Microbes associate in nature forming complex communities, but they are often studied in purified form. Here I show that neighbouring species enforce the re-distribution of carbon and antimicrobial molecules, predictably changing drug efficacy with respect to standard laboratory assays. A simple mathematical model, validated experimentally using pairwise competition assays, suggests that differences in drug sensitivity between the competing species causes the re-distribution of drug molecules without affecting carbon uptake. The re-distribution of drug is even when species have similar drug sensitivity, reducing drug efficacy. But when their sensitivities differ the re-distribution is uneven: The most sensitive species accumulates more drug molecules, increasing efficacy against it. Drug efficacy tests relying on samples with multiple species are considered unreliable and unpredictable, but study demonstrates that efficacy in these cases can be qualitatively predicted. It also suggests that living in communities can be beneficial even when all species compete for a single carbon source, as the relationship between cell density and drug required to inhibit their growth may be more complex than previously thought.

INTRODUCTION

The notion of pure culture is fundamental in microbiology. The isolation and growth of individual species is justified by the seemingly unpredictability [1] of in vitro assays when cultures contain multiple species. For antimicrobial sensitivity tests, this means the same microbe can show different sensitivities to the same drugs, depending on whether the cultures contain one or multiple species [2-4]. But microbes live in communities in nature, even when they are mostly competing for resources [5]. The question is, therefore, whether co-existing species can threaten drug efficacy in vivo with respect to standard sensitivity tests. Current data suggest they do [4, 6-9], notably reducing drug efficacy, and the result is that infections containing multiple species are more difficult to treat [10-12]—requiring alternatives to antibiotics altogether [10, 12]—and waters more difficult to treat [13]. This phenomenon has been associated with biofilm formation [14, 15], stochastic phenotypic variations of isogenic bacterial populations [14, 15], signalling molecules [14, 15] or enzymatic degradation of antimicrobials [14, 15]. But questions still remain about its predictability, and why, sometimes, drug efficacy seems to increase [6].

Below I present a model that provides a passive physical mechanism to explain and, perhaps more importantly, predict this phenomenon. The model relies on Fick’s first diffusion law and aims to understand how microbial growth changes the flow of carbon and antimicrobial molecules, and, thus, influence drug efficacy against all species exposed. Note that, when surrounded by neighbours, species attain lower densities within a community with respect to that in pure culture because carbon is shared between multiple species [16]. Now, the model predicts that carbon and antimicrobial molecules will distribute evenly among all species exposed if they have similar sensitivity to the antimicrobial. The result is relatively less drug molecules per cell of each species, and therefore lower drug efficacy. If one or more species are not sensitive to the antimicrobial, carbon molecules still distribute evenly as it is an active process [17, 18] but drug molecules are not: They flow back through diffusion from non-sensitive species into the environment, re-exposing those that are sensitive to the drug. Here, the model predicts relatively more drug per cell of sensitive species resulting thus in higher drug efficacy. These predictions were maintained across a range of parameter values for carbon uptake rate, carbon affinity, and biomass yield—fundamental components of the growth function in microbes [19].

To validate these predictions, I measured the efficacy of tetracycline against Escherichia coli Wyl in standard sensitivity assays using pure cultures, cultures with equal proportion of another microbe with similar drug-sensitivity (Salmonella typhimurium), and cultures containing equal proportion of another microbe now tolerant to tetracycline (Escherichia coli GB(c)). Consistently with the theoretical predictions, inhibiting Wyl required more tetracycline—with respect to pure cultures—in the presence of equally sensitive neighbours and less tetracycline in the presence of drug-tolerant neighbours.

