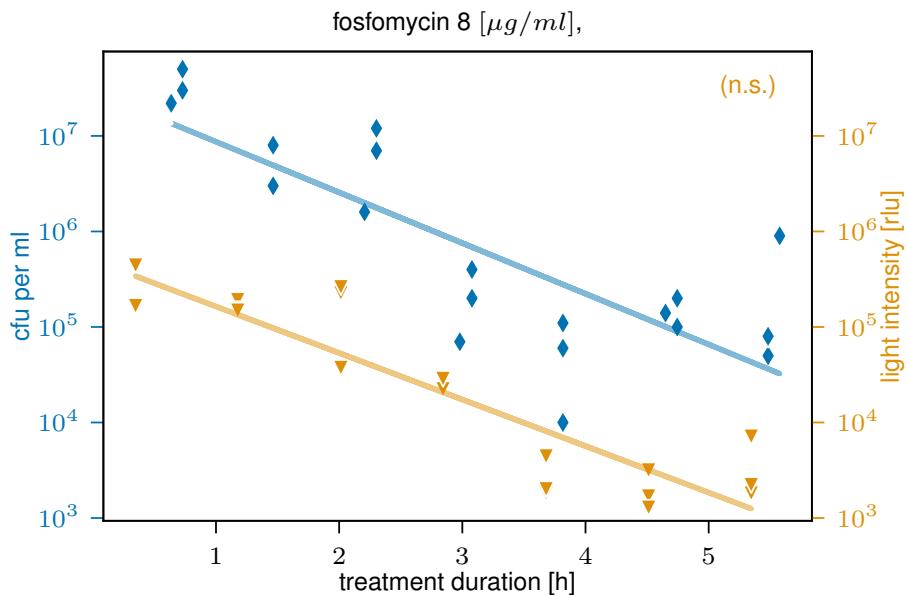


Fig. 3.13 Fosfomycin. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals.

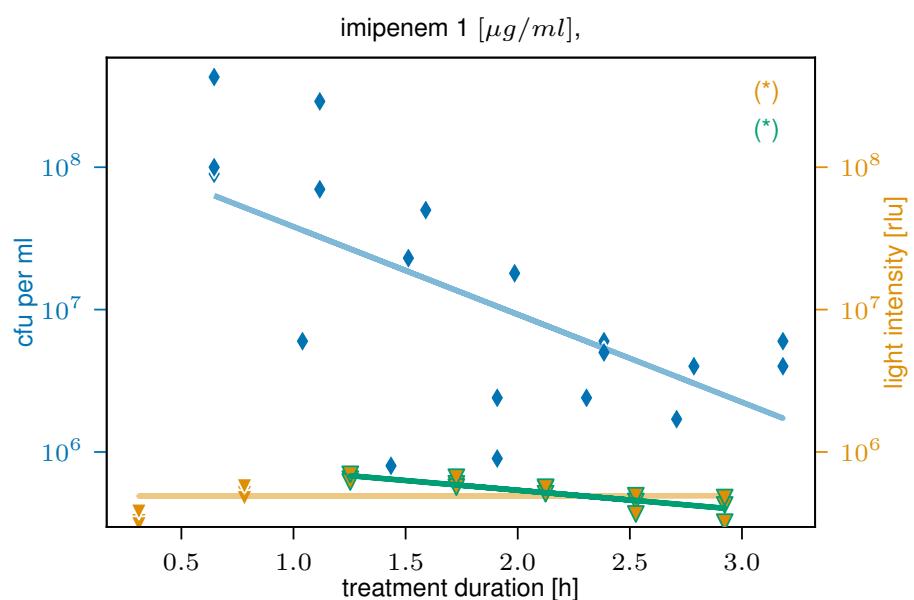


Fig. 3.14 Imipenem. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. '+' indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data).

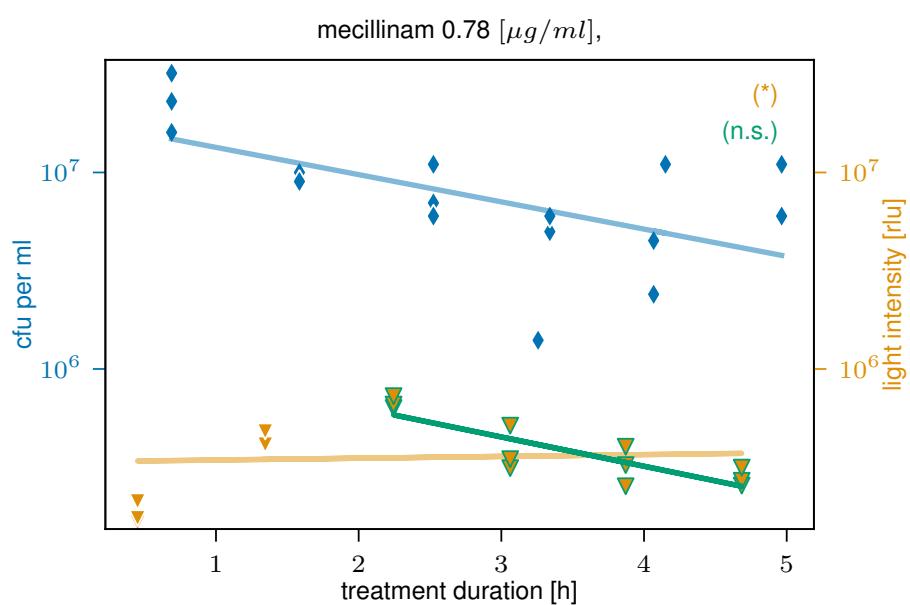


Fig. 3.15 Mecillinam. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals.

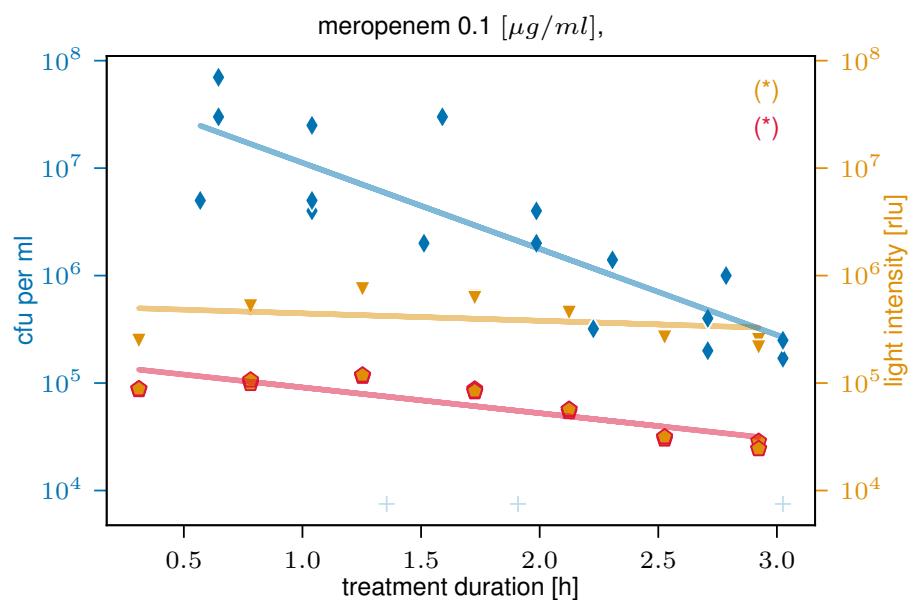


Fig. 3.16 Meropenem. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. The morphology-corrected luminescence signal is shown as orange pentagons with red frames, and the corresponding rate fit indicated by a red line. ‘+’ indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data).

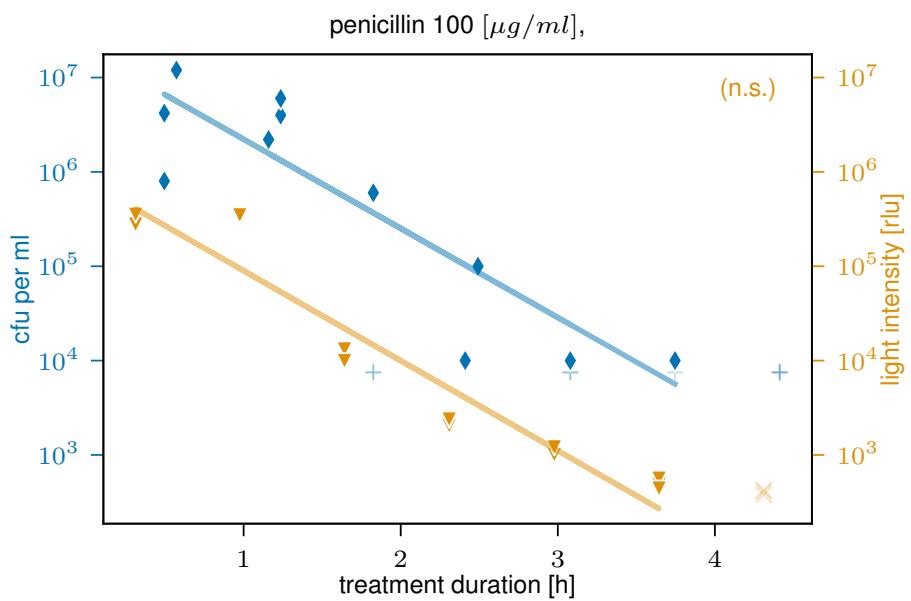


Fig. 3.17 Penicillin. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. ‘+’ indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data), and If a signal ends early (due to dropping below its detection limit), the corresponding data point of the other signal was cut to the same endpoint for a consistent comparison. These excluded data points are marked as ‘X’.

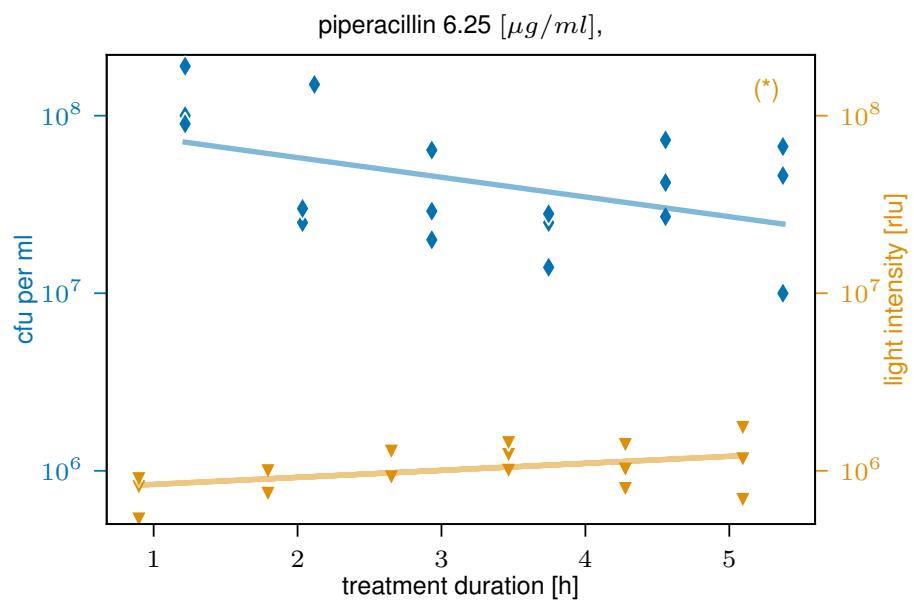


Fig. 3.18 Piperacillin. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals.

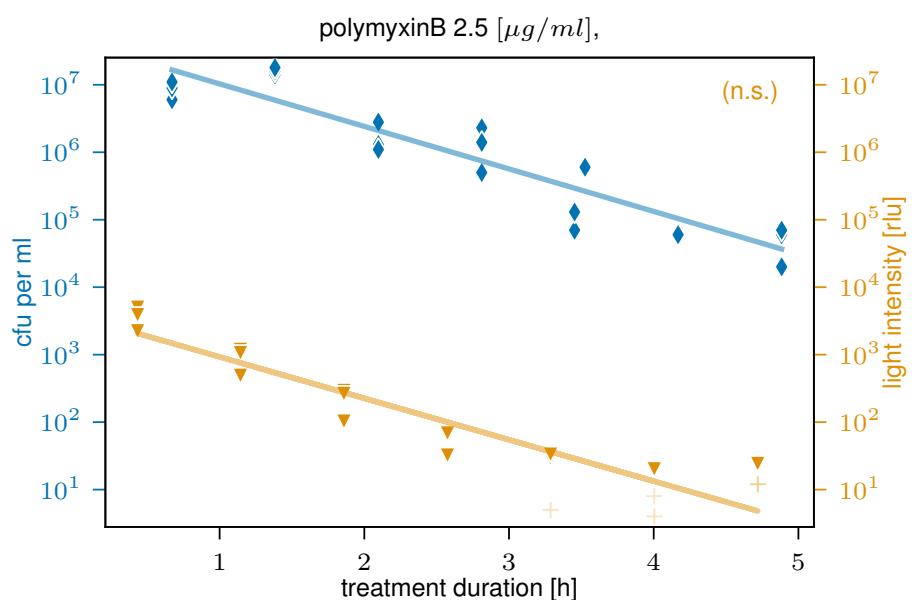


Fig. 3.19 Polymyxin B. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. ‘+’ indicates data points below detection limit or otherwise excluded (for CFU, symbolically plotted at  $10^4$  CFU/mL to visualize missing data).

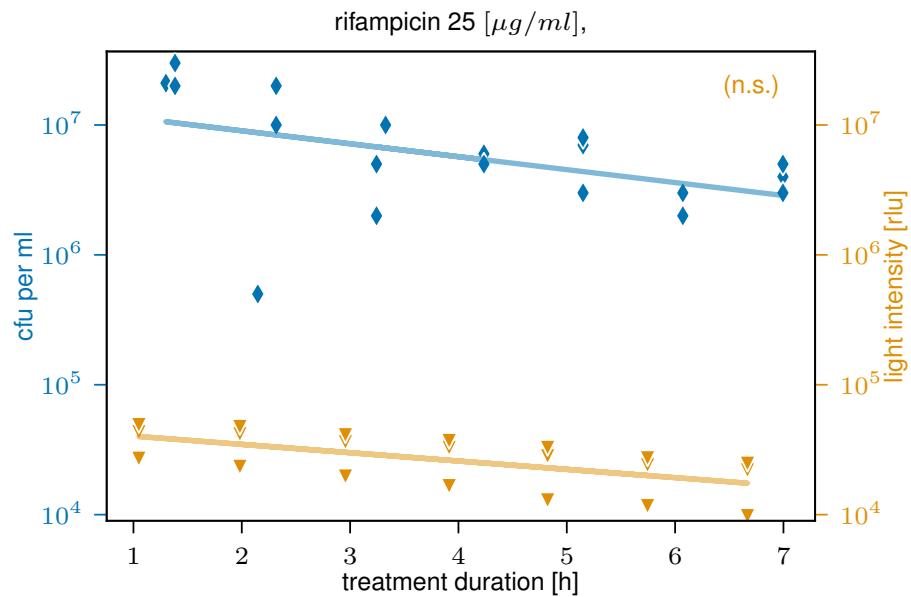


Fig. 3.20 Rifampicin (25  $\mu\text{g}/\text{mL}$ ). The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals.

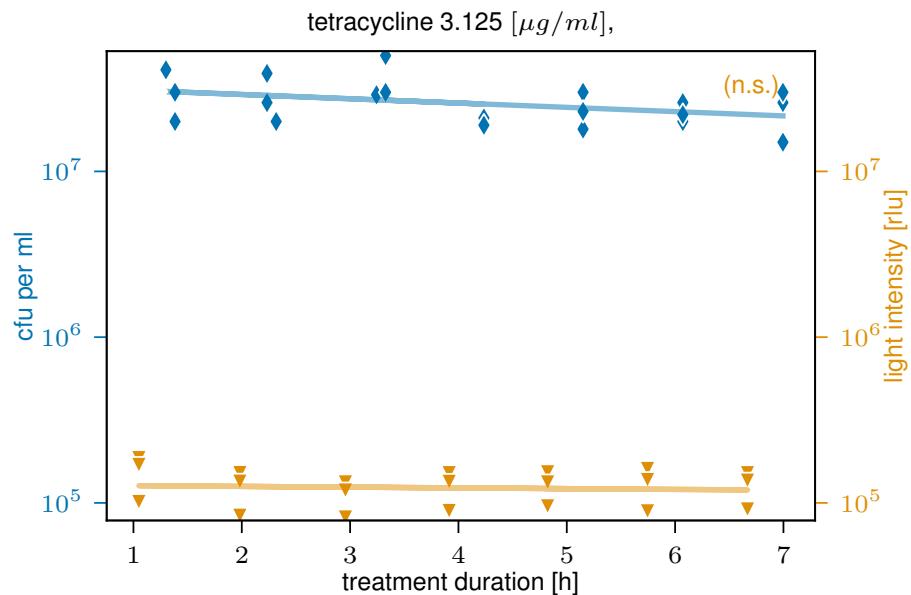


Fig. 3.21 Tetracycline. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals.

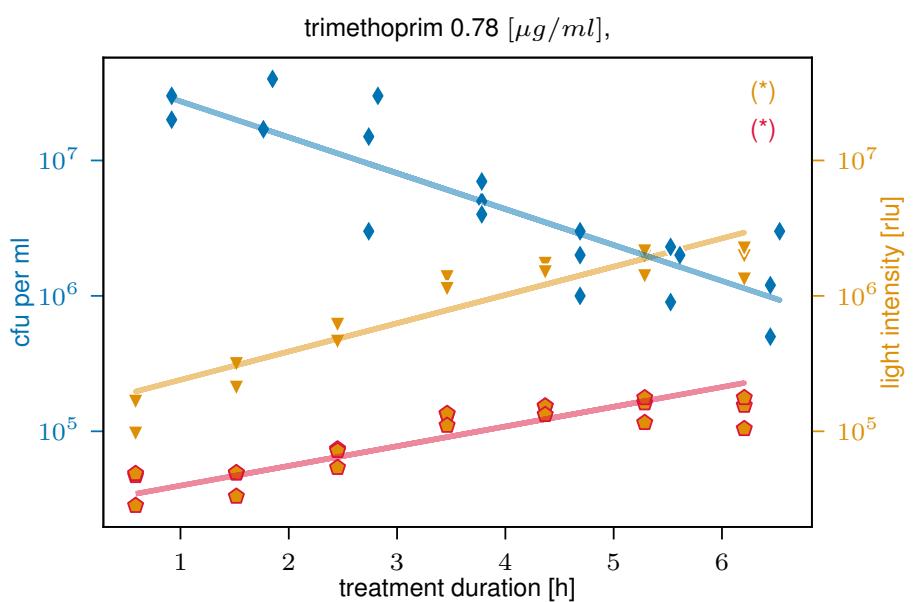


Fig. 3.22 Trimethoprim. The CFU signal is shown as blue diamonds and the light intensity as orange triangles for data points above their respective detection limits ( $10^4$  CFU/mL for CFU and 20 rlu for luminescence). Lines represent log-linear fits to the corresponding signals. The morphology-corrected luminescence signal is shown as orange pentagons with red frames, and the corresponding rate fit indicated by a red line.

