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Bacterial adaptation to sublethal antibiotic gradients can change the ecological properties of multitrophic microbial communities

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Antibiotics leak constantly into environments due to widespread use in agriculture and human therapy. Although sublethal concentrations are well known to select for antibiotic-resistant bacteria, little is known about how bacterial evolution cascades through food webs, having indirect effect on species not directly affected by antibiotics (e.g. via population dynamics or pleiotropic effects). Here, we used an experimental evolution approach to test how temporal patterns of antibiotic stress, as well as migration within metapopulations, affect the evolution and ecology of microcosms containing one prey bacterium, one phage and two protist predators. We found that environmental variability, autocorrelation and migration had only subtle effects for population and evolutionary dynamics. However, unexpectedly, bacteria evolved greatest fitness increases to both antibiotics and enemies when the sublethal levels of antibiotics were highest, indicating positive pleiotropy. Crucially, bacterial adaptation cascaded through the food web leading to reduced predator-to-prey abundance ratio, lowered predator community diversity and increased instability of populations. Our results show that the presence of natural enemies can modify and even reverse the effects of antibiotics on bacteria, and that antibiotic selection can change the ecological properties of multitrophic microbial communities by having indirect effects on species not directly affected by antibiotics.

1. Introduction

Antibiotics are frequently released into the environment in an active form, where they exert selective pressure on bacteria [1,2]. Already very low antibiotic concentrations (10–100 times below lethal conditions) can increase the relative abundance of resistant bacteria and select for *de novo* resistance by increasing the rate of adaptive evolution [1,3]. Furthermore, and much less studied, antibiotics could have indirect cascading effects on species that are not directly affected by antibiotics but that predate or parasitize bacterial cells. Antibiotics could thus have unexpected and indirect, community-wide consequences for the trophic dynamics and functioning of natural microbial ecosystems.

Previous studies have shown that, even in the simplest cases (e.g. a single species evolving in response to a single antibiotic), the rate of bacterial adaptation is affected by both the final concentration of the antibiotic (i.e. the strength of selection) and the rapidity with which the final antibiotic concentration is realized [4–6]. Antibiotics released into the environment are likely to exert even more complex patterns of selection gradients due to spatial and temporal variation in the leakage of antibiotics [1,6–9]. Fluctuations in antibiotic concentrations could affect resistance evolution by directly impacting population sizes, and therefore the supply of beneficial mutations [10]. First,

even if the mean antibiotic concentration is the same, population sizes will tend to be lower in fluctuating versus stable antibiotic environments because population declines in unfavourable antibiotic conditions might not be fully compensated by population increases in favourable conditions [11]. Second, the amplitude of the fluctuations will impact population sizes even if the mean level of antibiotic is held constant because increases in the amplitude of the fluctuations will expose populations to more extreme conditions [12]. Third, the period of unfavourable conditions will typically be longer in autocorrelated environments where conditions are more similar in time ('red noise'), probably resulting in lower population sizes [13]. Finally, migration among subpopulations can dampen the negative impacts of environmental fluctuations by rescuing local populations from extinction and by spreading advantageous mutations [13,14]. While temporal fluctuations could make bacterial populations more prone to stochastic extinctions [15], the negative effects of antibiotics could be overcome via adaptation [16]. Although these factors have been addressed individually for single populations, here we seek to assess the combined influence of these factors and their interactions in a community context.

Bacterial adaptation to antibiotics could be further affected by the biotic interactions with the surrounding microbial community. For example, predators and parasites reduce bacterial densities in natural environments [17] and could thus constrain the emergence of resistance mutations. In addition, bacterial consumers exert selection for bacterial defence evolution [18,19], which could conflict with antibiotic resistance selection due to clonal interference (all mutations need to arise in the same individual) or via trade-offs (antibiotic-resistance mutants might be more susceptible to predation). However, there is also potential for bacterial consumers to indirectly select for antibiotic resistance if the same genes positively affect bacterial growth in the presence of both consumers and antibiotics. For example, bacteria often reside in biofilms (mats of cells on surfaces), and this growth mode has been shown to increase bacterial fitness in the presence of both antibiotics [20] and protist predators [21,22]. Finally, concurrent selection by antibiotics and bacterial consumers could lead to higher pleiotropic growth costs, which could decrease the relative benefit of double-resistant versus single-resistant mutants when selection pressures fluctuate temporally. While migration could promote adaptation by increasing effective population sizes and spread of beneficial mutations, it could also constrain adaptation by creating mismatches between locally adapted subpopulations [23].

Here, we used a laboratory-based experimental evolution approach to study how temporal and spatial sublethal antibiotic gradients affect the evolution and ecology of multitrophic microbial communities. We set up experimental metacommunities with the bacterial strain *Pseudomonas fluorescens* SBW25, two protist predators (*Tetrahymena pyriformis* ciliate and *Chilomonas paramecium* flagellate) and a phage parasite (SBW25Φ2). Sublethal concentrations [24] of bacteria-specific gentamicin antibiotic were used to create a patchwork of environmental conditions that varied across space and over time, ranging from slightly (11% density reduction) to highly (99% density reduction) unfavourable from the perspective of the ancestral bacterium. We used a fully factorial design, and modified the mean (low, intermediate and high), variance (low or high amplitude around the mean) and autocorrelation structure (the similarity between antibiotic concentration changes as a function of the time: red noise = similar; white noise = random) of antibiotic in multi-habitat-patch communities that were connected or unconnected via migration in different treatments (electronic supplementary material, figure S1). In addition to tracking species population dynamics over the course of our 40-day-long experiment (estimated to be approx. 1000 bacterial generations), we also measured how bacterial fitness responded to the antibiotic and to all enemies at the end of the experiment.