RESULTS

Consider \( n \) phenotypically distinct species competing for a limited resource, \( C \), and exposed to a drug, \( A \), supplied at concentration \( A_0 \),
(0) = \alpha_0, cast as the following model:

\[ \dot{S}_j = \frac{G_j(C)S_j}{I_j(A)}, \]  
\[ \dot{A}_j = -dA_j + \phi_j(A_e - A_j)S_j, \]  
\[ \dot{A}_e = -rA_e - \sum_{j=1}^n \phi_j(A_e - A_j)S_j, \]  
\[ \dot{C} = -\sum_{j=1}^n U_j(C)S_j, \]  

where \( K_j \) is the half-saturation parameter—the affinity of individuals from species \( j \) for the limited resource \( C \) is therefore given by \( 1/K_j \). The growth rate of each species, at a given resource concentration is denoted by \( \dot{G}_j(C) := U_j(C) \cdot y_j \) where \( y_j \) is the biomass yield per unit of resource in individuals from species \( j \). Note that, for simplicity, I assume that \( y_j \) does not vary between pure and mixed culture conditions. Biomass yield depends on resource availability [19] and competition for a common carbon source will likely change \( y_j \) in all competing species, albeit these changes can be difficult to measure [20]. Their growth inhibition by drug \( A \) is described qualitatively by the Hill function [21].

\[ I_j(A) := \frac{1}{1 + (A_j/K_j)^a}, \]  
where \( 0 \leq I_j(A) \leq 1 \)  

Scaling the growth function \( G_j(S) \) similarly to bacteriostatic drugs [22]. This function is dimensionless and has two parameters. First, \( a \) is a dimensionless Hill coefficient which characterises the co-operativity of the inhibition. And second, \( K_j \) is the affinity of drug \( A \) for its target. It can be derived from the drug concentration required to half the maximal growth rate, so that \( A_{50} = 1/K_j \rightarrow K_j = 1/A_{50} \) [21]. For the sake of simplicity, I assumed that drug \( A \) diffuses from the environment into cells of species \( j \), and vice versa, following Fick’s first diffusion law [23] with a diffusion coefficient \( \phi_j \); and part of \( A \) being lost to chemical stability [24] at a rate \( d \). This is not an unreasonable assumption given that antimicrobial molecules can indeed diffuse through cell.

Fig. 1  \( S_1 \) drug sensitivity profiles in pure and mixed culture growth conditions alongside species \( S_2 \). A Growth of species \( S_1 \), with different parameter values (\( k_1, p_1, \) and \( y_1 \)), after 24 h of growth in the presence of different antibiotic concentrations. I aggregated the resulting dose-response profiles (blue) to create a density map from low predicted cell density (white) to high predicted cell density (black). B-D IC\(_{50}\) antibiotic concentration inhibiting 90% (IC\(_{90}\)) the growth predicted without drug, resulting with different parameters values for the half-saturation parameter \( k_1 \) (B), maximal carbon up-take \( p_1 \) (C), or biomass yield \( y_1 \) (D) in eq. 1 when species \( S_2 \) is drug-sensitive. The IC\(_{50}\) for species \( S_1 \) growing as pure cultures is shown in grey, and growing in mixed culture with \( S_2 \) are shown in black. The parameter values for species \( S_2 \) were fixed at a value noted by a black arrow on the y-axis, followed by a dotted black line. E–G Change in IC\(_{50}\) as in Fig. (B–C), when the competing species \( S_2 \) is not drug-sensitive (resistant or tolerant). Parameter values used can be found in Table 1.
membranes facilitated by membrane proteins [25]. Consequently, drug A is released in active form following the death of target and non-target species.