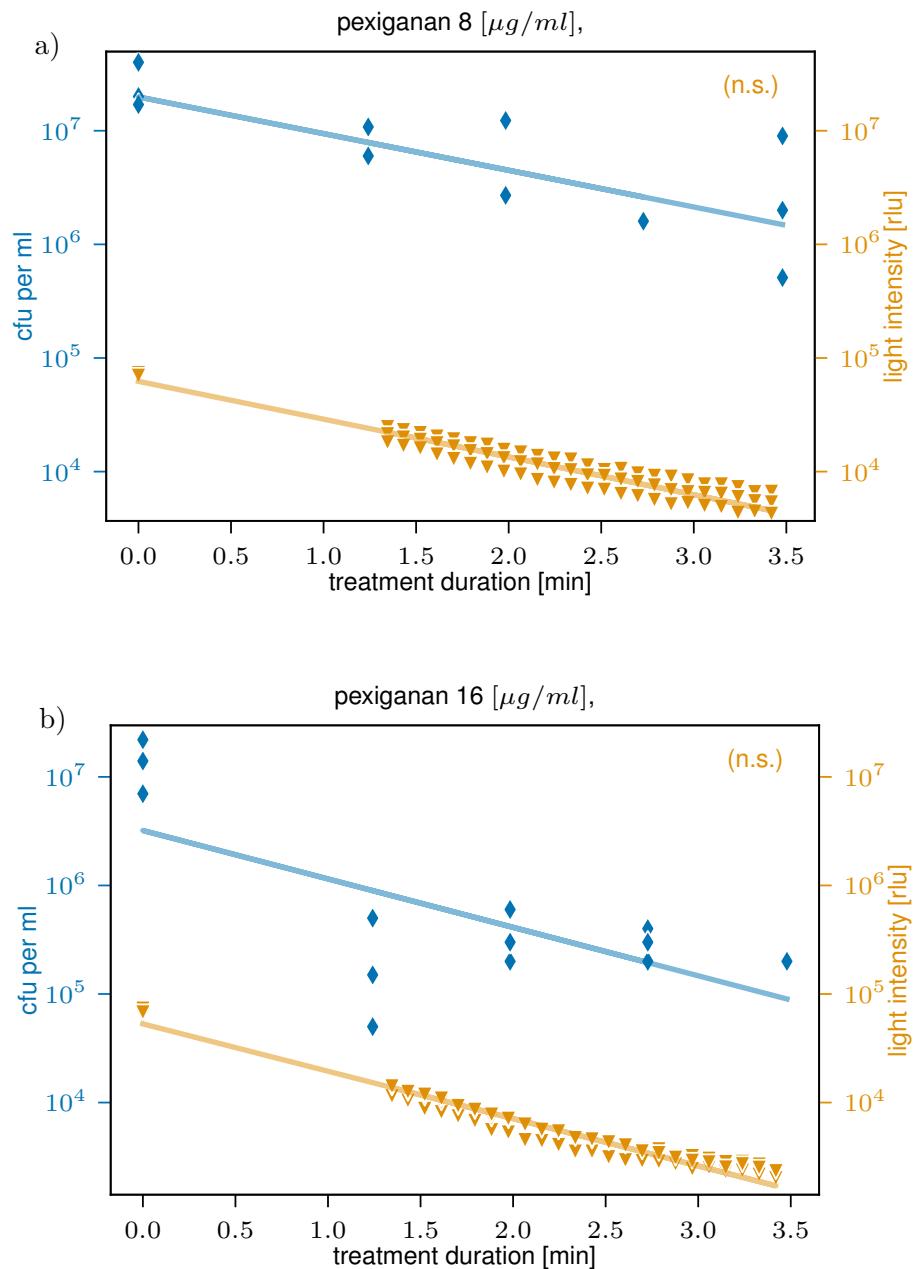


Fig. 3.23 Comparison of the CFU and luminescence signals from the pexiganan kill curve experiment using the liquid handling platform. The first data points at  $t_0$  represent the pretreatment CFU and light intensity values.

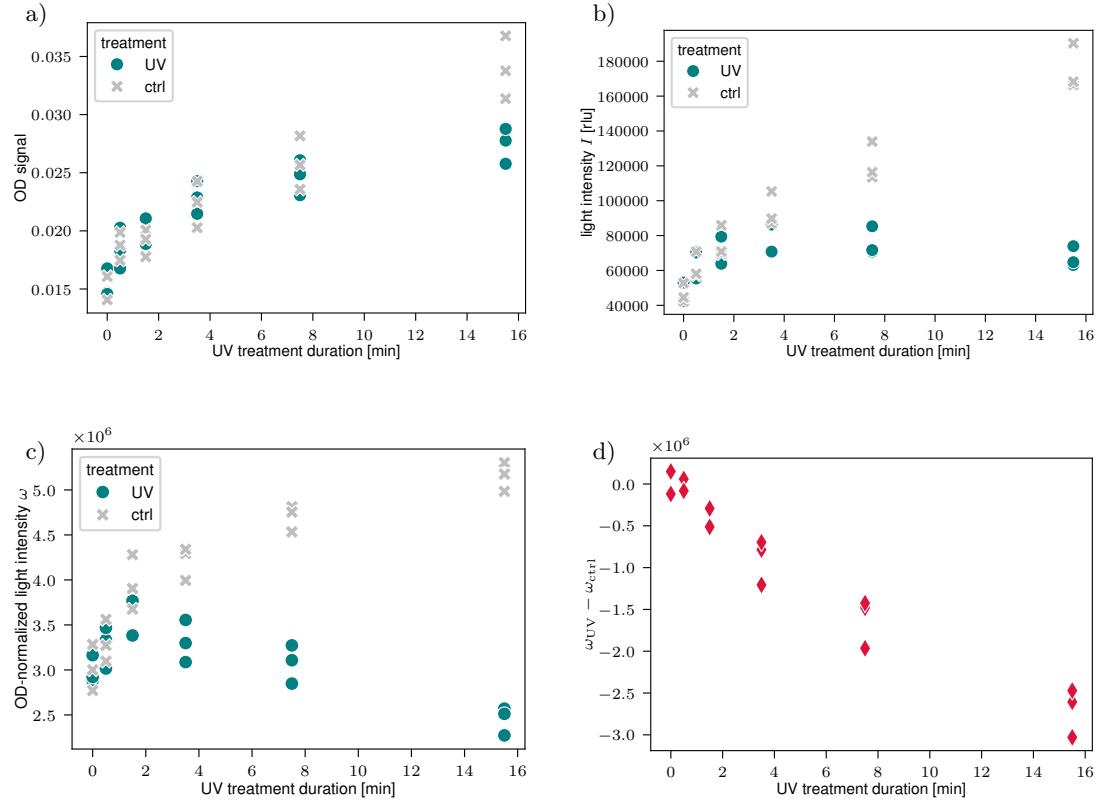


Fig. 3.24 Panel plot showing the effects of UV treatment on bacterial density (approximated by OD) and light intensity ( $I$ ) over time by comparing treated (UV) and untreated (ctrl) cultures. (a) Optical density (OD), (b) light intensity, (c) OD-specific light intensity  $\omega = I(t)/OD(t)$ , and (d) the difference in OD-specific light intensity between UV-treated and control.

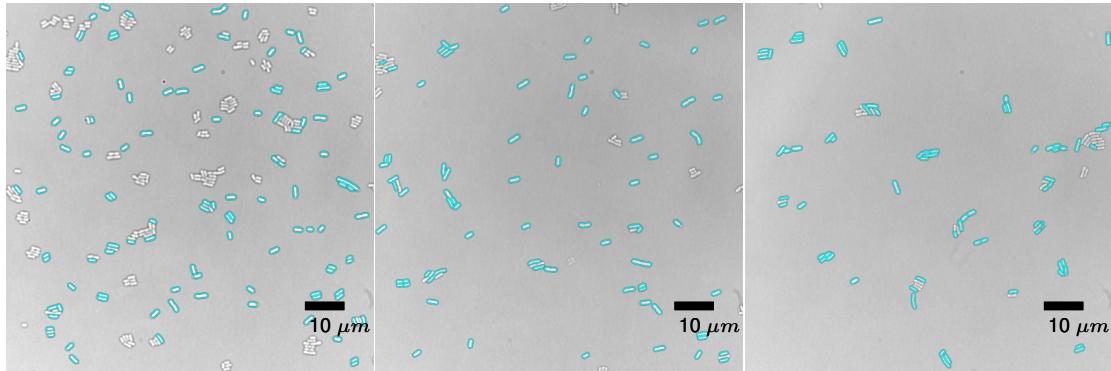


Fig. 3.25 Microscopy images of control (before treatment) samples. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

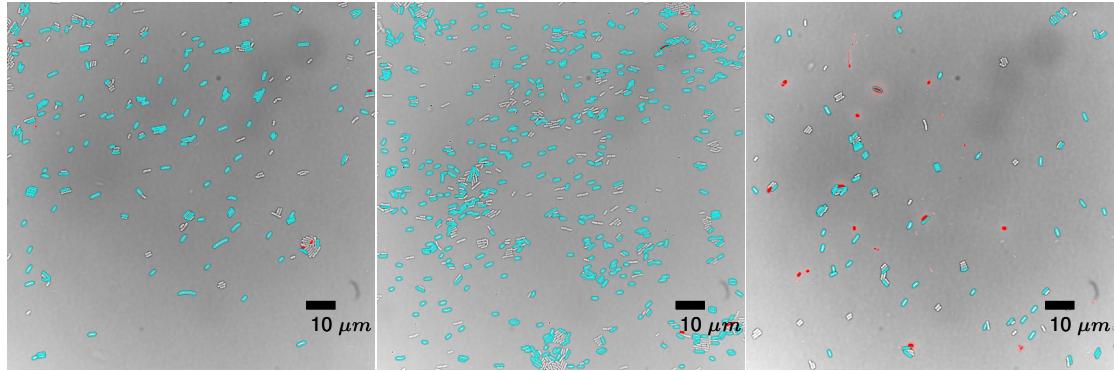


Fig. 3.26 Microscopy images of control (after 2 hours) samples. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

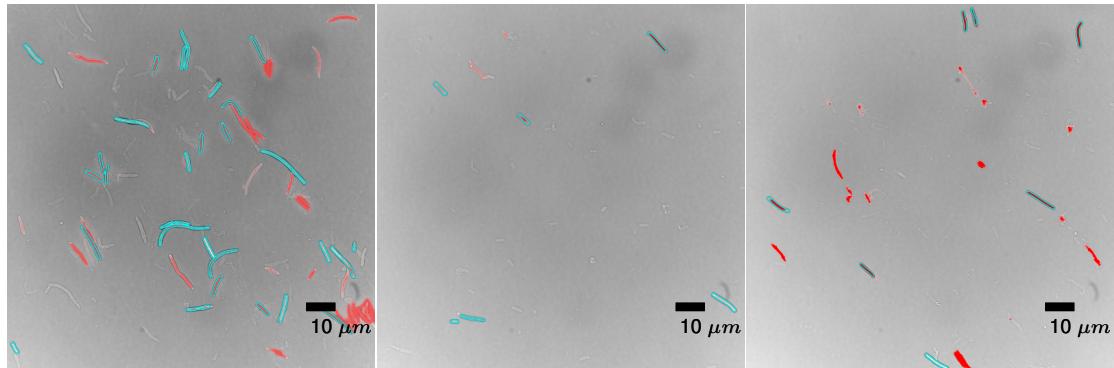


Fig. 3.27 Microscopy images of cells treated with ampicillin. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

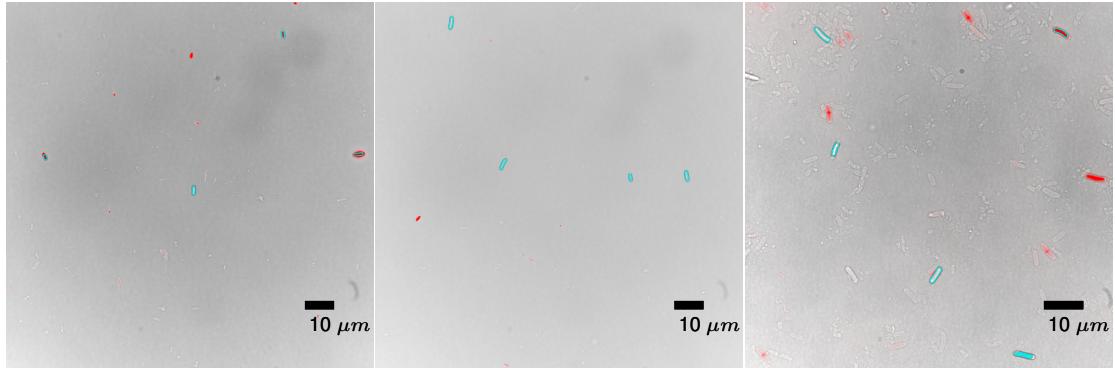


Fig. 3.28 Microscopy images of cells treated with amoxicillin. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

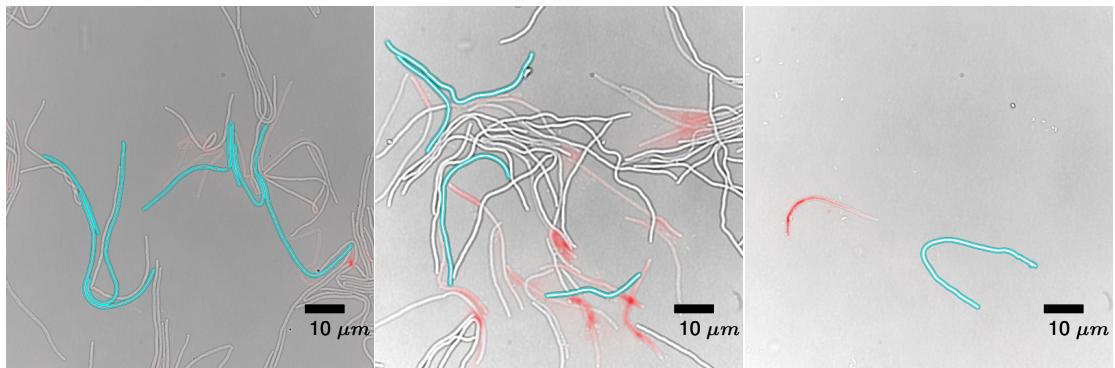


Fig. 3.29 Microscopy images of cells treated with ceftazidime. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

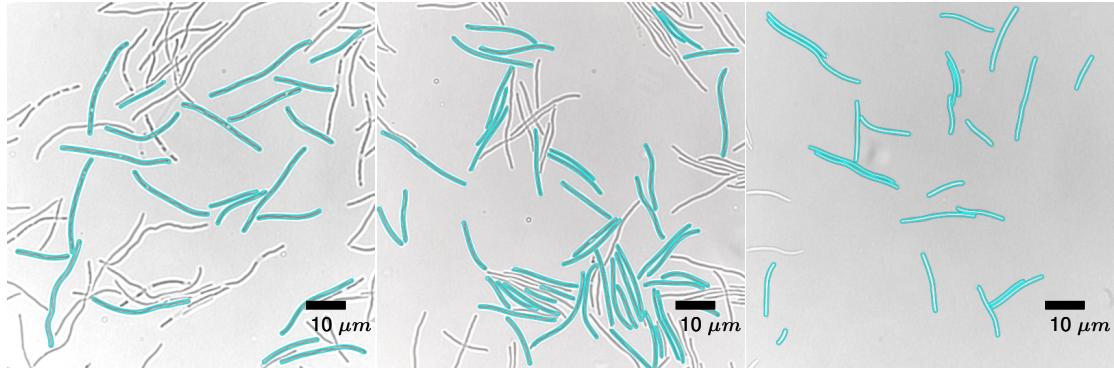


Fig. 3.30 Microscopy images of cells treated with ciprofloxacin. Each image represents a different replicate. The white gaps can indicate the start of cell division (cells are not fixed). We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

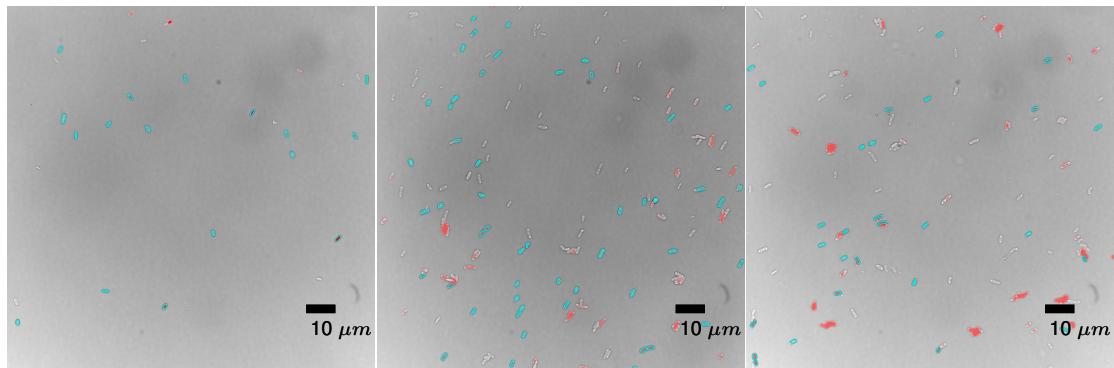


Fig. 3.31 Microscopy images of cells treated with colistin. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

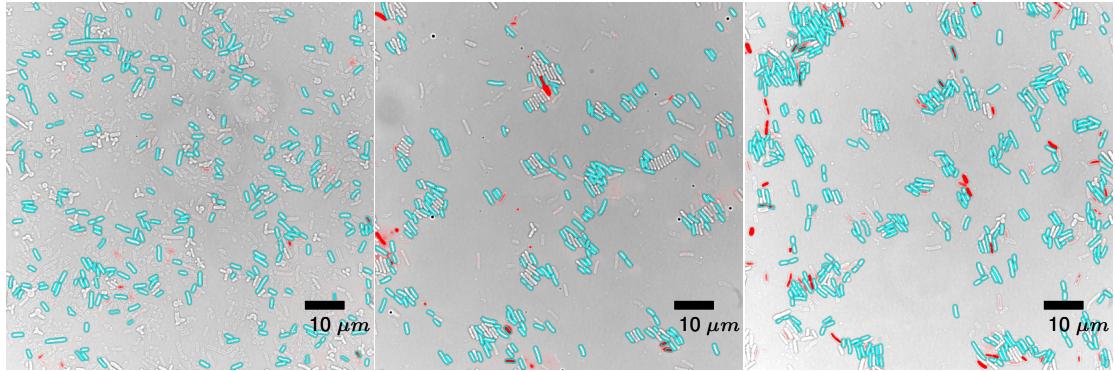


Fig. 3.32 Microscopy images of cells treated with fosfomycin. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