The experiment allowed us to address the four following questions. First, does spatial and temporal variation in sublethal antibiotic concentration select for bacteria with increased growth in the presence of antibiotics? Second, do bacteria evolve defences against all the enemies of the community? Third, does antibiotic selection interact with anti-predatory defence selection (e.g. so that there is a positive or negative correlation between evolving better at growing in the presence of antibiotics and evolving defences against enemies)? Fourth, does bacterial response to antibiotics and enemies reverberate through food webs, having indirect effects on the ecological properties of the whole microbial community?

2. Material and methods

(a) Experiment overview

Multitrophic communities containing bacteria, bacterivorous protozoa and parasitic bacteriophage were assembled in microcosms (2 ml deepwell microplates). Each microcosm contained 2 ml of media along with the bacterium *P. fluorescens* SBW25 [25], predatory protists *T. pyriformis* (CCAP 1630/1W) and *C. paramecium* (CCAP 977/2A), and bacteriophage SBW25Φ2 [25]. Bacterial populations were grown on a 0.75% Luria Broth (Sigma-Aldrich; 100% LB contains 10 g l^{-1} tryptone; 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl), in attempt to mimic relatively lower bacterial densities observed in natural versus laboratory environments. The media was amended with the antibiotic gentamicin sulfate (BioChemica). Gentamicin is an aminoglycoside bactericidal antibiotic that inhibits translation, meaning that it kills bacterial cells and therefore reduces bacterial abundance [26]. Gentamicin was chosen because it is widely used in clinical environments and because it affects bacterial evolution even at sublethal concentrations [1,24,27]. Preliminary assays of the gentamicin antibiotic on axenic protist cultures indicated that gentamicin did not directly affect protist growth (electronic supplementary material, figure S2). Antibiotic concentration was varied by replacing the old media on a daily basis with fresh media without removing bacterial or protist cells (details below).

The basic unit of the experiment was a six-microcosm metacommunity. Metacommunities were subjected to four experimental manipulations in a fully factorial design where we manipulated the mean (low, intermediate and high), variance (low or high amplitude around the mean) and autocorrelation structure (the similarity between antibiotic concentration changes as a function of time: red noise, similar; white noise, random) of antibiotic concentration in multitrophic metacommunities connected or unconnected with global migration (electronic supplementary material, figure S1). Each of the 24 treatment combinations was replicated in four metacommunities, yielding 96 metacommunities and 576 total microcosms.

(b) Microcosm set-up

Pseudomonas fluorescens bacterium was first grown overnight in Luria Broth (25°C), centrifuged (11 000*g*, 3 min) and washed in deionized water. The washed cells were then inoculated into

the microcosms (2-ml deepwell microplates) to yield a final titre of 1×10^5 cells ml^{-1}. Axenic protist cultures were centrifuged (760*g*, 8 min), washed in deionized water and inoculated into the microcosms (*C. paramecium*: 2150 cells ml^{-1}; *T. pyriformis*: 30 cells ml^{-1}). Phages were isolated from 24 h SBW25 cultures as described below and inoculated into the microcosms at titres of 370 phage particles ml^{-1}.

(c) Antibiotic temporal fluctuations

Altering the concentrations of gentamicin antibiotic daily total for 40 days was used to create temporal fluctuations in bacteria-specific antibiotic stress. Briefly, microcosms were centrifuged to pellet the cells, and 90% of the supernatant replaced with fresh media at a prescribed antibiotic concentration. According to preliminary experiments, 60% of bacteria, protists and phage were retained using this procedure. Gentamicin concentration fluctuated around three mean values (0.04, 0.2 and 0.4 µg gentamicin ml^{-1}) having low (±10% of mean) or high (±40% of mean) variance. While all antibiotic concentrations reduced ancestral bacterial densities (range: 11–99% density reduction at the initiation of the experiment), part of the bacterial population remained viable in all antibiotic concentrations. Randomly generated and normally distributed time series (mean = 0, s.d. = 1) were created to produce 12 temporally uncorrelated, 'white' time series. Using spectral mimicry [28], we created 12 corresponding 'red' time series, with identical properties (same mean and variance) but with autocorrelated time structure (lag-1 autocorrelation coefficient $\rho = 0.9$; electronic supplementary material, figure S3). All subpopulations within a metacommunity experienced different time series to ensure that the results were not biased by a particular set of time series.

(d) Migration

We imposed 10% global migration between all six subpopulations within half of the metacommunities daily prior to resource replacement: subsamples of all populations were mixed together and redistributed evenly among subpopulations within the metacommunity.