For my first computation I set the number of species \( j = 2 \), to facilitate later experimental validation, where \( i_1(A) = i_2(A) \) and \( G_1(C) = G_2(C) \). Thus, individuals from both species are sensitive to A and phenotypically identical. Given \( E_3 \), the density of individuals from either species as pure cultures, after 24 h of incubation, declines with higher drug concentrations consistently with standard clinical protocols [26] (Fig. 1A). To allow experimental validation, I calculated the concentration of A inhibiting the growth of the pure cultures by 90% (IC\(_{90}\)) as commonly used and non-parametric, Mann–Whitney U-test between IC\(_{90}\)s is shown in the inset. Raw data is shown as dots, whereas the boxes represent median (centre of the box), 25th, and 75th percentiles of the dataset. The whiskers show the most extreme data points that are not outliers. C. Reding

Table 1. Model parameters for Eqs. 1a–d, 2 and 3.

| Parameter       | Description                        | Value     |
|-----------------|------------------------------------|-----------|
| \( \mu_j \)     | Maximal carbon uptake rate          | 1.25 mg/OD/h |
| \( K_j \)       | Half-saturation constant            | 0.5 mg/mL  |
| \( Y_j \)       | Biomass yield                       | 0.65 OD/mg |
| \( d \)         | Drug degradation rate               | 10^{-1}/h  |
| \( k_j \)       | Affinity of drug A for species \( j \) | 0.1 mL/µg |
| \( \psi_j \)    | Diffusion coefficient               | 10 µL/OD/h |
| \( \alpha \)    | Hill coefficient                    | 2 (dimensionless) |
| \( A_0 \)       | Initial drug concentration          | 2 µg/mL    |
| \( C_0 \)       | Initial carbon concentration        | 2 mg/mL    |
| \( S_1(0) \)    | Inoculum size for species \( S_1 \) | 10^{-3} OD |
| \( S_2(0) \)    | Inoculum size for species \( S_2 \) | 10^{-3} OD |

With respect to the parameter values is nonmonotone, which means certain values for these parameters can maximise susceptibility for drug A. The neighbouring species shows similar, albeit not identical, changes in sensitivity (Fig. S2). This non-linearity is caused by cultures not reaching the equilibrium, for all drug concentrations and parameter values tested, within the standard 24 h incubation times. If, in the model, cultures are allowed to grow for longer and reach equilibrium, the IC\(_{90}\)-cell density profile can change (Fig. S1B). This phenomenon is exacerbated if both species grow in mixed culture conditions, where both become phenotypically more tolerant to drug A (Fig. 1B–D, black). If I were to target, say, individuals from species \( S_1 \), doing so when the species is surrounded by \( S_2 \) would require more drug. This is the case, for example, of pancreatic ductal adenocarcinoma treated with gemcitabine when bacteria grow within the tumour’s microenvironment [33]. More generally, genotypes analog to \( S_1 \) should increase their drug tolerance when they are surrounded by similarly sensitive species. Despite the freedom in parameter values in the model, this prediction is robust to changes in biomass yield (\( y_\) ) or maximal carbon uptake (\( y_\)) of species \( S_2 \) (Fig. S3).

To test this hypothesis, I mixed equal proportions (cell/cell) of Escherichia coli Wyl and Salmonella typhimurium SL1344 in minimal media supplemented with different concentrations of tetracycline (see Methods). Note that the inoculum effect is not linear with respect to the size of inocula [31, 32] unless the difference between inocula is tenfold or more, which is not the case here. Tetracycline can diffuse passively into cells of both gram-negative species [34], who also have similar sensitivity to this antibiotic: 0.232 ± 0.003 and 0.276 ± 0.016 µg/mL of tetracycline (mean IC\(_{90}\) = 95% confidence, with \( n = 8 \) replicates, see Figs. S2 and S4). This approximates to \( i_1(A) = i_2(A) \), as laid out by the theory above. The chromosome of E. coli Wyl carries yfp, gene encoding a yellow fluorescence protein (YFP), so I tracked its density in mixed culture conditions. Consistently with Eq. 1a–d, the bacterium was around 23% more tolerant to tetracycline when it grew in mixed culture with \( S. typhimurium \) (Mann–Whitney U-test \( p = 1.554 \times 10^{-4} \), ranksum = 36 with \( n = 8 \) replicates, Fig. 2A).