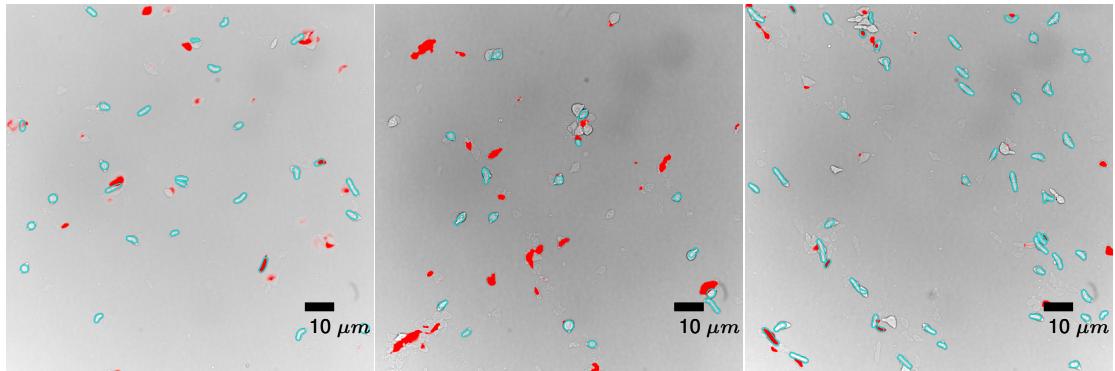


Fig. 3.33 Microscopy images of cells treated with meropenem. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

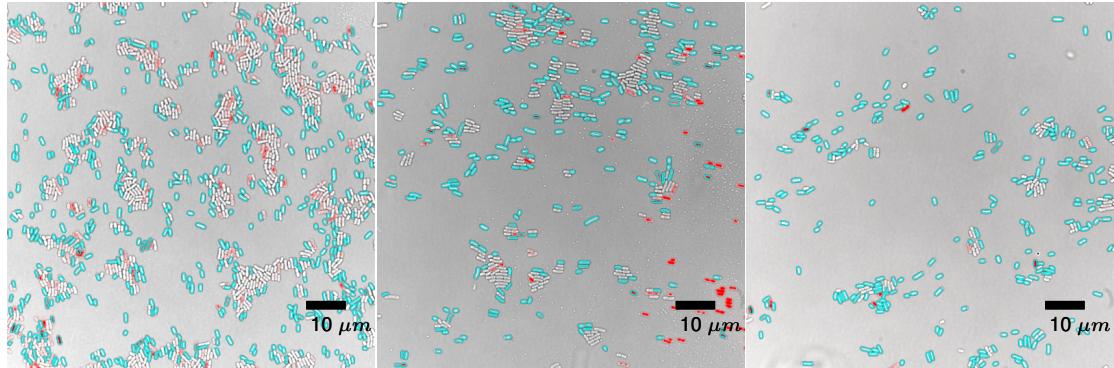


Fig. 3.34 Microscopy images of cells treated with pexiganan. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

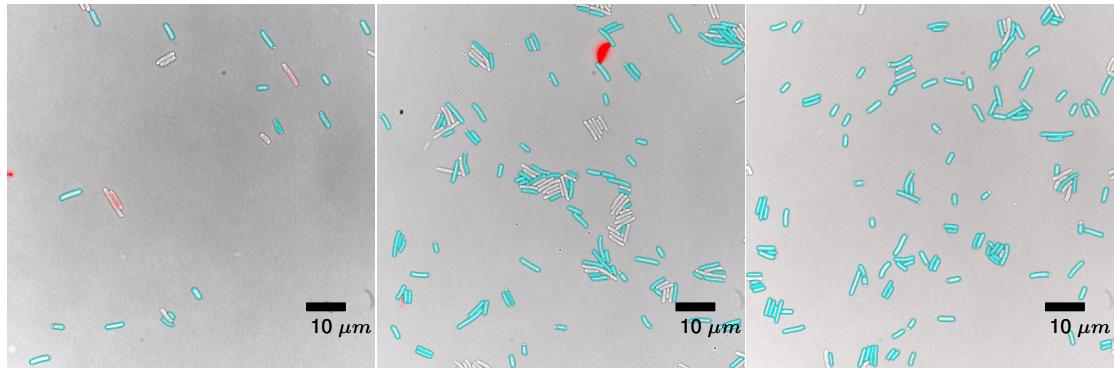


Fig. 3.35 Microscopy images of cells treated with rifampicin. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

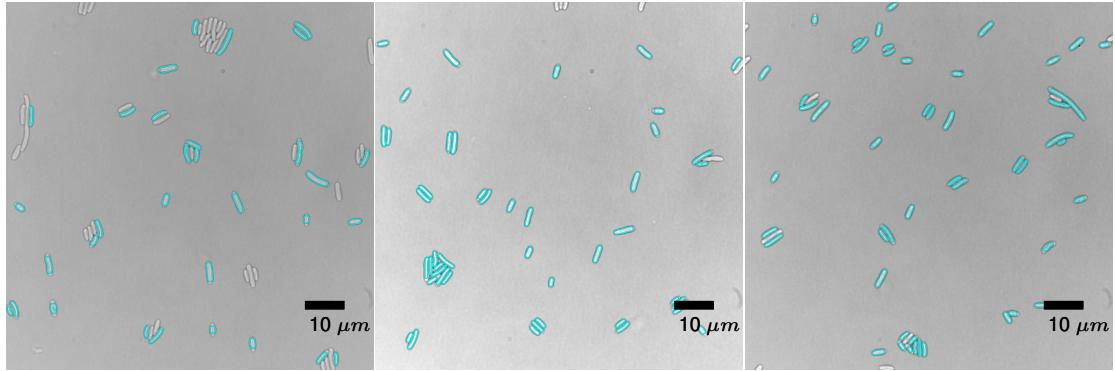


Fig. 3.36 Microscopy images of cells treated with tetracycline. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

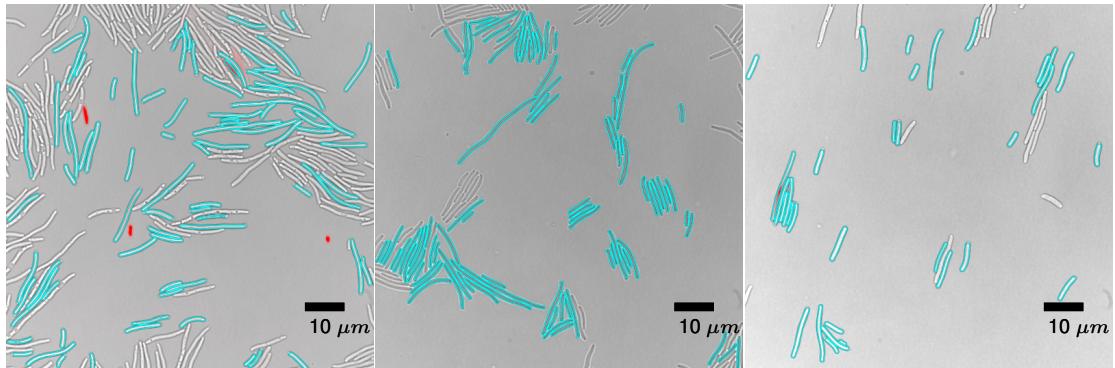


Fig. 3.37 Microscopy images of cells treated with trimethoprim. Each image represents a different replicate. We plotted the green channel of the recorded images in greyscale. The red channel, capturing propidium iodide activity, is overlaid in red. Bacterial shapes detected by the algorithm are outlined in cyan.

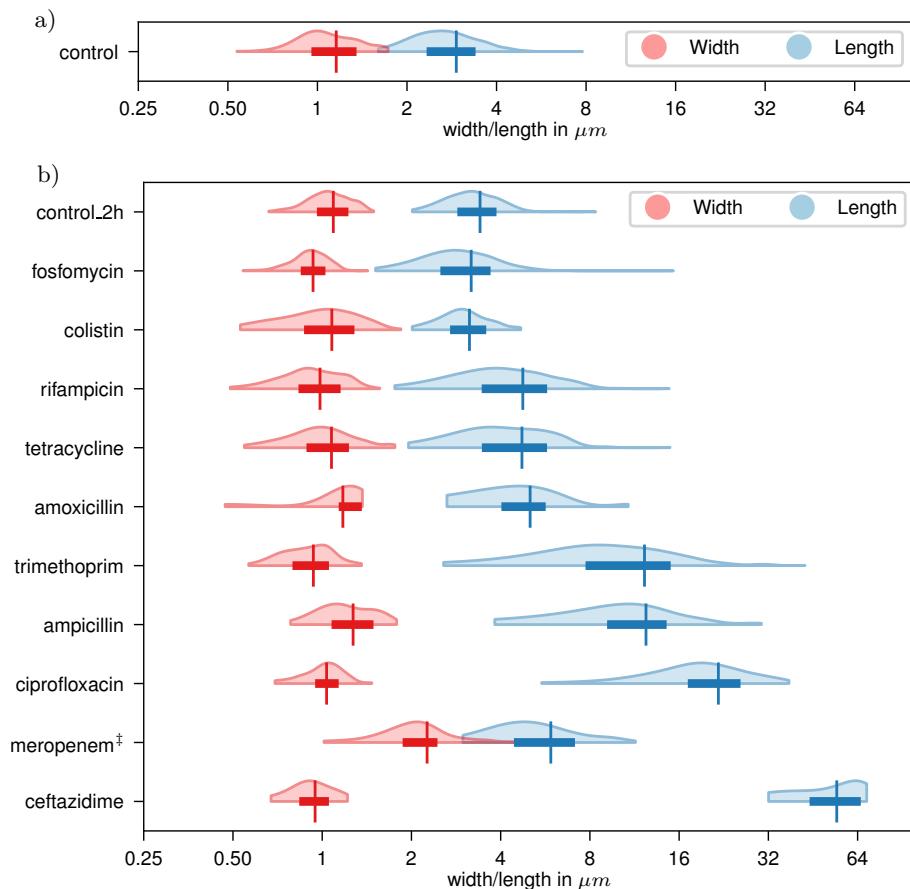


Fig. 3.38 Pooled density distributions (95% prediction intervals) of cell widths (red) and lengths (blue), obtained from microscopy images (a) before and (b) after 2 h of antibiotic treatment. Boxes indicate the interquartile range (Q1–Q3), and the mean is marked by (|). <sup>‡</sup> Poor fit quality for meropenem-treated cells, which adopt a lemon-like shape (Fig. 3.33), due to our algorithm assuming cylindrical geometry.

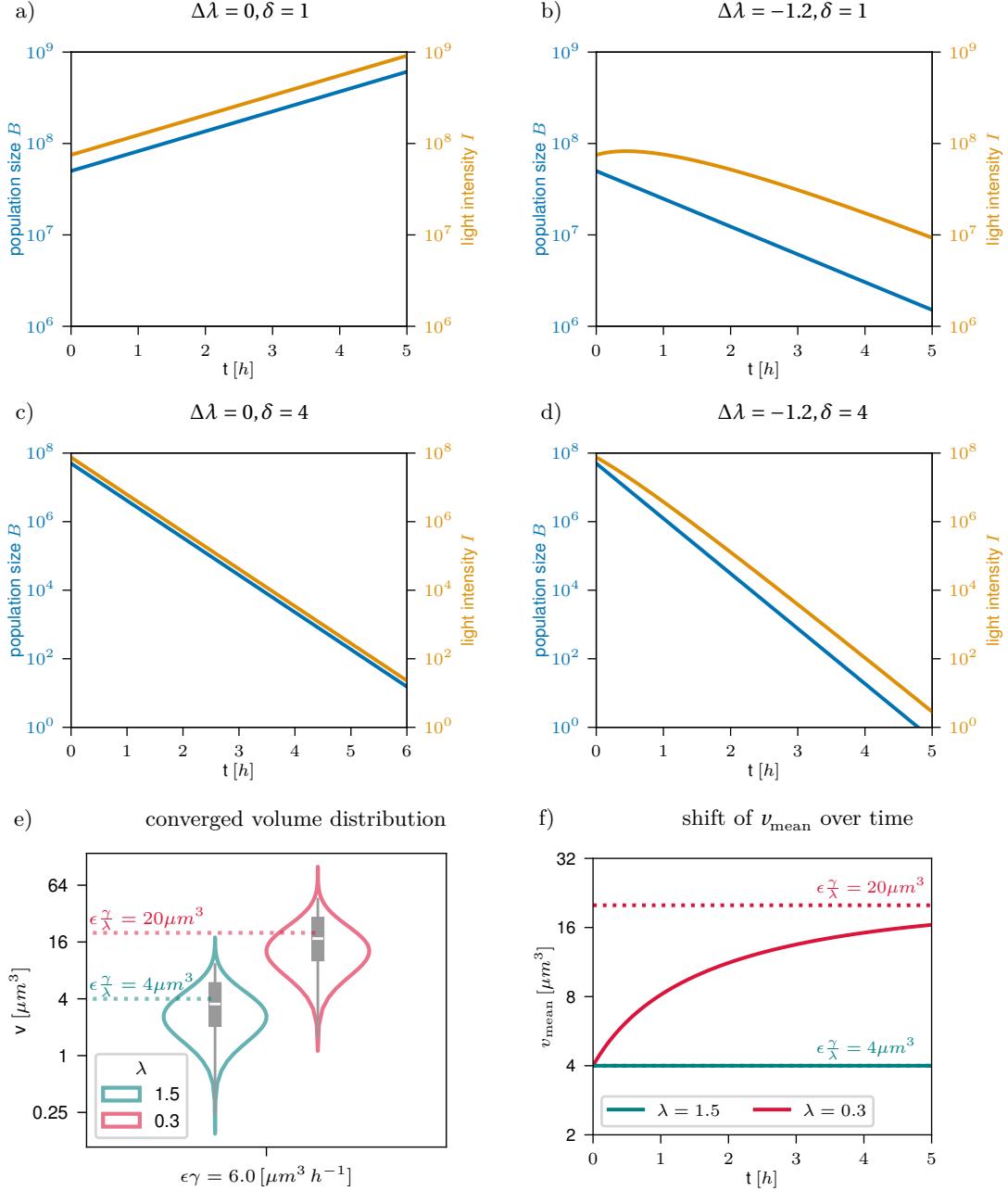


Fig. 3.39 Illustrative simulations using the filamentation model relating (a-d) bacterial population size (blue) and light intensity (orange) under different combinations of treatment-induced changes in division rate ( $\Delta\lambda$ ) and death rate ( $\delta$ ). Panel (e) shows the distributions of converged cell volumes for  $\lambda = 0.3$  and  $\lambda = 1.5$ . Panel (f) shows the shift of mean cell volumes over time for  $\Delta\lambda = -1.2$  and  $\Delta\lambda = 0$ . For all simulations, we used  $\lambda_0 = 1.5$ ,  $\gamma = 150$ ,  $\phi = 0.015$ , and  $\epsilon = 0.04$ . As shown in panel (b), treatment-induced filamentation can lead to a temporary discrepancy between luminescence- and CFU-based rates.

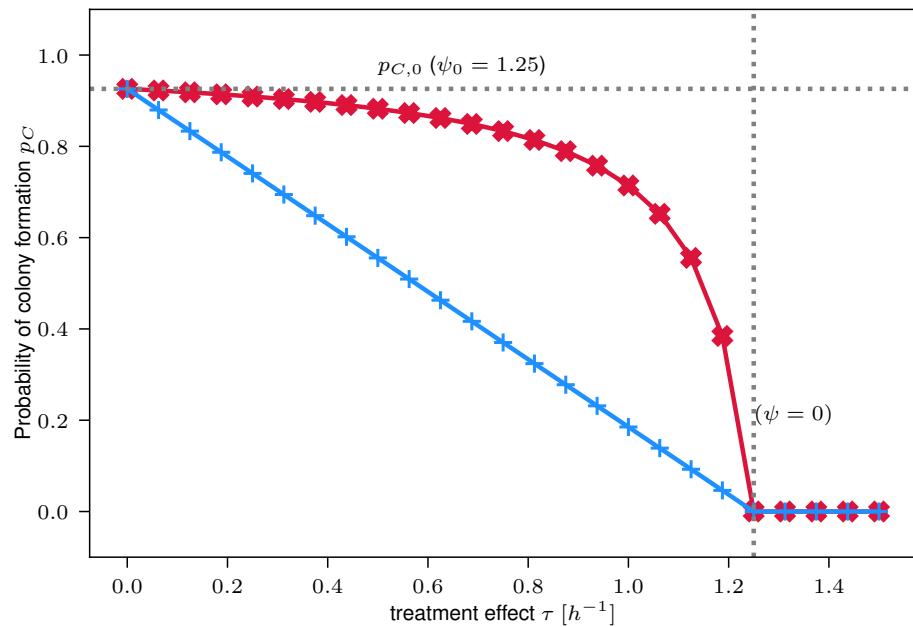


Fig. 3.40 Example of the probability of colony formation  $p_C = 1 - p_E$  (see Section 3.7) for a single plated bacterium. Blue shows  $p_C$  for a purely bactericidal drug ( $\lambda_T = 0$ ) and red for a purely bacteriostatic drug ( $\delta_T = 0$ ), plotted over the treatment effect  $\tau = \delta_T + \lambda_T$ . In this illustrative example we use  $\lambda_0 = 1.35 h^{-1}$  and  $\delta_0 = 0.1 h^{-1}$ .