(e) Population dynamics

We tracked the abundance of each species in the communities at 5-day intervals. Bacterial abundances were estimated using flow cytometry. Specifically, 25 µl of samples were analysed using the forward scatter detector on a Becton Dickinson Accuri C6 flow cytometer (flow rate = fast; minimum forward scatter threshold of 8000 based on negative control). The raw forward scatter output was analysed using the flowCore R package [29]. Bacterial cells were gated automatically as all particles within 1 s.d. of the mode, where the standard deviation was calculated as the square root of the mode. We ensured that cytometric measurements discriminated between bacterial cells and electronic and debris noise by comparing the results to sterile (but otherwise identical) media. Even though cytometry cannot discriminate between living and dead cells without specific labelling, it reliably reflects relative density differences between treatments and correlates well with colony counts on agar plates (electronic supplementary material, figure S4). We used a 96-pin replicator to plate all populations on LB agar plates at every time point to ensure that living bacterial cells could be recovered. *Chilomonas paramecium* and *T. pyriformis* protist densities were automatically estimated from three digitized microscope pictures for each microcosm with a Moticam 5 camera mounted on an inverted Motic AE2000 microscope. Image files were processed with CellProfiler [30] based on protist cell sizes. Finally, to estimate SBW25Φ2 phage densities, subsamples were amended with 10% chloroform (final concentration, v/v) and phage particles (plaques) were counted by inoculating the supernatant onto a lawn of ancestral bacteria.

(f) Bacterial adaptation

We measured adaptation of the final bacterial populations (day 40) by assaying their growth under the different predator and antibiotic treatments, and comparing their growth with the ancestral bacterial strain. Evolved bacteria were first isolated from phages by streaking on agar plates, after which we archived phage-free colonies at −80°C, which also kills the protist predators.

To estimate bacterial fitness changes in the presence of antibiotic, the growth of all evolved bacterial populations and the ancestral strain were measured in high antibiotic concentration by inoculating 2 µl of ancestral and each evolved population into 200 µl of 0.75% LB containing 0.4 µg ml^{-1} of gentamicin; bacterial densities were measured after 24 h by using flow cytometry (see above). Bacterial fitness was calculated as the growth of evolved bacterial populations relative to the growth of ancestral bacterium (values more than 1 indicate increased fitness of evolved bacteria).

To estimate bacterial adaptation to protists and phage, ancestral and all the evolved populations were grown in the presence of *C. paramecium*, *T. pyriformis* or phage SBW25Φ2 separately. Defence evolution was measured on the mean antibiotic concentration to which the bacterial populations adapted during the selection experiment (0.04 µg ml^{-1}, 0.2 µg ml^{-1} or 0.4 µg ml^{-1}). Briefly, 2 µl inoculum of all bacterial populations and ancestral bacteria were inoculated in 180 µl of media after 20 µl of each washed (see above) enemy (approx. 50 cells ml^{-1}) was added to the respective defence assay plate. The cultures were grown for 24 h (reflecting the temporal scale of antibiotic manipulation) at 25°C, after which we estimated bacterial densities using flow cytometry.

Formation of large cell aggregations has been previously connected to bacterial defence against protist predation [19,21,22]. Bacterial defence was therefore also determined as the mean number of bacterial cell aggregations observed in the digitized microscope pictures at the last sampling time point. We only included cell aggregates that were larger than the biggest protist species, *T. pyriformis*, in the analysis. *Tetrahymena pyriformis* uses a small orifice to filter in particles [22] and is thus unable to feed on cell aggregates larger than the orifice diameter. To estimate pleiotropic cost of bacterial adaptation, the growth of ancestral and all evolved bacterial populations were also measured in the absence of both antibiotics and enemies (see above).

(g) Statistical analyses

We used general linear mixed models for all data analyses. In all analyses, the dependent variable was explained with the main effects of migration, mean, autocorrelation and variability of antibiotic concentration, and all their two-way and three-way interactions. Metacommunity replicate was used as a random factor as one replicate of each treatment was found on each of four plates. We used repeated-measures analysis for time-structured data (population dynamics, predator-to-prey abundance ratio and predator community diversity data), in which metacommunity replicates were set as subject variables and sampling day as a repeated factor. Only the last sampling point was used for all 'evolutionary' models (fitness increases to both antibiotics and enemies and changes in pleiotropic growth cost) and initial bacterial densities were included as random covariates to statistically control slight density differences between different populations. Diversity was estimated as the mean Shannon's diversity index [31]. Temporal stability of populations was estimated from the time-series data as a coefficient of variation (CV: s.d./mean) of each evolving population. Excluding all three-way interaction effects