Next, I explored in the model the case where individuals from both species have different sensitivities to drug A (\( i_1(A) \neq i_2(A) \)). This scenario is akin to pathogens such as C. difficile growing alongside human cells [35] where the latter are unaffected by the drug (\( i_2(A) \approx 1 \)). The model now predicts a subset of values for \( K, y, \)
and \( \mu \) that make \( S_1 \) more sensitive to the drug in the presence of individuals from species \( S_2 \) (Fig. 1E–G), whether \( S_1 \) is not affected by drug \( A \) or is resistant to it for example through efflux pumps [36] (see Supplementary Text). To test this prediction, I mixed equal proportions (cell/cell) of two constructs of Escherichia coli with different sensitivities to tetracycline. One construct is Wyl, used above, who is sensitive to the antibiotic. The other construct is GB(c), harbouring a non-transmissible plasmid carrying the gene tet(36) [37] and, therefore, resistant to the drug. Tetracycline binds to the bacterial ribosome, inhibiting protein synthesis [38], and tet(36) provides ribosomal protection against tetracycline [37] without degrading the antibiotic. The IC\(_{90}\) for this construct was 6.106 ± 0.272 µg/mL of tetracycline (mean IC\(_{90}\) ± 95% confidence with \( n = 8 \) replicates). Now, \( I_1(A) \times I_2(A) \) satisfies the assumption above. The IC\(_{90}\) for \( E. coli \) Wyl was 0.232 ± 0.003 µg/mL of tetracycline as pure culture. Growing alongside drug-resistant GB(c), however, it was 0.112 ± 0.003 µg/mL (Fig. 2B).

It is noteworthy to highlight the two processes at play. On the one hand, the growth of each species is limited due to competition for the same resource—also known as competitive suppression [16]. It could be conjectured that the observed variations in IC\(_{90}\) are caused by variations in growth, with respect to pure culture conditions, caused by competitive suppression. Indeed, \( E. coli \) Wyl grows less, with respect to pure culture conditions, in the presence of tetracycline-resistant \( E. coli \) GB(c), and yet, drug efficacy against Wyl was highest in the presence of this competitor. Now, the relative abundance of each species during the competition determines the flow and distribution of antimicrobial molecules given they diffuse passively following Fick’s law. Since glucose is actively transported into the cytoplasm, even against concentration gradients [17, 18], the model predicts virtually no effect on the carbon uptake by each species as Fig. S5 illustrates. In other words, the carbon source C is depleted more rapidly when more species use it, limiting the growth of each competing species with respect to pure culture conditions.

On the other hand, the sensitivity to drug \( A \) determines the fraction of carbon that gets used by each species. When \( I_1(A) = I_2(A) \), Eq. 1a–d suggest that individuals from both species accumulate similar amounts of drug \( A \) (Fig. S7A–C). This means that, analog to the carbon, drug \( A \) is depleted more rapidly, limiting the exposure of the target species \( S_1 \). When combining both processes for carbon and drug molecules, the result is relatively fewer drug molecules per cell in both species with respect to that predicted in pure culture. However, when \( I_1(A) \neq I_2(A) \), by virtue of different affinities of drug \( A \) for each species \( (\kappa_2 \neq \kappa_3) \), the accumulation of drug molecules is uneven (Fig. S7D–I). Soon after the exposure to drug \( A \), the species \( S_2 \), with the least affinity for it, and the environment are in equilibrium. In other words, the number of drug molecules that diffuse into \( S_2 \)-cells are the same that diffuse from these cells back to the environment. These molecules, in turns, are accumulated by the species with the most affinity for the drug. Here, integrating both processes results in more molecules per cell in the species with highest drug-sensitivity (\( S_1 \)).