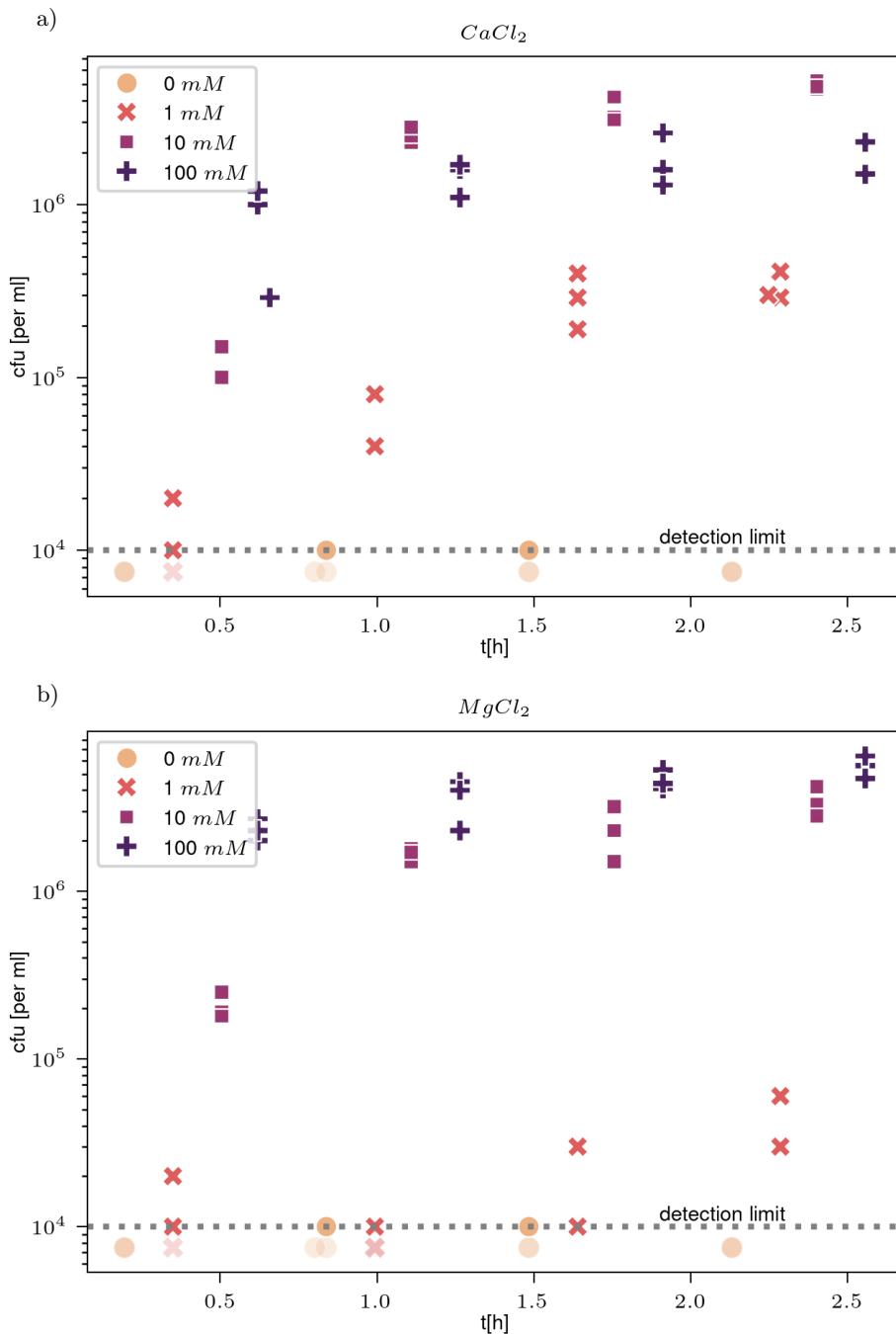


Fig. 3.41 CFU measured over time in supplemented dilution media. Bacterial cultures treated for 1 min with 16 µg/ml pexiganan were diluted (1:100) in PBS supplemented with varying concentrations of (a)  $CaCl_2$  and (b)  $MgCl_2$ . Diluted samples were repeatedly plated over time to test whether supplementation prevents further bacterial killing.

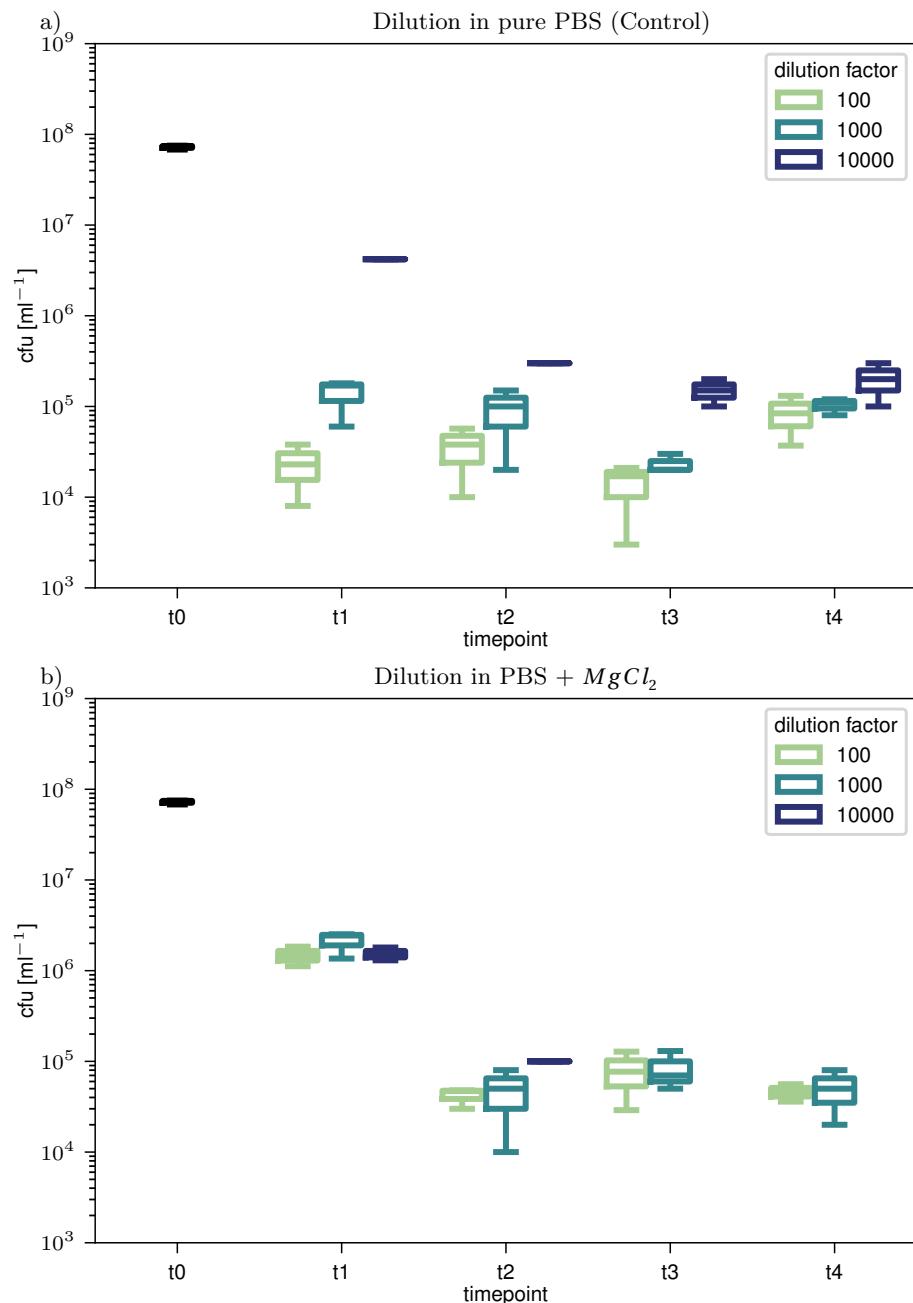


Fig. 3.42 CFU time-kill curves for pexiganan, performed manually, comparing dilution in (a) unsupplemented PBS and (b) PBS supplemented with 100 mM MgCl<sub>2</sub>. Timepoints correspond to: t<sub>0</sub> ≈ 0 s, t<sub>1</sub> ≈ 20 s, t<sub>2</sub> ≈ 2 min, t<sub>3</sub> ≈ 3 min 20 s, and t<sub>4</sub> ≈ 5 min. The black boxplot (t<sub>0</sub>) indicates the pre-treatment bacterial density.

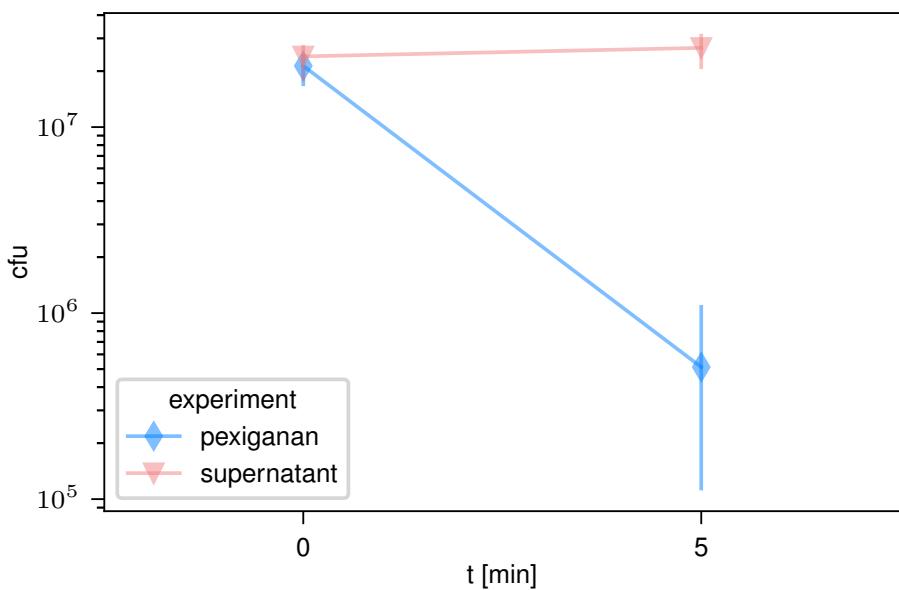


Fig. 3.43 Experiment AMP kill curve vs supernatant kill curve. Here we show the original AMP-Killcurve in blue and the change of CFU over time in the supernatant. The error bars show the min/max interval of the three replicates. Table 3.5 lists the confidence interval and mean for the bootstrapped rates. According to the significance criterion (defined in methods) these two rates are significantly different. The confidence interval of the rate of change of CFU in the supernatant includes zero.

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# 4

## Antimicrobial Combination Effects at Subinhibitory Doses do not Reliably Predict Effects at Inhibitory Concentrations

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### Abstract

Assessing how combinations of antibiotics inhibit bacterial growth above and beyond the individual constituent antibiotics is riddled with several difficulties: (i) measuring death rates at clinically relevant drug concentrations is challenging, (ii) there is no unifying definition of what constitutes synergistic or antagonistic interactions, and (iii) both synergism and antagonism may be concentration dependent. To assess how well sub-inhibitory measurements predict inhibitory behaviour, we quantified drug interactions for 15 pairwise drug combinations on a  $12 \times 12$  concentration checkerboard recording 8,640 time-resolved luminescence trajectories. To handle time-varying treatment effects and allow fair comparisons between drugs with distinct killing dynamics, we introduced a growth-integrated rate-like metric  $\psi$  to summarise each trajectory and assigned interaction types (synergistic/neutral/antagonistic) based on Bliss independence and Loewe additivity. We found that the interaction type frequently changes as the concentration increases from sub-inhibitory to inhibitory concentrations. Moreover, interaction type depended on the mixing ratio, implying that single-point sub-inhibitory measurements alone are not sufficient to predict interactions at clinically relevant concentrations.

### 4.1 Introduction

Antimicrobial resistance (AMR) poses a high global health burden, having caused an estimated 1.14 million deaths in 2021 [1], while the time until clinical resistance evolves against new drug classes is decreasing [2]. To develop a more sustainable approach to antibiotic use, a deeper understanding of the influence of treatment on the evolution of resistance is necessary.

One strategy that has been shown to slow the evolution of resistance in notoriously fast-evolving pathogens, such as HIV, malaria, and tuberculosis, is combination therapy [3, 4]. In theoretical

#### **4. ANTIMICROBIAL COMBINATION EFFECTS AT SUBINHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS**

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models, combination therapy seems to outperform other strategies (such as mixing or cycling) [5–7], and it also seems to perform best in in vitro simulations [8, 9]. However, clinical evidence for the efficacy of combination therapy is inconclusive, and a comprehensive meta-analysis did not identify a significant overall benefit [10].

Several factors contribute to the inconclusiveness of clinical studies, ranging from ethical constraints limiting the selection of focal pathogens (i.e. not HIV or M. tuberculosis) to the lack of statistical power to track resistance evolution [10]. Another potential reason is drug interactions. Drug combinations can exhibit synergistic or antagonistic interactions, depending on whether the combined effect is stronger or weaker than the expectation based on the effects of the single drugs. However, defining this expectation is non-trivial, as null models are numerous and the best choice to describe drug neutrality remains debated ([11, 12]). Here, we use two classical and widely applied reference frameworks to define neutrality: the response-based Bliss independence [13] and dose-based Loewe additivity [14].

Bliss independence is based on the assumption that the probability of being affected by one drug is statistically independent of being affected by the other [13]:

$$p_{AB}^{\text{Bliss}} = p_A + p_B - p_A p_B. \quad (4.1)$$

Under this assumption, Bliss independence is expected to be more appropriate for drug pairs with distinct and non-overlapping mechanisms of action [15].

In contrast, Loewe additivity – introduced as iso-additivity by Frei in 1913 [16] and later formalised by Loewe [14] – defines neutrality through dose equivalence: a combination is considered neutral if pairs of concentrations that produce the same effect  $\psi$  (i.e. a measure of net growth) lie on a linear isobole connecting the corresponding single-drug doses [14]:

$$\frac{c_A}{f_A^{-1}(\psi(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi(c_A, c_B))} = 1. \quad (4.2)$$

Here  $f_i^{-1}$  represents the inverse of a pharmacodynamic function  $f_i(c)$  that maps drug concentrations  $c_i$  to net growth rates  $\psi$  [17].

There are already many studies on drug interactions below the minimum inhibitory concentration (MIC) (e.g. [18–26]); or at the MIC level (e.g. [27–29]). Studies covering the arguably clinically more relevant inhibitory regime are scarce and often cover only a few points in the inhibitory range (e.g. [30–34]). The primary reason for this is methodological: under sub-inhibitory conditions,

growth rates can be easily assessed at high throughput using optical density measurements, whereas inhibitory conditions typically require labour-intensive, low-throughput colony-forming unit assays [35]. This lack of data on effects at high concentrations represents a problem, not only because inhibitory conditions are more therapeutically relevant but also because the right tail of the pharmacodynamic curve has a strong influence on resistance evolution [36].

Although it is well established that drug interactions can depend on dose and mixing ratio [19, 24, 37–39], it remains unclear how reliably sub-inhibitory interaction measurements predict interaction behaviour in the inhibitory regime. Here, we provide an extensive map of interactions for 15 pairs of antibiotics on  $12 \times 12$  concentration checkerboards that include inhibitory concentrations, using time-resolved bioluminescence trajectories. Using Bliss independence and Loewe additivity, we quantify how often sub-inhibitory interaction classifications generalise to inhibitory conditions and how often they fail, including cases of qualitative reversals across regimes.

## 4.2 Results

### 4.3 Discussion

### 4.4 Methods

### 4.5 Data, Materials, and Software Availability

Experimental datasets and code are available at Zenodo (DOI: 10.5281/zenodo.18374151).

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#### 4.6 Supplementary Information Chapter 4

## 4.7 SI TEXT

### Time weighted net growth rates

We define  $\psi$  the linearly time weighted average of the temporal net growth rate  $\hat{\psi}$ :

$$\psi = \int_0^T \hat{\psi}(t) w(t) dt, \quad w(t) = \frac{2}{T^2}(T-t), \quad (4.3)$$

where the kernel  $w(t)$  decreases linearly from  $T$  to 0 and is normalised such  $\int_0^T w(t) dt = 1$ . To derive  $\psi$  from the luminescence trajectories  $I(t) = I_0 e^{\int_0^t \hat{\psi}(u) du}$ , we first normalise

$$y(t) = \frac{I(t)}{I(0)}. \quad (4.4)$$

We then define the log-normalised trajectory

$$Y(t) = \ln y(t) = \ln I(t) - \ln I(0) = \int_0^t \hat{\psi}(u) du, \quad (4.5)$$

and obtain  $\psi$  directly from  $Y(t)$  by integrating over time. Applying Fubini's theorem yields

$$\int_0^T Y(t) dt = \int_0^T \left( \int_0^t \hat{\psi}(u) du \right) dt = \int_0^T (T-u) \hat{\psi}(u) du, \quad (4.6)$$

so that

$$\psi = \frac{2}{T^2} \int_0^T Y(t) dt. \quad (4.7)$$

The area between two curves (control and treatment) is

$$\int_0^T (Y_{\text{ctrl}}(t) - Y_{\text{treat}}(t)) dt = \int_0^T (T-t) (\hat{\psi}_{\text{ctrl}}(t) - \hat{\psi}_{\text{treat}}(t)) dt, \quad (4.8)$$

and we define the treatment effect as the difference between the corresponding time-weighted net growth rates,

$$\tau = \psi_{\text{ctrl}} - \psi_{\text{treat}} = \frac{2}{T^2} \int_0^T (Y_{\text{ctrl}}(t) - Y_{\text{treat}}(t)) dt. \quad (4.9)$$

Intuition for  $\psi$  and  $\tau$ .

Non-monophasic case. If rates vary over time, Equation 4.8 shows that  $\tau$  represents a time-weighted average of instantaneous rate differences,

$$\tau = 2/T^2 \int_0^T (T-t) (\hat{\psi}_{\text{ctrl}}(t) - \hat{\psi}_{\text{treat}}(t)) dt,$$

where the weighting factor  $(T-t)$  decreases linearly from  $T$  to 0. Early rate differences thus contribute more strongly than later ones. This weighting is desirable because an early

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population reduction has a bigger therapeutical impact than a delayed effect, even if both reach the same endpoint.

Constant-rate case. If the temporal net growth rate is constant  $\hat{\psi}(t) = \hat{\psi}$ , Equation 4.7 simplifies to:

$$\psi = \hat{\psi}.$$

Accordingly; if the temporal net growth rate of control and treatment condition are constant; the treatment effect (Equation 4.8) simplifies to:

$$\tau = \psi_{\text{ctrl}} - \psi_{\text{treat}}.$$

Hence, when trajectories are log-linear,  $\tau$  directly measures the difference in net rates.

### Quantifying Light Noise.

We quantified luminescence leakage by a calibration plate in which six source wells (E5, E12, E20, L5, L12, L20) contained 50  $\mu\text{L}$  of overnight culture and all other wells were empty. A single luminescence read yielded raw intensities  $\hat{I}$ . For each empty well  $i$  we calculated the ratio  $I_{\text{empty}}/I_{\text{culture}}$  relative to the nearest source well  $j$  at the distance of the grid  $d_{ij}$  (with diagonal neighbours at distance  $\sqrt{2}$ ). For each discrete distance, we summarized ratios by their median to obtain a kernel  $r(d)$  (Fig. 4.11). We estimate the light contribution from well  $j$  into well  $i$  as  $\hat{I}_{ij} = r(d_{ij}) \hat{I}_j$ . For each experimental plate and time point, we corrected each well by subtracting received light noise,

$$I_i = \hat{I}_i - \sum_j r(d_{ij}) \hat{I}_j, \quad (4.10)$$

where the sum is restricted to wells within  $d \leq 3$ .