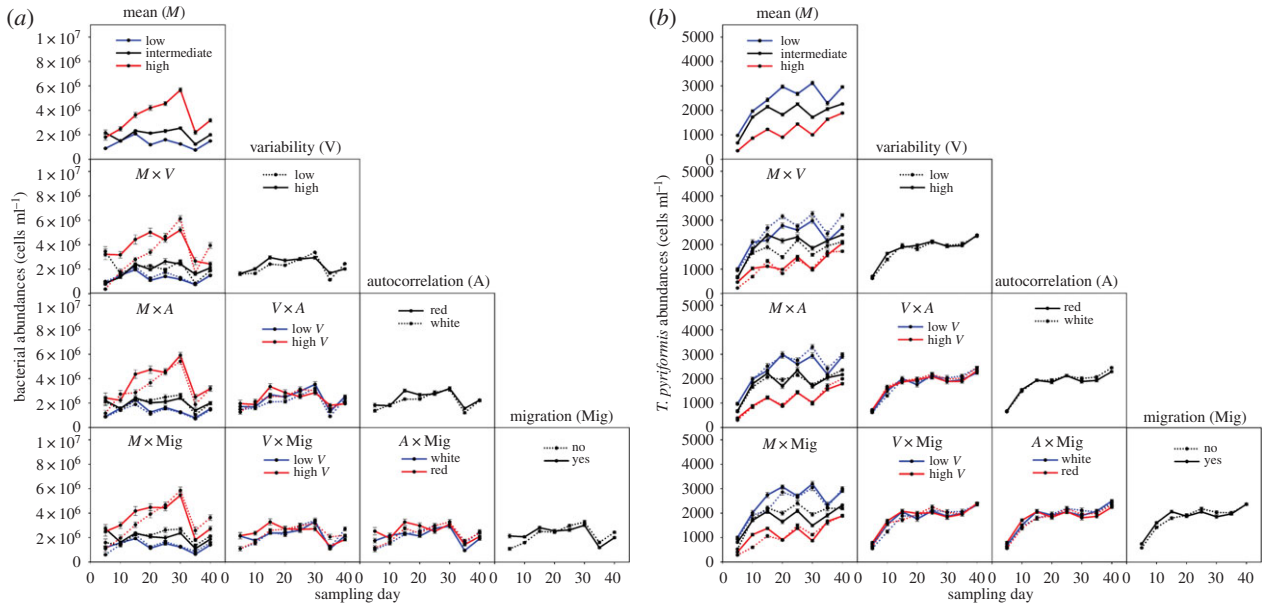

Figure 1. (*a*) Bacterial and (*b*) *T. pyriformis* ciliate abundances in different experimental treatments. The main effects of experimental factors are depicted on the diagonal, and two-way interactions between different factors below the diagonal. Line colours in interaction panels depict 'column' factors, and solid and dashed lines the effect of 'row' factor. All lines show ±1 s.e.m.

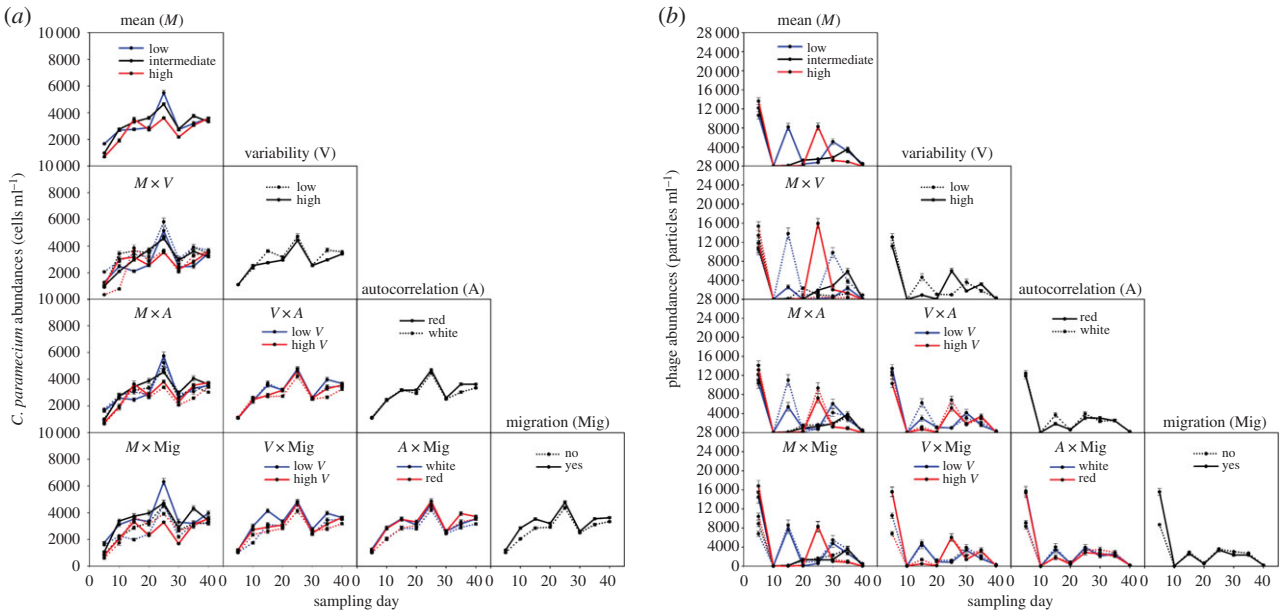

Figure 2. (*a*) *Chilomonas paramecium* flagellate and (*b*) phage Phi2 abundances in different experimental treatments. The main effects of experimental factors are depicted on the diagonal, and two-way interactions between different factors below the diagonal. Line colours in interaction panels depict 'column' factors, and solid and dashed lines the effect of 'row' factor. All lines show ±1 s.e.m.

from the models did not change the results, which confirms the robustness of our analysis.

3. Results

Results are presented in the text below, in figures 1–5, in electronic supplementary material, tables S1–S4, and summarized in electronic supplementary material, figure S5.

(a) Population dynamics

The mean antibiotic concentration had a substantial impact on bacterial growth. Counterintuitively, bacteria reached their highest densities in the high, second highest in the intermediate and lowest densities in the low antibiotic environments (figure 1*a* and electronic supplementary material, table S1). Both high variability and autocorrelation increased bacterial densities, and this was especially clear in the high antibiotic environment (figure 1*a*; electronic supplementary material, table S1). Despite having a non-significant main effect (figure 1*a*; electronic supplementary material, table S1), migration increased bacterial densities in autocorrelated populations evolving in high antibiotic environment (data not shown visually, but see significant interactions between mean, autocorrelation and migration in electronic supplementary material, table S1). All other higher-order interactions were non-significant (electronic supplementary material, table S1).