To verify this hypothesis, I estimated the content of tetracycline in \( E. coli \) Wyl by dividing the bacterium’s culture density, measured in relative fluorescence units to allow tracking in mixed culture conditions, by the concentration of tetracycline defining its IC\(_{90}\). The estimates closely resemble the theoretical predictions in Fig. 2C: \( E. coli \) Wyl contains ∼20% less tetracycline growing next to Salmonella typhimurium and 65% more tetracycline growing alongside drug-resistant GB(c) (Fig. 3).

**DISCUSSION**

Why do microbes form communities? It was recently [5] suggested that bacteria rarely work together based on the biological interactions between them. But fundamental physics could draw a different picture, and help answer the question. If, as my study suggests, two species growing together can tolerate tetracycline better than they would otherwise do in isolation, it is not unreasonable to hypothesise that forming such communities can help diminish the effect of toxic molecules in nature. For example, to grow closer to an antibiotic-producing microorganism and benefit from the carbon available or tolerate chemotherapies. Or, indeed, taking over a particular niche in case of drug-tolerant microbes without producing specialised molecules such as bacteriocins [39]. All as a by-product of a physical law.

It should be stressed that my theory is not specific to *Escherichia coli* and tetracycline, so I anticipate that other species and molecules obey the same principle described here. An example can be found in the interactions between tumours and bacteria, and the resulting tolerance to chemotherapies [8]; or between microbial pathogens [6]—albeit reliably predicting the outcome is still challenging [5]. Now, there are limitations in the model some of which are imposed by experimental conditions. For simplicity, the Fick’s term in Eq. 1b and c has no spatial derivative as it would be expected. However, during the experiment I shook the cultures to homogenise the distribution of tetracycline and nutrients. Here the aforementioned derivatives can be approximated as the difference in drug molecules between environment and cytoplasm. The model also assumes reliance on a single carbon source.

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**Fig. 3** Relative content in drug molecules changes as predicted by model. **A** Difference in drug content per \( S_1 \)-cell at the IC\(_{90}\) between pure culture and mixed culture conditions. Positive and negative values denote more drug in \( S_1 \)-cells in pure and mixed culture conditions, respectively. Lack of difference is shown as a horizontal, dotted line. **B** Experimental estimation of the difference in relative cell content in the bacterium *Escherichia coli* Wyl. Raw data for Wyl is shown as red dots. Lack of difference is shown as a horizontal, black line and the 95% confidence of the lack of difference as a horizontal, dotted line.

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In reality, however, I used two: Glucose as the main carbon source, and casamino acids. The latter can indeed be used as a carbon source when glucose is scarce [40]. The concentration I used (0.1% w/v) was low enough to enable the growth of the microorganisms without showing diauxic growth dynamics typical from cultures that are sustained on multiple carbon sources [41]. While speciation might indeed occur over time to avoid competition [42], is it really important to my conclusions?

The model that I present here is, like all models, wrong in some ways but also useful in others. The goal of my study is to demonstrate that growing alongside other microorganisms can predictably change the efficacy of a drug used against one—or against all—of them. It is, from the model standpoint, indifferent which carbon type is used to grow so long the relative frequency of the microbes surrounding the target is sufficiently high. Figure 1 shows the changes in IC_{50} are consistent regardless of the differences in growth parameters, akin to those existing between microorganisms using different carbon sources [43] or metabolic efficiencies [19]. Moreover, the model and experiment use two species mixed in the same proportion for the purpose of pinpointing the physical mechanism. In nature, communities are more complex and this phenomenon is likely to be more nuanced, if not even stronger, based on the ratio between the number of cells of the target species and its neighbours.

My contribution with this model is to demonstrate that, contrary to current belief, the change in drug efficacy derived from the growth of other microorganisms is not arbitrary. There is, at least, one simple physical mechanism behind the changes in efficacy and, therefore, the changes can be qualitatively predicted—a necessary step if competition is to be harnessed [5] and used either in the clinic or treatment of pollutants in the soil.