### Drug conditions and response functions

**Single-drug pharmacodynamic curves.** For each drug  $i$  we describe the relationship between concentration  $c$  and net growth rate by the fitted pharmacodynamic curve

$$f_i(c) = \psi_0 - \frac{(\psi_0 - \psi_{\min,i})(c/z_{\text{MIC},i})^{\kappa_i}}{(c/z_{\text{MIC},i})^{\kappa_i} - (\psi_{\min,i}/\psi_0)}, \quad [0, \infty) \rightarrow [\psi_{\min,i}, \psi_0]. \quad (4.11)$$

On this domain,  $f_i$  is monotone and therefore invertible, with inverse  $f_i^{-1} : [\psi_{\min,i}, \psi_0] \rightarrow [0, \infty)$ .

**Closed-form inversion.** Using Equation 4.11, the inverse  $f_i^{-1}$  can be written in closed form as

$$f_i^{-1}(\psi) = z_{\text{MIC},i} \left( \frac{(\psi_0 - \psi) \psi_{\min,i} / \psi_0}{(\psi_0 - \psi) - (\psi_0 - \psi_{\min,i})} \right)^{1/\kappa_i}, \quad \psi \in [\psi_{\min,i}, \psi_0]. \quad (4.12)$$

**Cartesian conditions.** A condition is defined by a set of drug concentrations  $\{c_i\}$ , with drug  $i \in \{\text{AMO, CHL, COL, PEN, POL, TET}\}$ . The control condition corresponds to  $\{c_i = 0 \ \forall i\}$  and is denoted by  $\emptyset$ .

Throughout this work, quantities characterizing a treatment are written as functions of the underlying condition  $\{c_i\}$ . For example,  $\psi(\{c_i\})$  denotes the effective growth rate associated with a condition. For brevity, we write  $\psi(c_A)$  when only one drug is present,  $\tau(c_A, c_B)$  when two drugs are present, and  $\psi_0$  for the untreated control.

**Polar pharmacodynamic curves.** For two-drug combinations, conditions are parameterized in polar coordinates  $(z, \phi)$ , and the corresponding effective growth rate is written as

$$\psi(z, \phi) = \psi_0 - \frac{(\psi_0 - \psi_{\min}(\phi))(z/z_{\text{MIC}}(\phi))^{\kappa(\phi)}}{(z/z_{\text{MIC}}(\phi))^{\kappa(\phi)} - \psi_{\min}(\phi)/\psi_0}. \quad (4.13)$$

### Bliss independence

Bliss independence is defined by assuming that the probabilities of a cell being killed by drugs  $A$  and  $B$  within a fixed observation window  $T > 0$  are statistically independent [1]. Under this assumption,

$$p_{AB}^{\text{Bliss}} = p_A + p_B - p_A p_B, \quad (4.14)$$

where  $p_A$  and  $p_B$  denote the single-drug kill probabilities within  $T$ .

**Time-varying hazards.** Let  $\hat{\tau}_i(t) \geq 0$  denote a time-dependent kill hazard under treatment  $i$  on  $t \in [0, T]$ . The survival fraction at time  $T$  is  $S_i(T) = \exp(-\int_0^T \hat{\tau}_i(u) du)$ . Bliss independence implies multiplicative survival  $S_{AB}(T) = S_A(T) S_B(T)$ , hence additive cumulative hazards:

$$\int_0^T \hat{\tau}_{AB}^{\text{Bliss}}(u) du = \int_0^T \hat{\tau}_A(u) du + \int_0^T \hat{\tau}_B(u) du. \quad (4.15)$$

To connect this survival formulation to the growth-based log-trajectories, we interpret the instantaneous treatment effect as the reduction in temporal net growth relative to the untreated control, i.e.  $\hat{\tau}_i(t) = \hat{\psi}_{\text{ctrl}}(t) - \hat{\psi}_i(t)$ . With  $Y_i(t) = \int_0^t \hat{\psi}_i(u) du$  from Equation 4.5, it follows that

$$Y_i(t) = Y_{\text{ctrl}}(t) - \int_0^t \hat{\tau}_i(u) du.$$

Using additive cumulative hazards under Bliss then yields

$$Y_{AB}^{\text{Bliss}}(t) = Y_A(t) + Y_B(t) - Y_{\text{ctrl}}(t). \quad (4.16)$$

Integrating Equation 4.16 over time yields the Bliss prediction for the effective treatment effect:

$$\tau^{\text{Bliss}}(c_A, c_B) = \tau(c_A) + \tau(c_B). \quad (4.17)$$

**Bliss-based interaction index  $\mu$ .** Based on the Bliss expectation for a combination at concentrations  $(c_A, c_B)$ , we define the interaction index

$$\mu(c_A, c_B) = \frac{\tau(c_A) + \tau(c_B) - \tau(c_A, c_B)}{\text{median}(\tau(c_A)) + \text{median}(\tau(c_B))}. \quad (4.18)$$

To avoid division by zero, normalization is performed using the medians of the monotherapy effects. The index is only evaluated where a condition  $(c_A, c_B)$  is classified as eligible if the combined effect  $\tau(c_A, c_B)$  is significant and at least one of the two monotherapy treatment effects,  $\tau(c_A)$  or  $\tau(c_B)$ , is significant.

Interpreting  $\mu$ .

Positive values of  $\mu$  indicate synergy ( $\tau(c_A, c_B) > \tau(c_A) + \tau(c_B)$ ), negative values indicate antagonism, and  $\mu = 0$  corresponds to neutral interactions.

### Loewe additivity

Loewe additivity is a form of dose additivity and formalizes self-additivity, i.e. that a drug combined with itself should behave like a higher dose of the same drug. This concept is independent of the particular choice of effect measure, provided the effect can be represented on a shared, monotone dose-response scale. In our case, this effect scale is the effective net growth rate  $\psi$  (see Equation 4.7).

For a combination with observed effect  $\psi(c_A, c_B)$ , Loewe additivity requires that the fractions of the corresponding monotherapy-equivalent concentrations sum to one,

$$\frac{c_A}{f_A^{-1}(\psi(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi(c_A, c_B))} = 1, \quad (4.19)$$

where  $f_A$  and  $f_B$  are the single-drug pharmacodynamic curves mapping concentrations to  $\psi$ , and  $f_A^{-1}$  and  $f_B^{-1}$  map an effect level  $\psi$  to the corresponding monotherapy-equivalent concentration. As pharmacodynamic function  $f_i$ , we use the fitted single-drug pharmacodynamic curve (Equation 4.11) for the well-based interaction analysis, whereas for the surface-based analysis we infer  $f_i$  from the monotherapy edge profiles of the chequerboard surface splines. The Loewe prediction  $\psi_{\text{Loewe}}(c_A, c_B)$  is obtained by numerically solving Equation 4.19 for  $\psi$ .

**Loewe interaction index ( $\nu$ ).** We quantify deviations from Loewe additivity by the centered dose-equivalence index

$$\nu(c_A, c_B) = \frac{c_A}{f_A^{-1}(\psi(c_A, c_B))} + \frac{c_B}{f_B^{-1}(\psi(c_A, c_B))} - 1. \quad (4.20)$$

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The index is only evaluated where a condition  $(c_A, c_B)$  is classified as eligible if the combined effect  $\tau(c_A, c_B)$  is significant and at least one of the two monotherapy treatment effects,  $\tau(c_A)$  or  $\tau(c_B)$ , is significant.

Because  $f_A^{-1}$  and  $f_B^{-1}$  are only defined on  $\text{Im}(f_A) = [\psi_{\min,A}, \psi_0]$  and  $\text{Im}(f_B) = [\psi_{\min,B}, \psi_0]$ ,  $\nu$  is only defined if

$$\psi(c_A, c_B) \in \text{Im}(f_A) \cap \text{Im}(f_B) = [\max(\psi_{\min,A}, \psi_{\min,B}), \psi_0]. \quad (4.21)$$

Interpreting  $\nu$ .

By construction  $\nu = 0$  corresponds to Loewe additivity,  $\nu < 0$  indicates synergy, and  $\nu > 0$  indicates antagonism.

Because Loewe additivity requires monotherapy-equivalent concentrations for both drugs at the observed effect level,  $\nu$  can only be evaluated when  $\psi(c_A, c_B)$  lies in the overlap of the two monotherapy effect ranges, i.e.  $\psi \geq \max(\psi_{\min,A}, \psi_{\min,B})$ .

### Peptide--antibiotic interaction model

We consider two drugs,  $A$  and  $B$ , acting on a population with size trajectory  $x(t)$ . Drug  $A$  represents a peptide-like effect that causes an instantaneous multiplicative reduction in population size by a factor  $\alpha \in (0, 1]$ , whereas drug  $B$  represents a conventional antibiotic that changes the subsequent net growth rate but does not cause an initial drop. We assume that the combination of two drugs induces a sharp drop by the factor  $\alpha$  followed by net growth, determined by drug  $B$ :

$$\begin{aligned}x_{\text{ctrl}}(t) &= x_0 \exp(\hat{\psi}_{\text{ctrl}} t), \\x_B(t) &= x_0 \exp(\hat{\psi}_B t), \\x_A(t) &= \alpha x_{\text{ctrl}}(t), \\x_{AB}(t) &= \alpha x_B(t),\end{aligned}$$

and:

$$\begin{aligned}Y_{\text{ctrl}}(t) &= \ln x_0 + \hat{\psi}_{\text{ctrl}} t, \\Y_B(t) &= \ln x_0 + \hat{\psi}_B t, \\Y_A(t) &= Y_{\text{ctrl}}(t) + \ln \alpha, \\Y_{AB}(t) &= Y_B(t) + \ln \alpha.\end{aligned}$$

Using the definition of effective-rate  $\tau_i = \frac{2}{T^2} \int_0^T (Y_{\text{ctrl}}(t) - Y_i(t)) dt$ , we obtain the following.

$$\tau_A = \frac{2}{T^2} \int_0^T (-\ln \alpha) dt, \quad \tau_B = \frac{2}{T^2} \int_0^T (\hat{\psi}_{\text{ctrl}} - \hat{\psi}_B) t dt,$$

and

$$\tau_{AB} = \frac{2}{T^2} \int_0^T ((\hat{\psi}_{\text{ctrl}} - \hat{\psi}_B) t - \ln \alpha) dt. \quad (4.22)$$

Separating the two terms in Equation 4.22 yields

$$\tau_{AB} = \tau_A + \tau_B, \quad (4.23)$$

which matches the Bliss expectation for additive treatment effects.

### Condition-wise interaction inference on the checkerboard

Each condition  $\{c_i\}$  is realized by a pool of replicate wells  $\mathcal{W}(c_A, c_B)$ , from which effective growth rates are inferred as described above. Treatment effects are estimated by bootstrap resampling

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from the pools of replicate wells. We draw  $B = 200$  pairs consisting of one control well and one condition well, sampled with replacement from  $\mathcal{W}(\emptyset)$  and  $\mathcal{W}(c_A, c_B)$ , respectively. For each draw  $b$ , the treatment effect is defined as

$$\tau_b(c_A, c_B) = \psi_b(\emptyset) - \psi_b(c_A, c_B),$$

yielding a bootstrap distribution of treatment effects.

For combination conditions, the corresponding mono-treatment growth rates  $\psi_b(c_A, 0)$  and  $\psi_b(0, c_B)$  are sampled analogously and included in each bootstrap draw, as they are required for interaction analysis.

**Regime-wise aggregation across checkerboard conditions.** To summarise interactions for a given drug pair within a regime (sub-inhibitory or inhibitory), we combine the condition-wise bootstrap interaction estimates from all eligible checkerboard cells. In each aggregation draw, we sample 200 eligible conditions with replacement, using sampling probabilities proportional to

$$w(\phi) = \sin(2\phi). \tag{4.24}$$

This excludes monotherapy edges and gives the highest weight to near-equal mixing. For every sampled condition, we draw one interaction score from its condition-wise bootstrap distribution. Repeating this procedure yields an aggregated distribution, from which we report the median and central 95% interval for the regime-level interaction estimate.

### Continuous interaction surfaces and geometric exploration

**Density-based restriction of the surface domain.** For some drugs, the empirical data density decreases at high concentrations as trajectories drop below the detection limit and are excluded. We therefore restrict the chequerboard to the high-density subdomain. For each drug pair, we compute, for every grid cell  $(i,j)$ , the valid fraction  $f(i,j) = n_{\text{valid}}(i,j)/4$  (with  $n_{\text{valid}}$  counting non-NaN values of  $\psi$ ). We then fit a monotone-decreasing helper surface  $f_{\text{dens}}(i,j)$  by alternating one-dimensional isotonic regression sweeps (`sklearn.isotonic.IsotonicRegression`) along both index axes, followed by bivariate spline interpolation on the  $(i,j)$  grid (`scipy.interpolate.RectBivariateSpline`). Cells with  $f_{\text{dens}}(i,j) < f_{\text{min}}$  are excluded, and all subsequent bootstrap resampling and surface-spline fitting are performed only on the remaining cells.

**Bootstrap datasets and coordinate transform.** For each pair of drugs  $(A,B)$  we first collect all wells belonging to that pair together with their estimated effective net growth rates  $\psi$ . Each well is indexed by a normalised concentration pair  $(z_A, z_B)$ . To stabilise interpolation near zero, we transform the axes to  $Z_A = \log_2(z_A + 1)$  and  $Z_B = \log_2(z_B + 1)$ . For each bootstrap draw  $b$  we generate a resampled surface dataset by sampling, with replacement, one well for every occupied grid point, and retaining its triplet  $(Z_A, Z_B, \psi)$ .

**Monotone surface construction.** For each bootstrap dataset we enforce a monotone-decreasing  $\psi$ -surface by alternating one-dimensional isotonic regressions along rows and columns. This is important to ensure that the backtransformation  $z_\phi(\psi)$  is unique. Specifically, for each column and then for each row we apply an isotonic regression with a nonincreasing constraint using `sklearn.isotonic.IsotonicRegression` [2]. On each monotone grid we fit a bivariate spline  $s_{AB}^{(b)}(Z_A, Z_B)$  using `scipy.interpolate.RectBivariateSpline` with a linear basis functions in both directions. For each drug combination we calculate a consensus surface  $\psi^{\text{cons}}$  based on the median of the single splines.

**Bliss and Loewe interaction indices on the surface.** Given the consensus surface  $\psi^{\text{cons}}(z_A, z_B)$ , we evaluate Bliss and Loewe interaction measures on the same effect scales defined above. In particular, we use the surface-derived treatment effects  $\tau(\cdot)$  (see Equation 4.9) to compute the

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Bliss expectation

$$\tau_{\text{Bliss}}(z_A, z_B) = \tau(z_A) + \tau(z_B),$$

where  $\tau(z_A)$  and  $\tau(z_B)$  are obtained from the consensus edge profiles  $\psi^{\text{cons}}(z_A, 0)$  and  $\psi^{\text{cons}}(0, z_B)$ , with  $\psi^{\text{cons}}(\emptyset) = \psi^{\text{cons}}(0, 0)$ . We then define a normalized Bliss interaction index on the surface by

$$\mu_{\text{surf}}(z_A, z_B) = \frac{\tau_{\text{Bliss}}(z_A, z_B) - \tau(z_A, z_B)}{\tau_{\text{Bliss}}(z_A, z_B)},$$

using the same sign convention as Equation 4.18.

For Loewe additivity, we evaluate  $\nu(z_A, z_B)$  in the same way as Equation 4.20, using the consensus monotherapy edges to obtain the monotherapy-equivalent doses  $z_A^*$  and  $z_B^*$  at the observed effect  $\psi^{\text{cons}}(z_A, z_B)$ :

$$\nu(z_A, z_B) = \frac{z_A}{z_A^*} + \frac{z_B}{z_B^*} - 1, \quad \psi^{\text{cons}}(z_A^*, 0) = \psi^{\text{cons}}(0, z_B^*) = \psi^{\text{cons}}(z_A, z_B).$$

Single-drug inoculum effect analysis

During data analysis, we noted that inocula varied more than expected, which we traced to variability induced by the pintool. Across all wells, the pre-treatment luminescence standard deviation was ( $\sigma(I_0) = 6.89 \times 10^4$  RLU), between experiments ( $\sigma_{\text{between}} = 4.33 \times 10^4$  RLU) and within experiments ( $\sigma_{\text{within}} = 5.47 \times 10^4$  RLU). To investigate whether the observed net growth rates  $\psi$  systematically depended on inoculum size, we used the pre-treatment luminescence signal ( $I_0$ ) as a proxy for initial cell density.

For all monotherapy drug concentration conditions with at least  $n \geq 5$  replicates, we fitted an ordinary least-squares regression of  $\psi$  on  $I_0$  to obtain a concentration-specific slope  $\beta_{\text{inoculum}}$  together with a confidence interval, the value  $p$  and the determination coefficient  $R^2$ . These slopes and their uncertainty summaries were visualised as concentration–slope profiles with 95% intervals in Fig. 4.12. Across AMO, CHL, PEN, and TET, the estimated slopes were negligible across concentrations, whereas COL and POL showed the strongest dependence on  $I_0$  at intermediate concentrations (around  $1 \frac{\mu\text{g}}{\text{mL}}$ ).

To assess whether inoculum size varies systematically along the one-dimensional concentration index, we pooled all single-drug wells and mapped each well to its concentration index. We then performed a permutation test on the ordinary least-squares slope of  $\log_{10} I_0$  versus index by permuting  $\log_{10} I_0$  across indices to obtain a null distribution. We found no evidence for a systematic

trend of inoculum size with index in our data ( $p = 0.092$ ), suggesting that inoculum variation contributes noise but does not introduce a strong directional bias along the concentration series. Consistent with this, the inoculum effect primarily adds scatter at intermediate concentrations of COL and POL, contributing to the increased spread in the corresponding single-drug PD curves.