Tetrahymena pyriformis was the second most abundant enemy in the experiment overall (relative abundance: 31.0% ± 0.3%, mean ± s.e.m. throughout). In contrast to bacteria, *T. pyriformis*

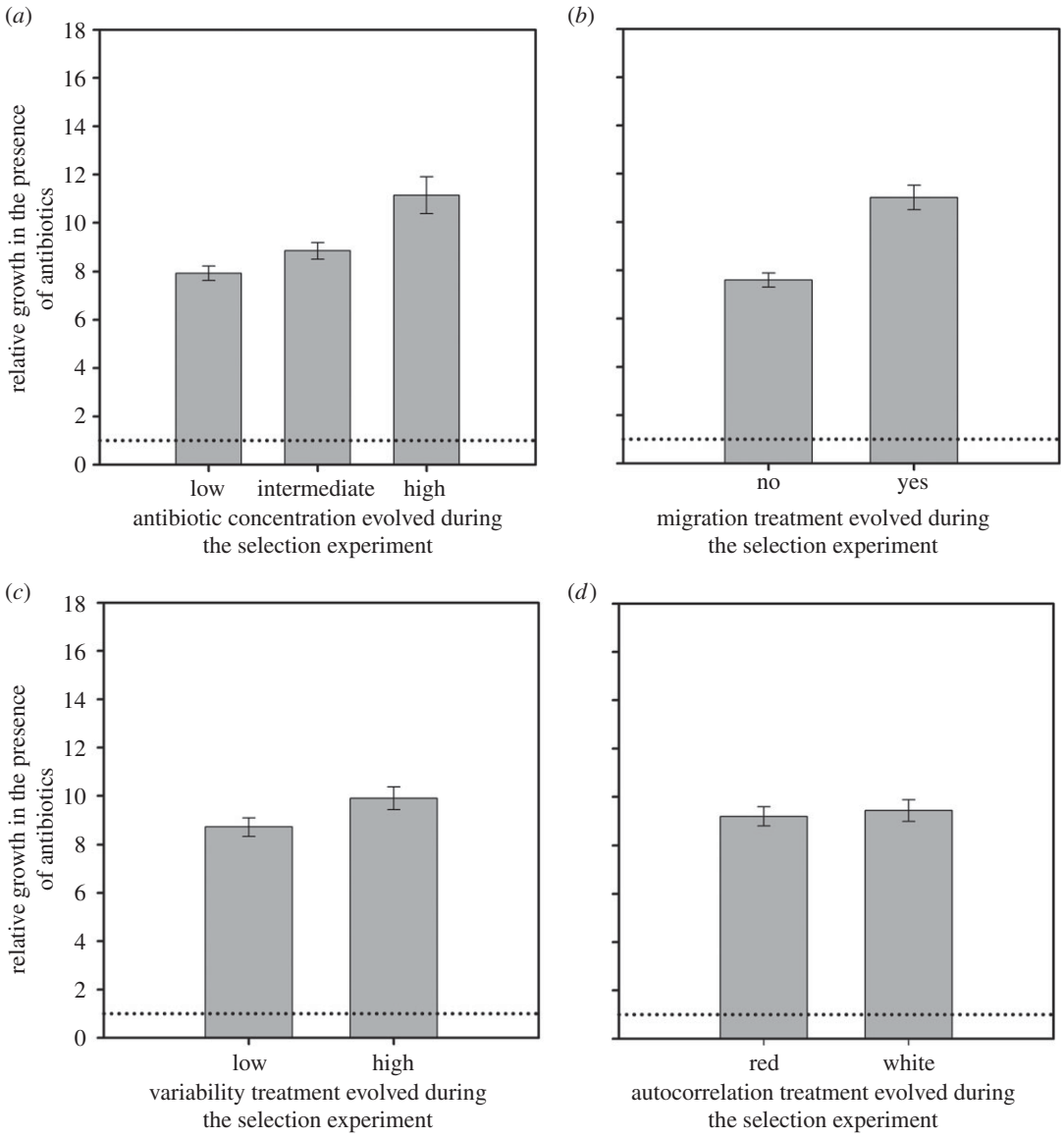

Figure 3. Bacterial adaptation to antibiotics. The effect of mean antibiotic (*a*) concentration, (*b*) migration, (*c*) variability and (*d*) autocorrelation structure for the fitness of evolved bacteria relative to ancestral bacterium. All bars show ± 1 s.e.m. These assays were performed at high antibiotic concentration.

reached their highest densities in the low, second highest in the intermediate and lowest densities in the high antibiotic environment (figure 1*b*; electronic supplementary material, table S1). High variability increased *T. pyriformis* densities only in the intermediate antibiotic environment, while no effect was found in the high, and the opposite effect was found in the low antibiotic environments (figure 1*b*; electronic supplementary material, table S1). Migration slightly increased *T. pyriformis* densities during the three first sampling points (figure 1*b*; electronic supplementary material, table S1). All other main effects or higher order interactions were non-significant (electronic supplementary material, table S1).

Chilomonas paramecium was the most abundant enemy in the experiment overall (relative abundance: 48.0% $\pm$ 0.3%). Similar to *T. pyriformis*, *C. paramecium* were at their lowest densities in the high antibiotic environment, but *C. paramecium* densities did not differ between low and intermediate antibiotic environments (figure 2*a*; electronic supplementary material, table S1). While high variability had a positive effect on *C. paramecium* densities in the high antibiotic environment, it had negative effects in the low antibiotic environment. Migration increased *C. paramecium* densities (figure 2*a*; electronic supplementary material, table S1), especially when the mean antibiotic concentration was low and when there was low variability (electronic supplementary material, table S1). All other higher-order interactions were non-significant (electronic supplementary material, table S1).