**METHODS**

**Media and strains**

The strains of *Escherichia coli* GB(c) and Wyl were a gift from Remy Chait and Roy Kishony, and non-labelled *Salmonella typhimurium* SL1344 a gift from Markus Arnoldini and Martin Ackermann. Experiments were conducted in M9 minimal media supplemented with 0.4% glucose (Fisher Scientific #G/0500/53) and 0.1% casamino acids (Duchefa #C1301.0250), supplemented with tetracycline. M9 minimal media (SOX) was made by mixing equal proportions (vol/vol) of two parts, part A and part B, and diluted accordingly to 1X. Part A (SOX) contains 350 g/L of K2HPO4 (Sigma #P3786) and 100 g/L of KH2PO4 (Sigma #P9791); whereas part B (SOX) contains 29.4 g/L of trisodium citrate (Sigma #S1804), 50 g/L of (NH4)2SO4, 10.45 g/L of MgSO4 (Fisher Scientific #M/1050/53), I made tetracycline stock solutions from powder stock (Duchefa #0150.0025) at 5 mg/mL in deionised water. Subsequent dilutions were made from this stock and kept at 4 °C.

**Sensitivity assay**

Using a 96-pin replicator, I inoculated a 96-well microtitre plate, containing 150 μL of media supplemented with 0–0.5 μg/mL of tetracycline (for *E. coli* Wyl and *S. typhimurium*) or 0–15 μg/mL (for *E. coli* GB(c)), with 1 μL from an overnight culture of each strain (~2·10⁸ cells) to measure drug sensitivity in pure cultures. For sensitivity assays of Wyl in mixed culture conditions I inoculated the microtitre plate, containing 150 μg/mL of media supplemented with 0–0.5 μg/mL of tetracycline, with equal number of cells from two overnight cultures from Wyl and *S. typhimurium* using 8 technical replicates for each drug concentration. For the competition between Wyl and GB(c), however, I used 0–0.2 μg/mL to measure drug efficacy more reliably given the increased drug efficacy observed against Wyl. Figure 58 illustrates that mixing 1 μL of each species, therefore doubling the inoculum size with respect to pure culture conditions, or mixing 0.5 μL to maintain the same inoculum size has no impact on the sensitivity assays consistently with the nonlinear relationship reported between inoculum size and drug efficacy [31, 32].

I incubated the microtitre plate at 30 °C in a commercial spectro-photometer, shaking at 750 rpm to ensure uniform mixing, and measured the optical density of each well at 600 nm (OD_{600}, yellow fluorescence for Wyl (YFP excitation at 505 nm, emission at 540 nm), and cyan fluorescence for GB(c) (CFP at 430 nm/480 nm) every 20 min for 24 h. I defined the minimum inhibitory concentration as the tetracycline concentration able to inhibit 90% of the growth observed in the absence of antibiotic after the 24 h incubation period.

**Culture readings**

Fluorescence protein genes were constitutively expressed with an approximately constant fluorescence to optical density ratio (Fig. 59). The number of colony forming units is positively correlated with optical density measured at 600 nm (OD_{600}) (Fig. S10). Thus, I normalised fluorescence readings with respect to optical density readings, using the ratio optical density to fluorescence that I in pure culture conditions, to track the relative abundance of Wyl in mixed culture conditions. This metric is referred to as ‘Estimated optical density’ or ‘eOD’ in the main text. Time series dataset were blank corrected prior to calculating the minimum inhibitory concentration.

**DATA AVAILABILITY**

Data in Figs. 2B, 2C, and 3B can be found in a supplementary spreadsheet. A non-parameterised, python3 implementation of Eq. 1a-d is hosted in "github.com/rc-reding/papers/tree/master/MicrobCommunities_ISMECOMMS_2022" with the parameter values used in the paper being listed in Table 1.

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AUTHOR CONTRIBUTIONS
CR developed and implemented the mathematical model, designed and executed the experimental assays, the corresponding data analysis and visualisation, and wrote the paper.

COMPETING INTERESTS
The author declares no competing interests.

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