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#### 4.8 SI tables

Table 4.1 Fitted single-drug pharmacodynamic (PD) curve parameters for Equation 4.11. For each antibiotic, we report the point estimates of  $\psi_0$ ,  $\psi_{\min}$ ,  $\kappa$ , and  $z_{\text{MIC}}$ , together with the corresponding lower (L) and upper (U) uncertainty bounds (95% intervals).

drug	$\psi_0$	$\psi_0^L$	$\psi_0^U$	$\psi_{\min}$	$\psi_{\min}^L$	$\psi_{\min}^U$	$\kappa$	$\kappa^L$	$\kappa^U$	$z_{\text{MIC}}$	$z_{\text{MIC}}^L$	$z_{\text{MIC}}^U$
polymyxinB	1.70	1.47	1.94	-4.43	-5.37	-3.48	1.48	1.01	1.95	0.51	0.42	0.59
tetracycline	1.77	1.75	1.79	-0.24	-0.28	-0.20	1.15	1.07	1.22	15.15	13.80	16.49
amoxicillin	1.73	1.70	1.77	-3.93	-4.02	-3.84	1.69	1.60	1.77	8.42	8.18	8.66
colistin	1.78	1.60	1.96	-6.08	-6.97	-5.18	1.48	1.18	1.78	0.58	0.52	0.65
chloramphenicol	1.78	1.75	1.81	-0.93	-1.02	-0.84	0.96	0.88	1.04	54.11	50.45	57.77
penicillin	1.78	1.73	1.83	-4.54	-4.84	-4.24	1.07	0.99	1.16	153.86	146.77	160.94

Table 4.2 This table summarises interaction classifications (N: neutral, S: synergistic, A: antagonistic) for each drug combination based on Loewe additivity ( $\mu$ ) and Bliss independence ( $v$ ), evaluated separately for sub-inhibitory and inhibitory concentration regimes based on bootstrap confidence intervals.

combination	loewe		bliss	
	sub-inhibitory	inhibitory	sub-inhibitory	inhibitory
amoxicillin_chloramphenicol	A	N	N	A
amoxicillin_colistin	A	A	N	A
amoxicillin_penicillin	N	N	S	A
amoxicillin_polymyxinB	A	A	A	A
amoxicillin_tetracycline	A	N	A	A
chloramphenicol_colistin	N	S	N	S
chloramphenicol_penicillin	A	N	N	A
chloramphenicol_polymyxinB	S	S	S	S
chloramphenicol_tetracycline	S	S	N	A
colistin_penicillin	A	A	A	A
colistin_polymyxinB	A	N	N	N
colistin_tetracycline	A	S	N	S
penicillin_polymyxinB	A	N	N	N
penicillin_tetracycline	A	N	N	A
polymyxinB_tetracycline	A	S	N	S

#### 4.9 SI figures

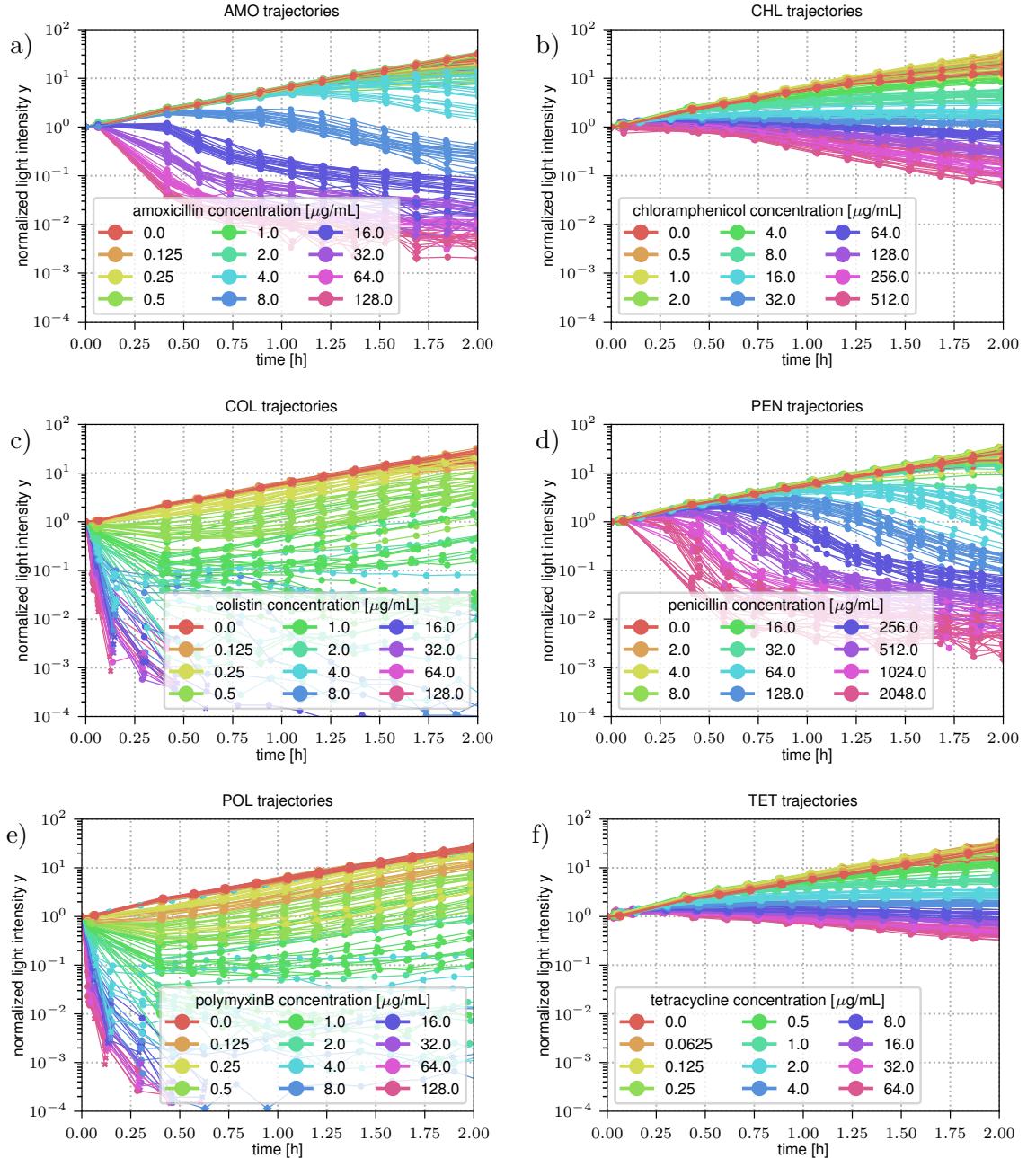


Fig. 4.1 Single-drug timecourse data for the six antibiotics used in this study. Panels (a–f) show the single-drug trajectories across 12 concentrations for amoxicillin, chloramphenicol, colistin, penicillin, polymyxin B, and tetracycline, respectively. For each concentration we aggregate 20 replicate curves, obtained from five independent combination experiments with four technical replicates each. Lines represent the concentration-wise median over replicates at each measurement cycle, and shaded bands indicate the central 50 % percentile interval (25th–75th percentiles). Time on the horizontal axis corresponds to the per-cycle mean acquisition time across wells to account for 200 ms per-well readout staggering.

4. ANTIMICROBIAL COMBINATION EFFECTS AT SUBINHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS

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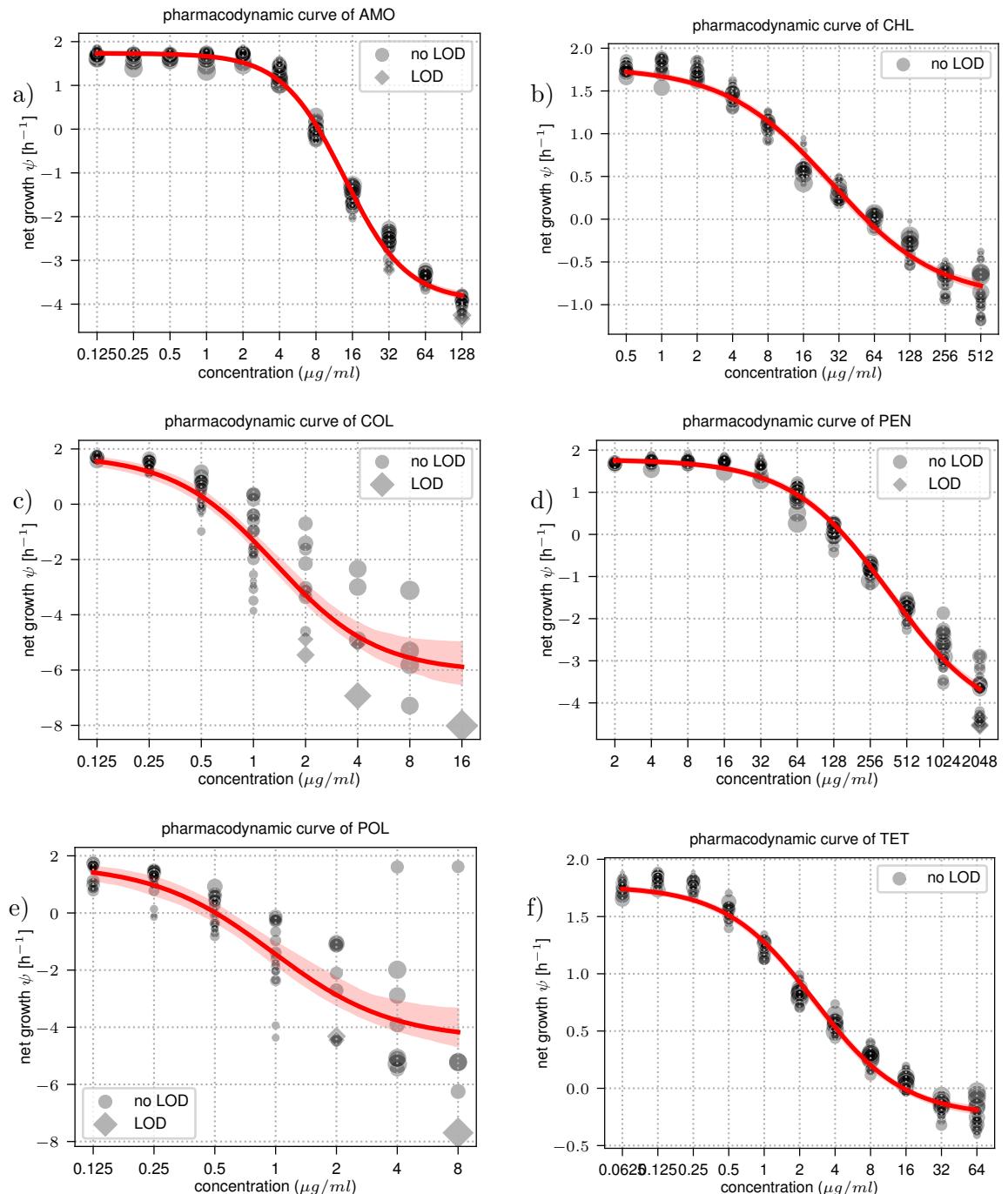


Fig. 4.2 Single-drug pharmacodynamic (PD) curves corresponding to the timecourse data in Fig. 4.1. Panels (a–f) show the net rate of population change as a function of drug concentration for amoxicillin, chloramphenicol, colistin, penicillin, polymyxin B, and tetracycline, respectively. Curves are fitted to the same replicate-aggregated datasets as in Fig. 4.1, and intervals indicate uncertainty in the fitted PD relationship.

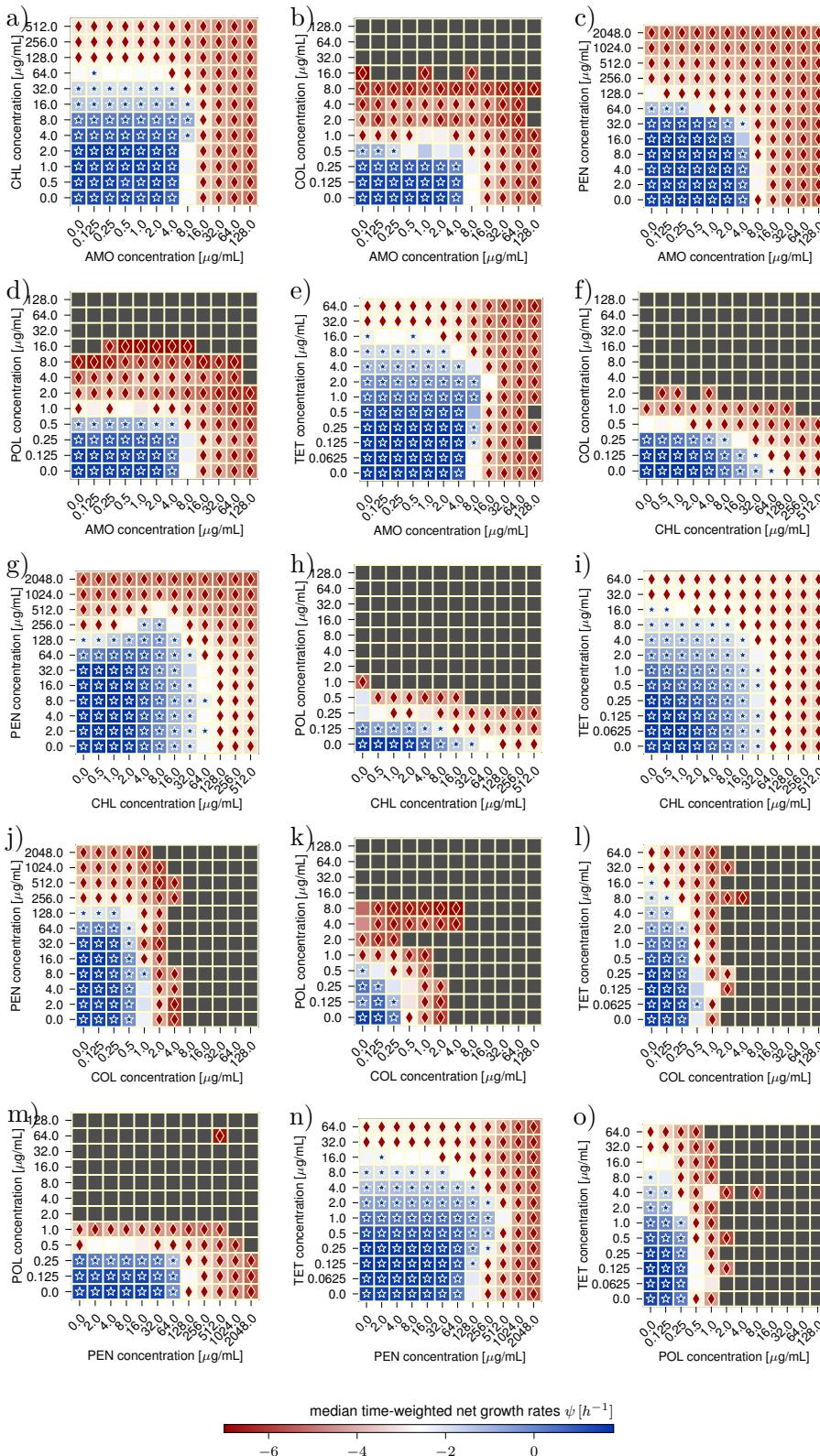


Fig. 4.3 Distribution of the effective net growth rate  $\psi$  across all drug combinations measured in checkerboard assays. Each panel corresponds to one drug pair, and each cell represents a single concentration condition. Colors indicate the median estimate of  $\psi$  across bootstrap resamples. Symbols indicate whether  $\psi$  differs significantly from zero: stars denote significantly positive growth, diamonds significantly negative growth. These effective growth rates are inferred from time-resolved luminescence trajectories as described in the Methods..

4. ANTIMICROBIAL COMBINATION EFFECTS AT SUBINHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS

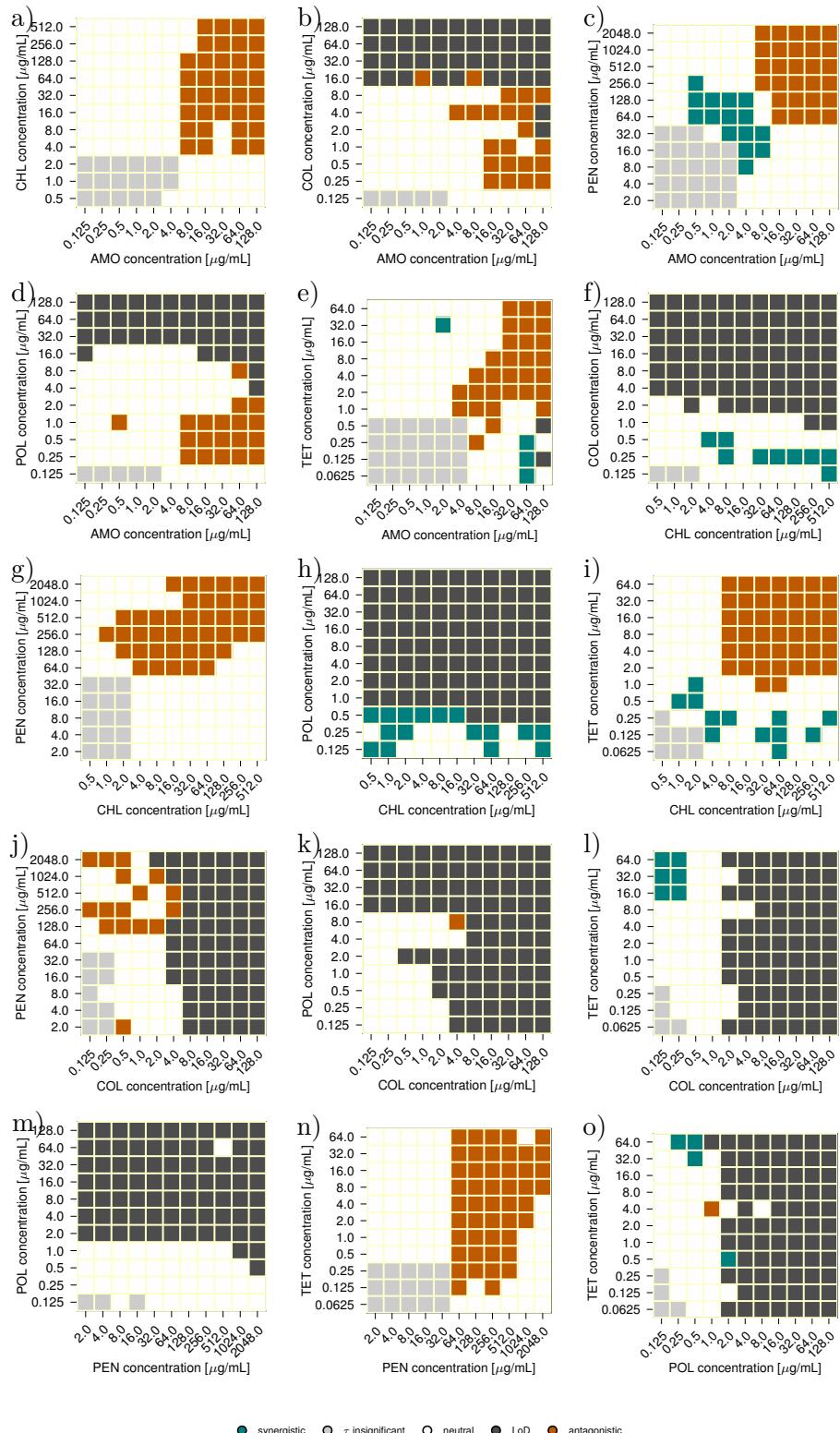


Fig. 4.4 Bliss interaction classification based on bootstrap inference of treatment effects. For each concentration pair, treatment effects were resampled to obtain a distribution of the Bliss interaction index  $\mu$ . Cells are colored according to statistical significance: teal indicates synergistic interactions (95% confidence interval entirely below zero), orange indicates antagonistic interactions (95% confidence interval entirely above zero), and white indicates no significant deviation from additivity. All estimates are based on effective growth rates  $\psi$  inferred from checkerboard experiments..