Phages were the least abundant enemy in the experiment overall (21.0% $\pm$ 0.5%). Phage reached their highest densities in the low antibiotic environment (figure 2*b*; electronic supplementary material, table S1), while phage densities did not differ between intermediate and high antibiotic environments (figure 2*b*). Both high variability and autocorrelation decreased phage densities (figure 2*b*; electronic supplementary material, table S1). Further, high variability increased phage densities in the intermediate and high antibiotic environments (figure 2*b*) but decreased phage densities in the low antibiotic environment (figure 2*b*; electronic supplementary material, table S1). Migration slightly increased phage densities, mainly due to the first sampling point (figure 2*b*). All other higher order interactions were non-significant (electronic supplementary material, table S1). In general, phage population dynamics varied considerably between different sampling points (figure 2*b*).

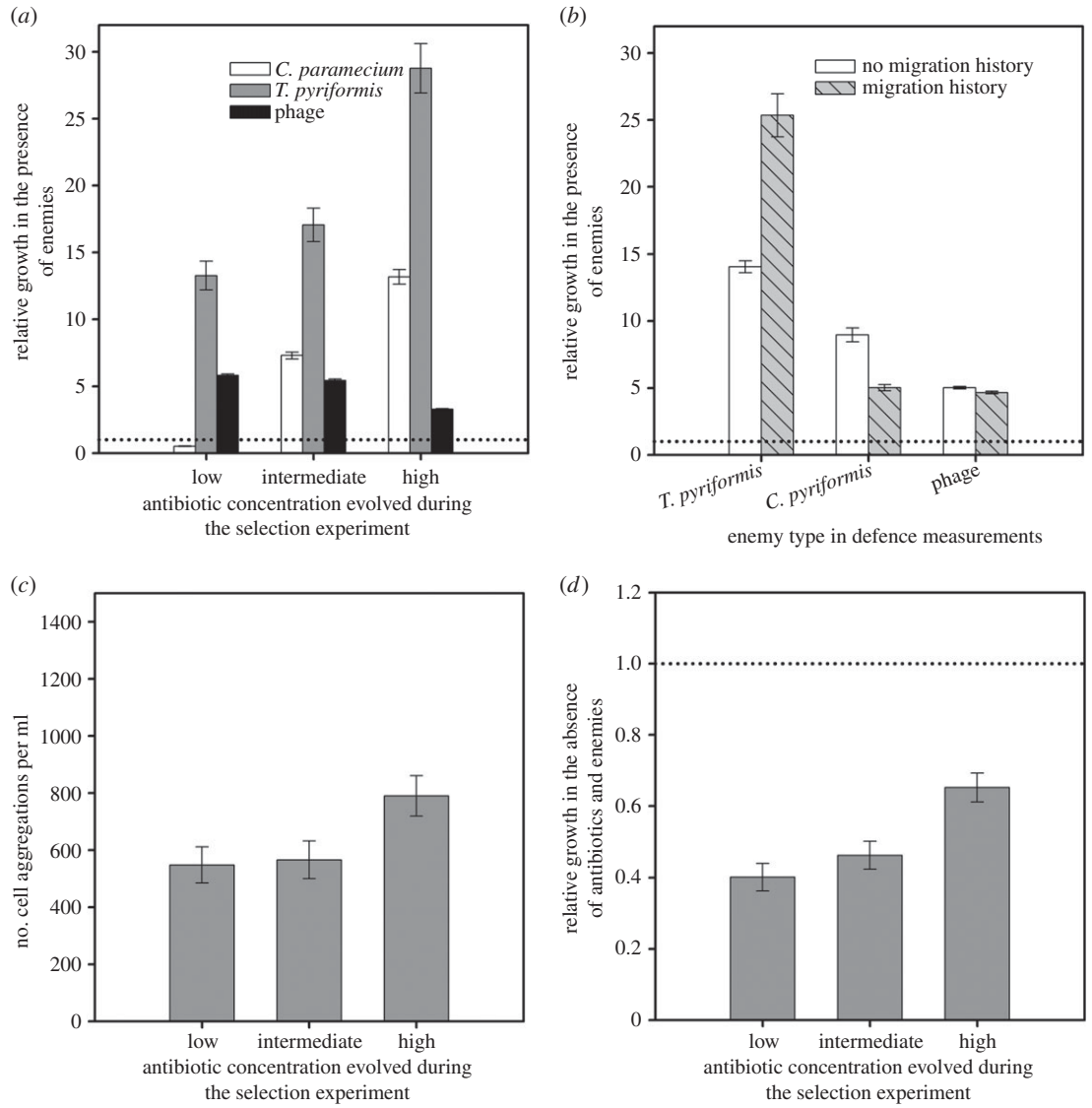

Figure 4. Bacterial adaptation to enemies. (*a*) Defence evolution against different enemies for bacteria evolved in different antibiotic environments during the selection experiment. (*b*) Defence evolution against different enemies for bacteria evolved in the absence and presence of migration during the selection experiment. (*c*) Bacterial cell aggregate formation evolution for bacteria evolved in different antibiotic environments during the selection experiment. (*d*) Pleiotropic growth cost measured in the absence of enemies and antibiotics for bacteria evolved in different antibiotic environments during the selection experiment. All bars show ± 1 s.e.m. In (*a,b,d*), quantities are relative to the ancestral strain.