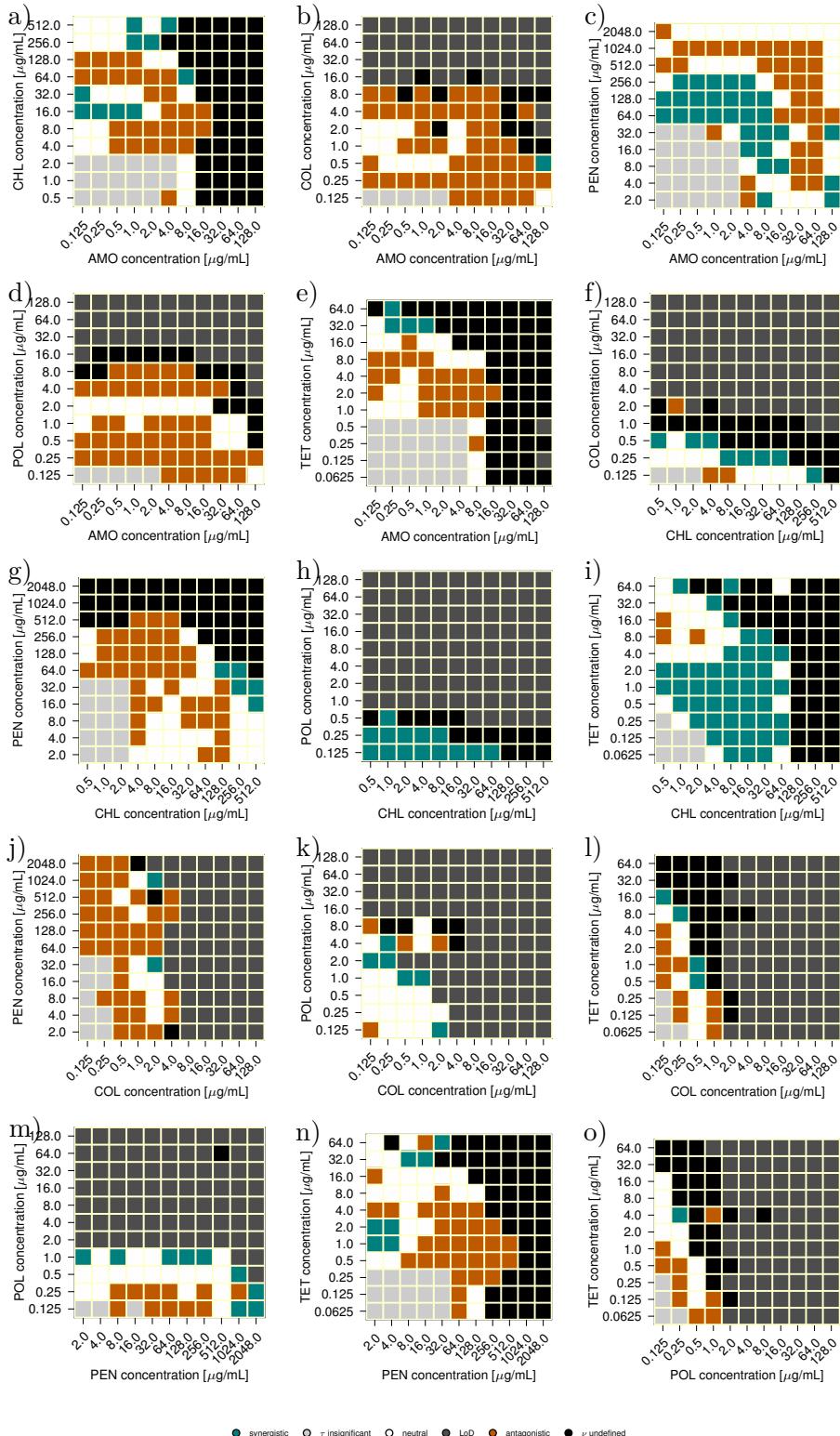


Fig. 4.5 Loewe interaction classification based on bootstrap inference of treatment effects. For each concentration pair, bootstrap samples were used to compute a distribution of the fractional inhibitory concentration index (FICI). Teal indicates significant synergy (95% confidence interval entirely below 1), orange indicates significant antagonism (95% confidence interval entirely above 1), and white indicates no significant deviation from Loewe additivity. All estimates are derived from effective growth rates  $\psi$  obtained from checkerboard assays..

4. ANTIMICROBIAL COMBINATION EFFECTS AT SUBINHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS

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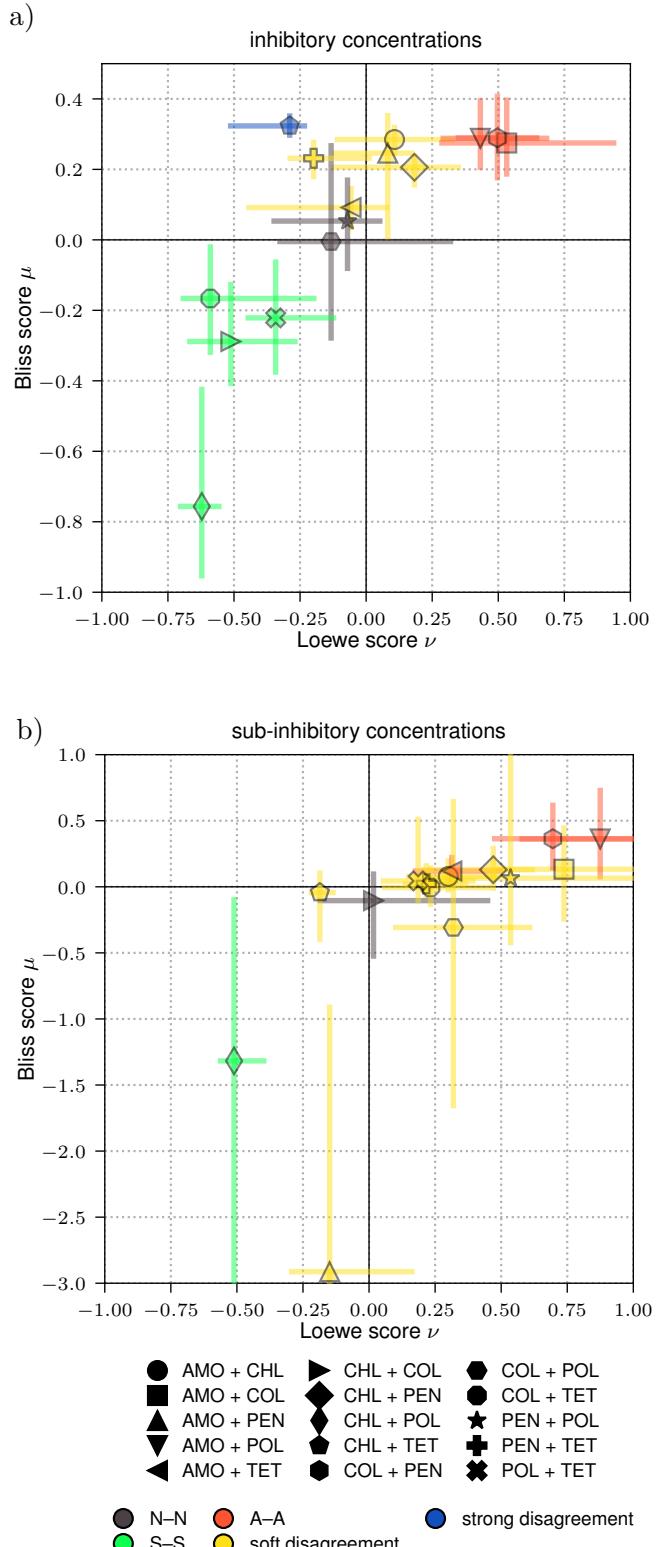


Fig. 4.6 Comparison of Loewe and Bliss interaction scores in two growth regimes. (a) Inhibitory (super-MIC) concentrations. (b) Sub-inhibitory (sub-MIC) concentrations. Each point represents a drug combination with error bars indicating bootstrap confidence intervals. Colors encode agreement or disagreement between Loewe and Bliss classifications.

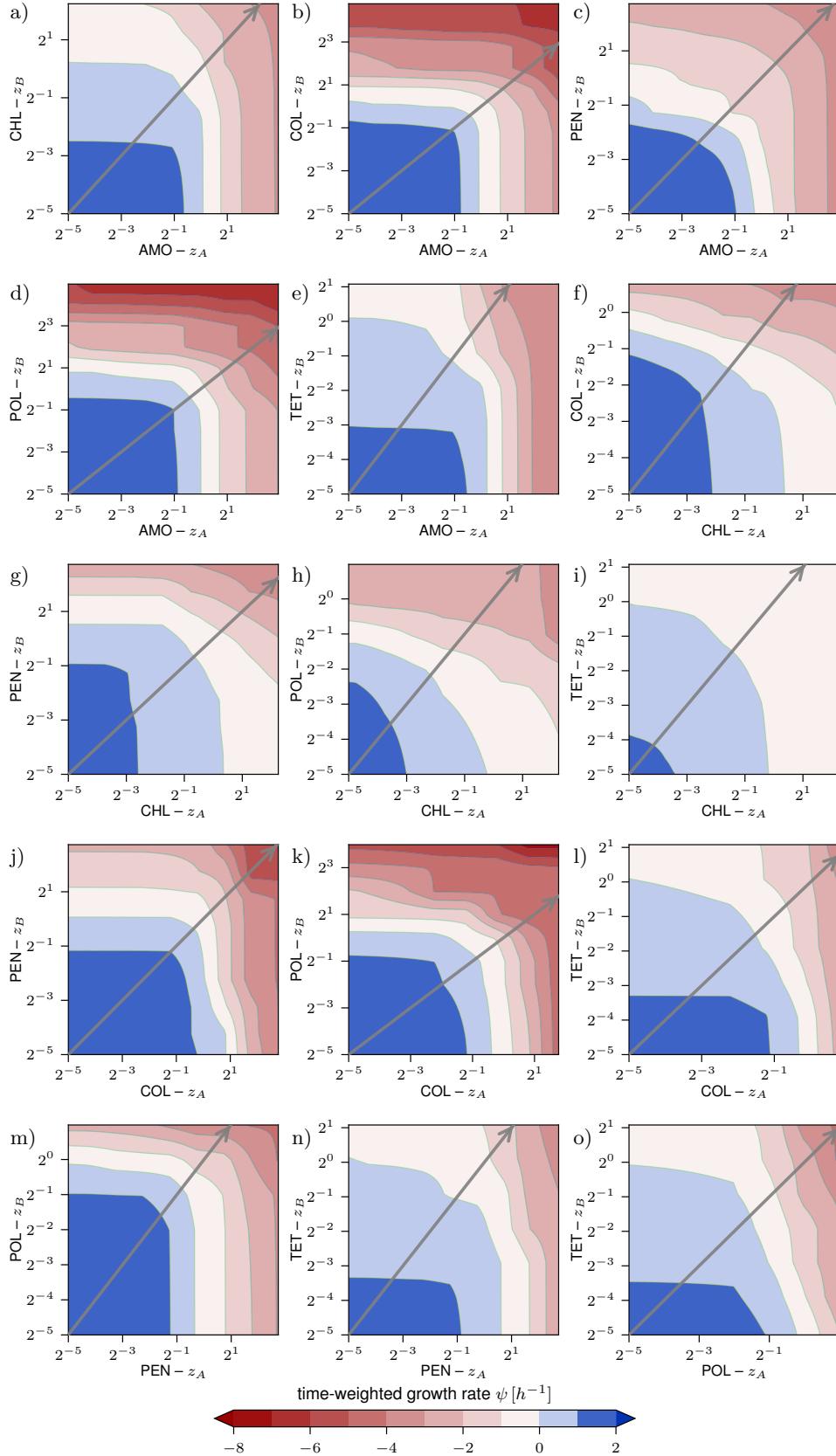


Fig. 4.7 Effective net growth rate surfaces  $\psi$  for all drug combinations. Each panel shows the median effective growth rate estimated from time-resolved luminescence trajectories across the concentration grid. Colors indicate the magnitude of  $\psi$ , with negative values corresponding to net killing and positive values to net growth. All panels share a common color scale to allow direct comparison across drug pairs.

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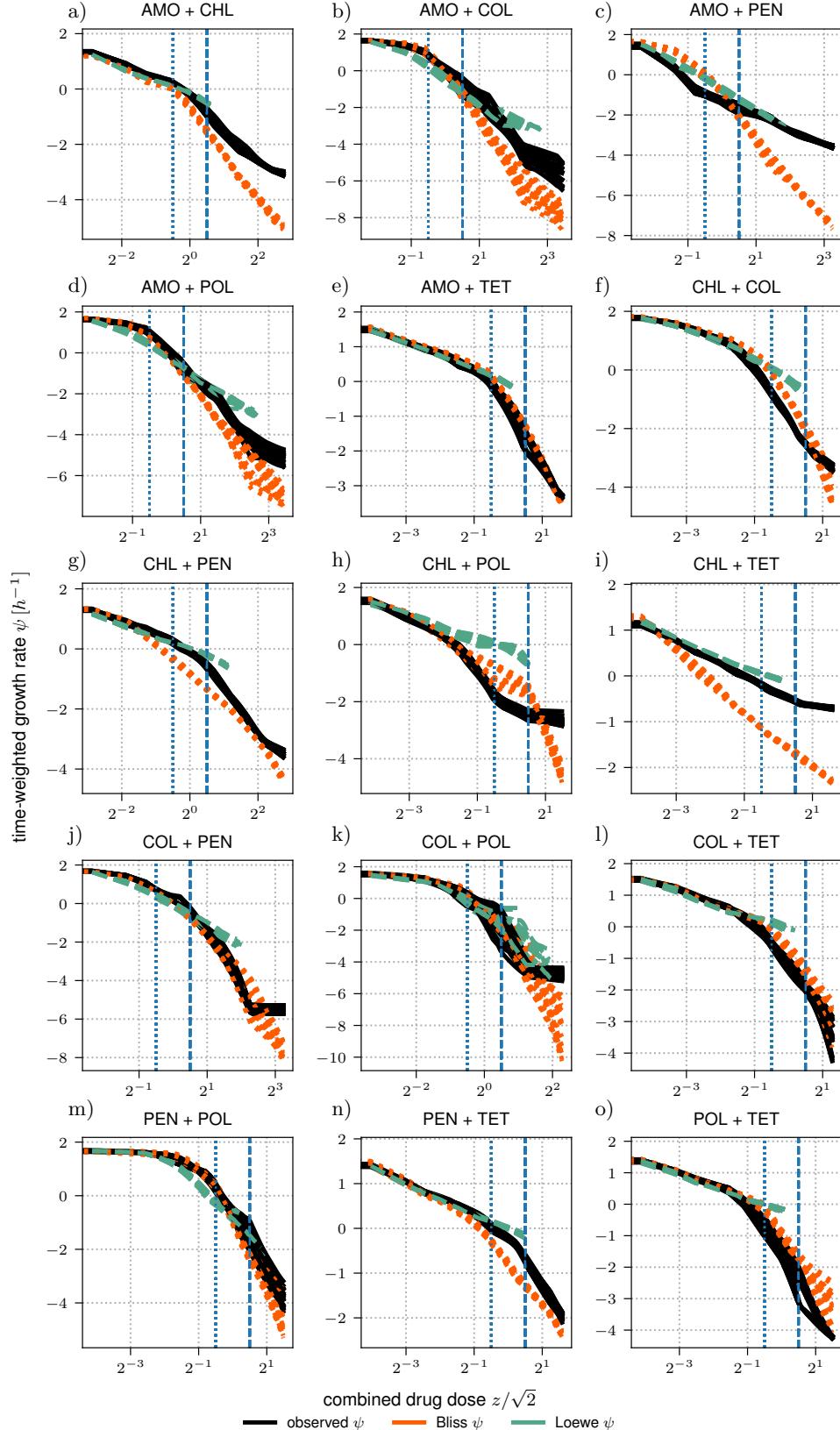


Fig. 4.8 Polar pharmacodynamic curves for all drug combinations. For each drug pair, we show one-dimensional cuts through the  $\psi$ -surface splines at  $\phi = 45^\circ$  (equal mixing in units of zMIC), plotting  $\psi$  as a function of the combined dose  $z$ . The blue dotted line marks  $z = 1/\sqrt{2}$ , corresponding to both single-drug doses being at  $0.5\text{zMIC}$ , and the blue dashed line marks  $z = \sqrt{2}$ , corresponding to both being at  $1\text{zMIC}$ . Solid curves show the median surface-derived  $\psi$ , with corresponding Bliss- and Loewe-based predictions evaluated along the same trajectory.