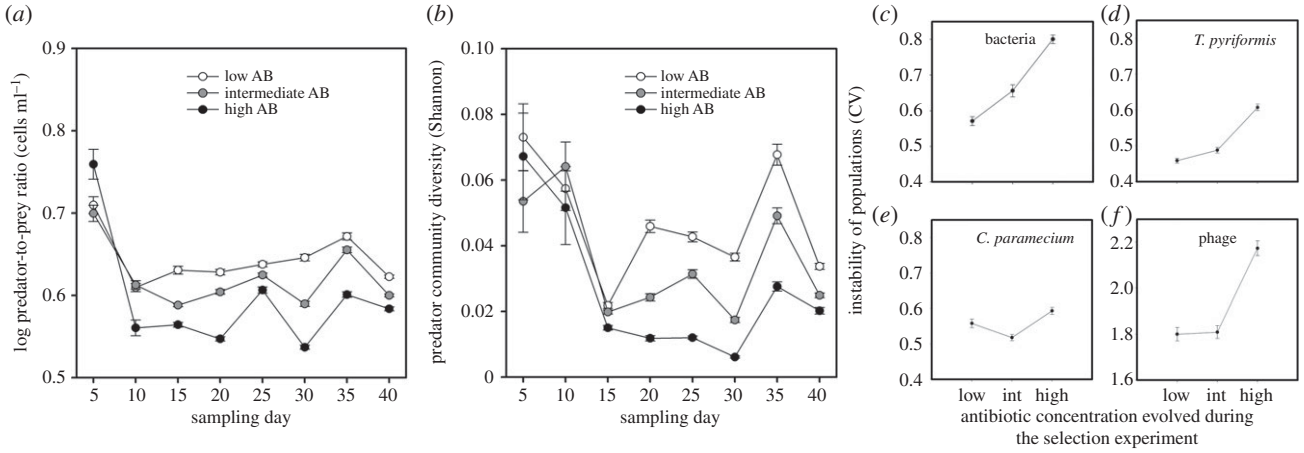

Figure 5. Differences in ecological properties of communities. (*a*) Predator-to-prey abundance ratio, (*b*) predator community diversity (alpha diversity, Shannon index) and (*c–f*) instability of (*c*) bacterial, (*d*) *T. pyriformis*, (*e*) *C. paramecium* and (*f*) phage populations evolved in different antibiotic environment during the selection experiment. AB denotes for antibiotic concentration in panels (*a,b*) and all bars show ± 1 s.e.m.

Together, these results show that, in contrast to the initial effect of antibiotics on the ancestral bacterium in monocultures, high antibiotic stress increased bacterial densities in the presence of community interactions without concomitant increases in enemy densities. Furthermore, high variability, autocorrelation and migration enhanced bacterial growth especially in the high antibiotic environment, while these same manipulations had more varied effects on enemy densities, except for migration, which has consistently positive effect on enemies (electronic supplementary material, figure S5).

(b) Bacterial adaptation to antibiotics

We first compared the growth of evolved versus ancestral bacterial populations and found that evolved bacteria reached approximately nine times higher population densities in the presence of antibiotics (mean of relative fitness: 9.3 ± 1.6). We next concentrated on evolved bacteria and found that prior evolution in high antibiotic environment led to the highest fitness increase in the presence of antibiotics (figure 3*a*; electronic supplementary material, table S2, assays performed at high antibiotic concentration). While autocorrelation or variability had no effects (electronic supplementary material, table S2), migration increased bacterial fitness in the presence of antibiotics (figure 3*b*; electronic supplementary material, table S2). All higher-order interactions were non-significant (electronic supplementary material, table S2). Together, these results show that bacteria evolved highest fitness increase to antibiotics in the high antibiotic environment and that this adaptation was further promoted only by migration.

(c) Bacterial adaptation to enemies

We found that evolved bacteria grew more efficiently in the presence of all enemies compared with ancestral bacteria: bacteria evolved the highest levels of defence to *T. pyriformis* (mean relative fitness: 19.7 ± 6.1), second highest to *C. paramecium* (mean relative fitness: 6.9 ± 0.8) and the lowest to SBW25Φ2 phage (mean relative fitness: 4.8 ± 0.2; figure 4*a*). Bacteria evolved the highest levels of defence against *T. pyriformis* in the high, second in the intermediate and lowest in the low antibiotic environments (figure 4*a*; electronic supplementary material, table S3). Further, only migration promoted bacterial defence evolution against *T. pyriformis*, while all other main effects and interactions were non-significant (figure 4*b*; electronic supplementary material, table S3). As for *T. pyriformis*, bacteria evolved the highest level of defence against *C. paramecium* in the high antibiotic environment, followed by the intermediate and low antibiotic environments (figure 4*a* and electronic supplementary material, table S3). In contrast to *T. pyriformis*, migration constrained bacterial defence evolution against *C. paramecium*, especially when bacteria had evolved in the high antibiotic concentration (figure 4*b*; electronic supplementary material, table S3). All other main effects and interactions were non-significant. In contrast to the protists, bacteria evolved the highest levels of defence to phage in the low, second highest in the intermediate and the lowest in the high antibiotic environment (figure 4*a*; electronic supplementary material, table S3). Similar to *C. paramecium*, migration constrained bacterial defence evolution against phage (figure 4*b*; electronic supplementary material, table S3), while all other main effects and interactions were non-significant.