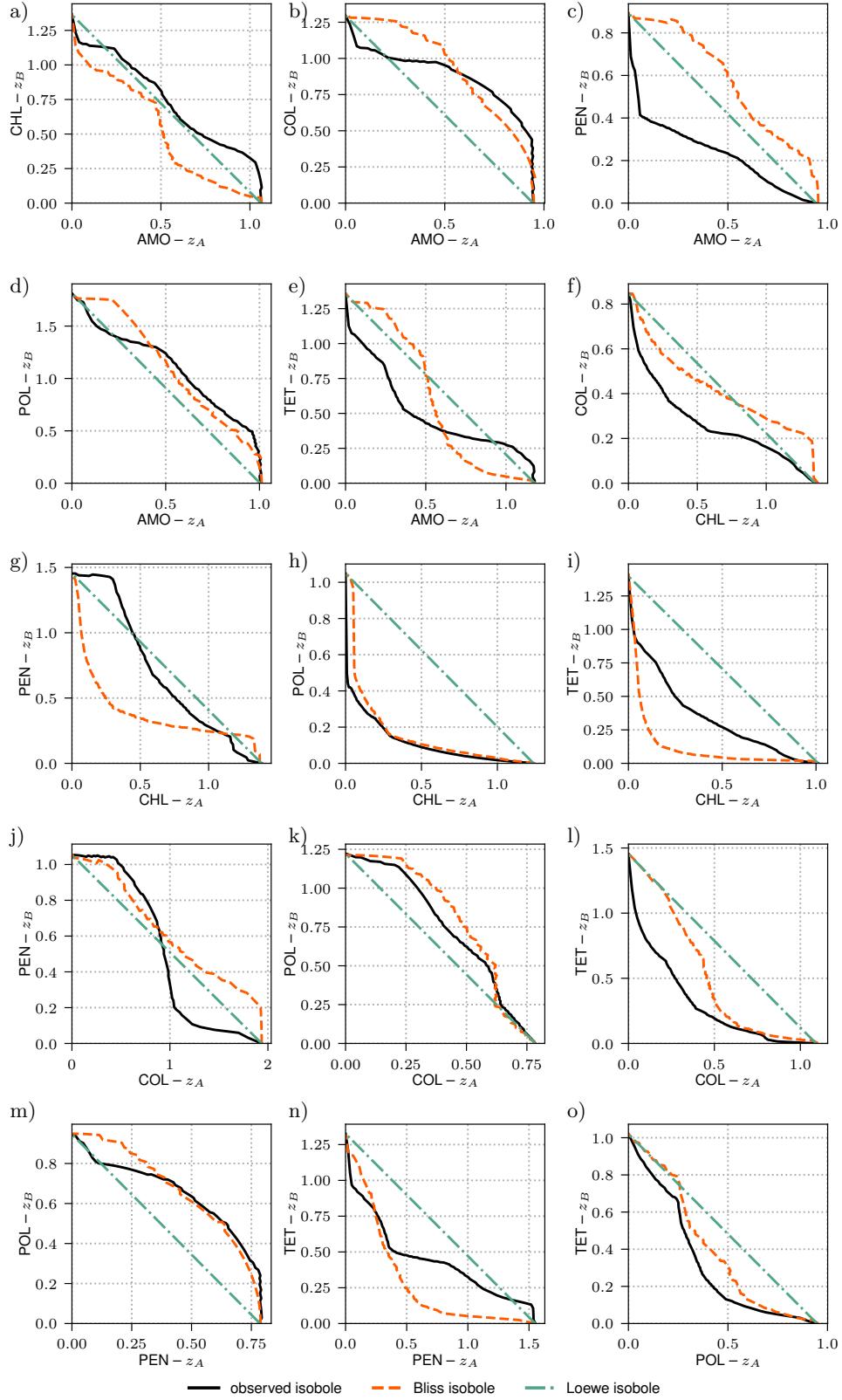


Fig. 4.9 Isoboles of constant effective growth rate  $\psi$  derived from the interpolated response surfaces. Each panel shows curves of equal  $\psi$  in concentration space, illustrating deviations from linear additivity. Bending toward the origin indicates synergistic interactions, whereas outward bending indicates antagonism.

4. ANTIMICROBIAL COMBINATION EFFECTS AT SUBINHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS

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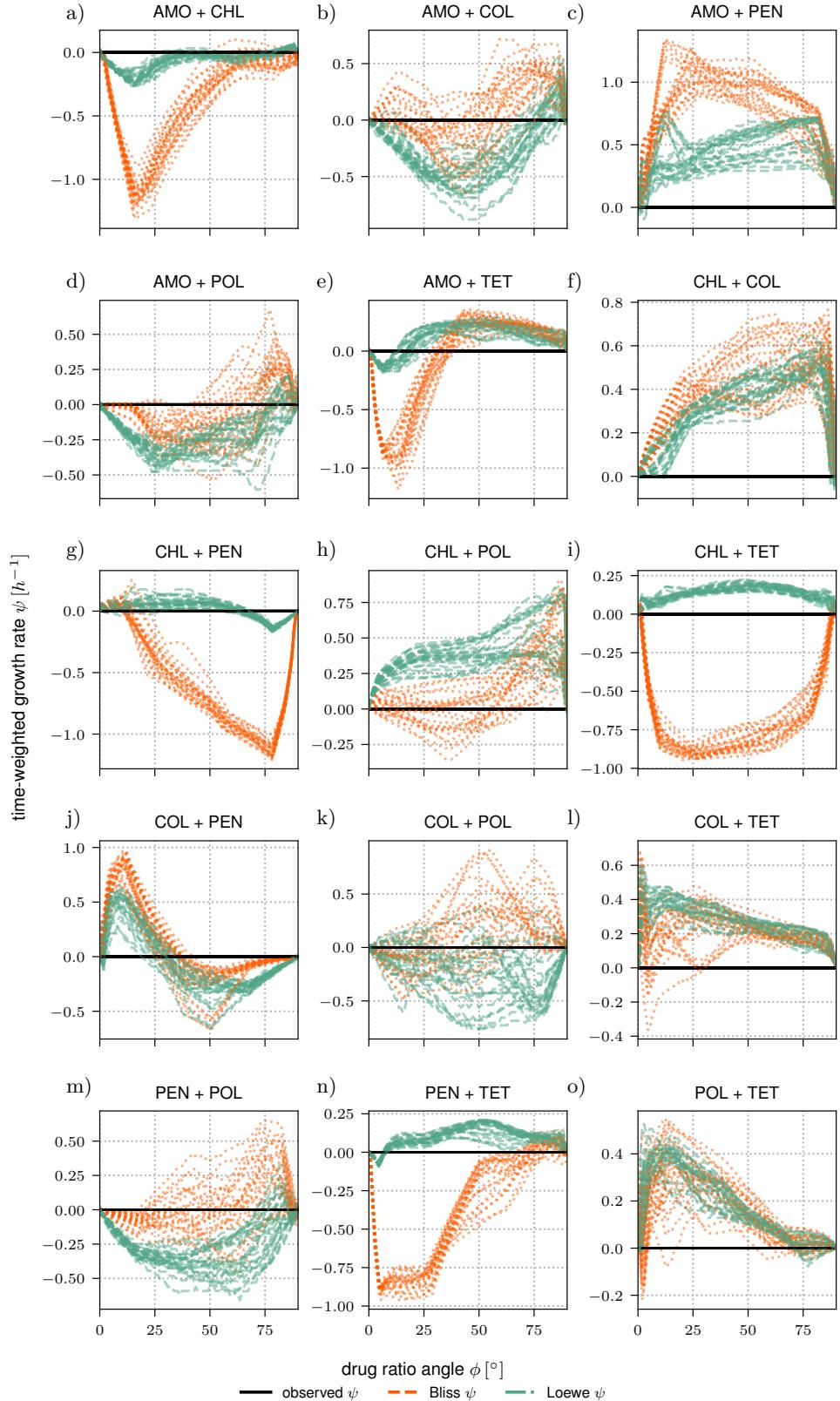


Fig. 4.10 Angular interaction profiles for all drug combinations. For each drug pair, we extract isoboles (paths of constant  $\psi$ ) from the consensus  $\psi$ -surface and evaluate Bliss- and Loewe-based predictions for  $\psi$  along these paths ( $z, \phi$ ), plotted as a function of the mixing angle  $\phi$ . Each combination is shown for one sub-inhibitory isobole, the MIC isobole, and one inhibitory isobole.

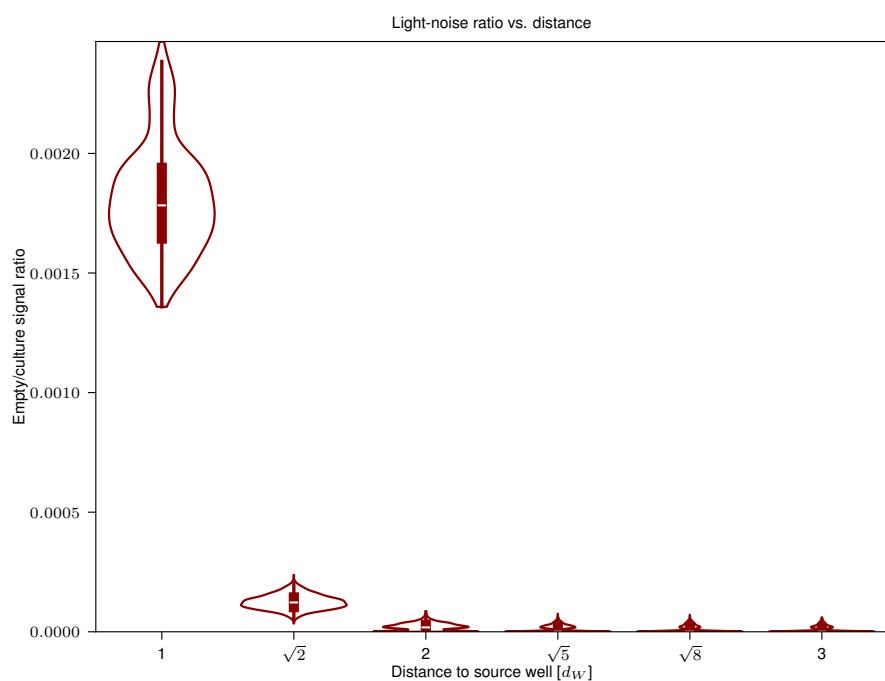


Fig. 4.11 Light noise distribution (shows the fraction of the original light intensity arriving in neighbouring wells; dependent on the distance to the source well; in well lengths  $d_w$ .

4. ANTIMICROBIAL COMBINATION EFFECTS AT SUBINHIBITORY DOSES DO NOT RELIABLY PREDICT EFFECTS AT INHIBITORY CONCENTRATIONS

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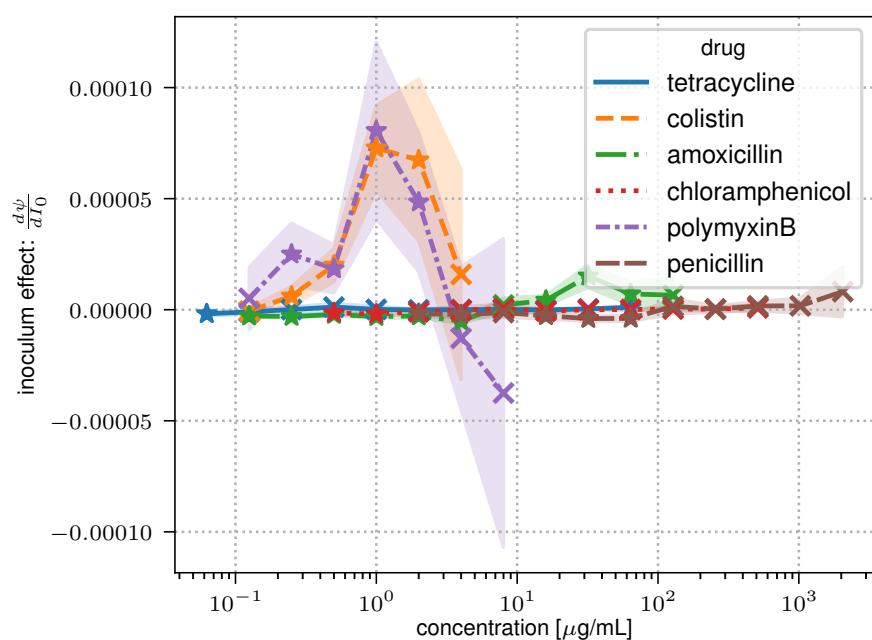


Fig. 4.12 Slope of the inoculum effect ( $d\psi/dI_0$ ) as a function of drug concentration. For each drug and concentration, slopes ( $d\psi/dI_0$ ) are obtained by regressing the observed net growth rates across inocula. Crosses denote concentrations with non-significant inoculum effects, whereas stars indicate statistically significant effects.

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# 5

## Concluding Remarks

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It is clear that antimicrobial resistance (AMR) will remain a significant health crisis for the foreseeable future, underscoring the importance of understanding the evolutionary forces shaping bacterial population dynamics under antibiotic pressure. This thesis addressed several interlinked questions centered around how antimicrobial treatment affects microbial populations at both within- and between-host scales. Our attempt to answer these questions inevitably led to new insights and further inquiries: How should antibiotics be deployed when the resistance phenotype of the infecting pathogen is unknown? Is combination therapy consistently superior to alternative strategies in suppressing resistance? How does horizontal gene transfer, especially mediated by plasmids, alter the evolutionary landscape compared to purely chromosomal resistance? What are the pharmacodynamic implications of combining antibiotics? And finally, how can we reliably quantify bacterial population dynamics under inhibitory conditions to adequately explore these drug interactions?

In the following sections, we revisit these core questions and summarize the key insights gained throughout this thesis.

### 5.1 Is combination therapy the best treatment strategy?

Determining whether combination therapy is the optimal regimen is not straightforward, because the very definition of therapeutic “success” remains contested and aspects such as tolerability are rarely built into current models and population level success is not always the best outcome for each individual. In Chapter 2 we therefore focused on three population-level outcomes that also benefit individual patients, well knowing that these do not cover the full complexity: (i) prevention of double resistance, (ii) suppression of single resistance, and (iii) reduction of the overall infection burden.

Using an in-vitro hospital-ward model we tested these endpoints under three scenarios. In the

prevention setting (no pre-existing double resistance) and the containment setting (double resistance already present), both parametrised with realistic basic reproduction numbers ( $\mathbb{R}_0$ ), combination consistently achieved the lowest levels of single and double resistance; mixing and cycling performed nearly as well, whereas monotherapies allowed single resistance to accumulate. A sensitivity analysis based on a stochastic model calibrated to the experiments confirmed the dominance of combination across a wide parameter range.

Because double resistance appeared only rarely in those realistic scenarios, we created an intentionally extreme emergence scenario in which every incoming patient carried a single-resistant strain. Even under this strong selection pressure, combination therapy generated the fewest new double-resistant infections, while the untreated control produced the most.

At first sight these findings contradict two common reservations: (i) that combination inevitably selects pre-existing double resistance, and (ii) that using more drugs must create more resistance. In our containment scenario small minorities of double-resistant bacteria indeed persisted for long periods—even without treatment—so their eradication seems unlikely under any regimen. Crucially, however, the practical challenge is to prevent new double-resistant strains, and here combination outperformed all alternatives. Likewise, the correlation between total antibiotic use and resistance [Gregory2018] does not hold when both drugs are applied simultaneously to block plasmid transfer: in our experiments ‘‘more drugs’’ meant less resistance.

The mechanistic explanation hinges on the contrast between purely vertical (chromosomal) and horizontally transferable (plasmid) resistance. For chromosomal genes the probability of double resistance scales with mutation rate and the number of bacteria carrying single resistances. For plasmids it scales with the probability that A- and B-resistant bacteria meet and conjugate. Combination therapy tackles both facets: it reduces the prevalence of single-resistant infections across the ward and suppresses plasmid exchange within super-infected hosts, whereas withholding treatment maximises both processes.

Admittedly, it is not surprising that our in-vitro results align with theoretical predictions: both approaches share the same core assumptions and omit major real-world complexities such as host heterogeneity, variable pharmacokinetics, microbiome interactions, and treatment tolerability. Nevertheless, the experimental-epidemiology framework offers a compelling middle ground. By using real bacterial strains and clinically relevant antibiotics, it adds biological realism that pure

modelling lacks, yet it still controls key confounders and remains far more affordable and ethically straightforward than large-scale clinical trials.

What I take away from this work is that the observation that more antibiotic use leads to more resistance should not discourage appropriate treatment. While this may hold at large scales, it does not necessarily apply in individual settings like hospitals. There, it is more important to treat effectively—especially to eliminate resistant bacteria. Combination therapy does this most reliably. Used appropriately, antibiotics can sometimes reduce resistance rather than promote it.

## 5.2 Can luminescence serve as a proxy for net growth?

To analyse drug interactions on a large scale, we adopted the bioluminescence assay of [1] and tested its suitability at inhibitory concentrations in Chapter 3.

To understand when luminescence can approximate net growth, we formalized the dynamics of light emission in simplified equations. In this framework, the change in light intensity approximates: (i) the change in cell number, if the per-cell light emission (cell-specific luminosity) remains constant, and (ii) the change in biomass, if the cell-specific luminosity scales with cell mass. In our experiments, we observed that the change in light intensity consistently matched the estimated change in biomass more closely than the estimated change in cell number, making (ii) the better assumption. This means we can typically use the change in luminescence as a proxy for biomass change, but only approximates cell number when changes in cell size are slow compared to changes in population size.

Conducting this study also highlighted the importance of knowing what each assay actually measures. CFU estimates the fraction of culturable cells and is sensitive to loss of culturability or antibiotic carry-over; OD combines signals from both live and dead biomass; and luminescence assays respond to changes in cell-specific luminosity. These distinctions are crucial when interpreting decline rates based on these read-outs, as each method implies a different operational definition of “death.”

These insights provided the basis for Chapter 4, where luminescence enabled us to quantify the population dynamics of antibiotic combinations across sub- and super-MIC ranges.

### 5.3 Can interaction data from sub-MIC concentrations be extrapolated to the killing range?

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### 5.4 General concluding note

This thesis explores the selection pressures shaping antimicrobial resistance evolution within hosts and across host populations.

Throughout, I have tried to be explicit about the limitations of the models and assays employed. Rather than dwelling on individual data points, I have focused on the patterns that persist across systems. Some of the most valuable lessons arose from serendipitous observations masquerading as obstacles; learning to pause and analyse such “nuisances” proved more fruitful than pushing past them.

Methodologically, I hope the insights gained with bioluminescence assays will assist others who wish to quantify population dynamics at inhibitory drug levels. At the same time, the pitfalls encountered with both CFU counting and bioluminescence should serve as a cautionary tale.

We found supporting evidence that, in concordance with results for chromosomal resistance, the rise of plasmid-mediated resistance can best be faced using combination therapy. Any strategy, however, must ultimately be validated in clinical settings, where host heterogeneity, patient-specific pharmacokinetics, and pathogen evolution can substantially alter outcomes.

While it is still not clear whether combination therapy is categorically superior to alternative strategies, our work on the pharmacodynamics of drug combinations demonstrates that specific drug interactions can influence population dynamics, and that interactions at inhibitory concentrations are not always identical to those at sub-inhibitory levels. Considering this in future studies—by explicitly distinguishing synergistic from antagonistic combinations—could help clarify the inconclusive outcomes of current study designs.

However, the “perfect” treatment strategy alone will not curb AMR. While this thesis focused on uninformed (empirical) therapy, the informed treatment guided by rapid diagnostics and real-time genomic surveillance is preferable and As point-of-care sequencing and resistance-gene panels mature, these systems will become more available in the future. At the population level, relatively simple and scalable public-health interventions have already demonstrated their impact.

England's national infection-control programme—including mandatory MRSA reporting, patient screening, isolation protocols, and care bundles—was associated with a 97 % reduction in ICU-acquired MRSA bloodstream infections between 2007 and 2012 [2]. Israel's centrally coordinated outbreak response, led by the Ministry of Health and involving mandatory case reporting, patient cohorting, and active oversight, achieved an approximately 80 % decline in the incidence of carbapenem-resistant *K. pneumoniae* within two years [3]. Conjugate vaccine roll-outs offer a complementary success story: following the introduction of PCV7 in the United States, penicillin-nonsusceptible invasive pneumococcal disease declined by over 80 % in children under two, and by nearly 50 % in adults aged 65 [4].

Together, these examples demonstrate that coordinated, evidence-led action—often straightforward to implement—can profoundly reshape the trajectory of antimicrobial resistance evolution.

## 5. CONCLUDING REMARKS

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