Lastly, we looked at whether there were differences among treatments in the number of SBW25 cell aggregations that were larger than the protists. Bacteria formed the highest number of cell aggregations in the high antibiotic environment, whereas no difference was found between intermediate and low antibiotic environments (figure 4*c*; electronic supplementary material, table S3). Further, high variability increased, and migration decreased, the number of cell aggregations, especially in highly variable environments (electronic supplementary material, figure S5 and table S3). Autocorrelation decreased the number of cell aggregations in the high antibiotic environment (electronic supplementary material, figure S5 and table S3). All other main effects and interactions were non-significant.

Together, these results indicate that bacteria evolved their highest levels of defence against protists in high antibiotic environment, and their highest levels of defence to phages in lower antibiotic concentrations. While migration promoted defence evolution against *T. pyriformis*, it constrained defence evolution against *C. paramecium* and phage.

(d) Costs of adaptation

Bacterial fitness increases were costly leading to reduction in growth (maximum density) in the absence of antibiotics and enemies (mean relative fitness compared with ancestral bacteria: 0.56 ± 0.14; figure 4*d*). Surprisingly, adaptation in the low and intermediate antibiotic environments led to relatively higher growth cost compared with the high antibiotic environment (figure 4*d*; electronic supplementary material, table S2). Only high variability alleviated the growth cost: all other main effects and interactions were non-significant (electronic supplementary material, table S2).

(e) The effect of evolution on ecological properties of the community

We first explored the impact of bacterial evolution on predator-to-prey abundance ratios. The relative abundance of predators was highest in the low, intermediate in the intermediate and lowest in the high antibiotic environment (figure 5*a*; electronic supplementary material, table S4). Variability and autocorrelation slightly decreased and migration increased the ratio of enemies to bacteria (electronic supplementary material, table S4). The positive effect of migration was especially clear in the intermediate and low antibiotic environments (electronic supplementary material, table S4). Variability and autocorrelation had most negative effects in the low antibiotic environment (electronic supplementary material, table S4).

We next studied the impact of bacterial evolution on predator community diversity. Enemy communities reached their highest diversities in the low, intermediate in the intermediate and lowest in the highest antibiotic environments (figure 5*b*; electronic supplementary material, table S4). Migration increased, while variability and autocorrelation decreased the enemy community diversity (electronic supplementary material, table S4). Moreover, the positive effects of migration and the negative effects of variability were especially clear in the intermediate and low antibiotic environments. All other interactions were non-significant (electronic supplementary material, table S4).

Lastly, we examined the impact of the treatments on the population stability. We found that increasing antibiotic concentration destabilized population dynamics of all species

(figure 5*c–f*; electronic supplementary material, table S4). Only migration stabilized bacterial population densities; all other main effects and interactions were non-significant (electronic supplementary material, table S4). Migration stabilized also protist population densities, but destabilized phage densities (electronic supplementary material, table S4). Variability destabilized *C. paramecium* densities in the intermediate and high antibiotic environments, but had stabilizing effect in the low antibiotic environment (electronic supplementary material, table S4). While variability had no effect on *T. pyriformis*, it stabilized phage densities in the intermediate and high antibiotic environments, and destabilized phage densities in the low antibiotic environment, especially in populations connected by migration (electronic supplementary material, table S4). Both *T. pyriformis* and *C. paramecium* densities were stabilized in the high and destabilized in the low and intermediate antibiotic environments by autocorrelation (electronic supplementary material, table S4).

Together, these results show that increased fitness to antibiotics and protist enemies correlated with decreased enemy species abundances, low enemy community diversity and more variable population fluctuations. These ecological changes were further shaped by migration, and to a lesser degree by interactive effects between mean antibiotic concentration, variability and autocorrelation.

4. Discussion

We found that selection by antibiotics and enemies affected bacterial adaptation and changed the ecology of multitrophic microbial communities via secondary effect on species that were not directly affected by antibiotics. To answer our four original questions, we found that, first, even though increasing antibiotic concentration initially increased the mortality of the ancestral bacterium, bacteria evolved to grow better in the presence of antibiotics in all of the antibiotic environments. Second, bacteria evolved defences to all enemies in the community. Third, bacteria evolved highest fitness increases both to antibiotics and to protists in the high antibiotic environment, which is indicative of positive pleiotropy. Fourth, the ecological changes in enemy communities were clearest in the high antibiotic concentration environment, where the evolutionary changes in bacterial fitness were also the greatest. In general, mean antibiotic concentration and migration were strongest drivers of bacterial evolution, while temporal antibiotic fluctuations affected mainly species' population dynamics.

Bacterial fitness to antibiotics increased in all environments and this increase was clearest when bacteria had evolved in the high antibiotic environment, where the initial selection was also the strongest (99% density reduction of ancestral bacterium). It is known that bacteria can evolve resistance to gentamicin (aminoglycoside) via three different mechanisms: reduced uptake, alterations at the ribosomal binding sites or production of aminoglycoside modifying enzymes [26]. Different resistance mechanisms confer different degrees of resistance: reduced uptake leads to moderate resistance [32], while enzymatic modification leads to high levels of resistance [33]. Therefore, increased bacterial fitness to antibiotics could have evolved via different mechanisms in different antibiotic concentrations. Bacterial adaptation to antibiotics was further enhanced only by migration, probably due to spread of beneficial resistance mutations between populations [6,34].

The mean and migration could thus have relatively larger effects on bacterial fitness to antibiotics compared with antibiotic fluctuations, at least over the temporal scales investigated here.

Bacteria evolved the greatest level of defence against *T. pyriformis* and second highest level of defence to *C. paramecium*. Evolving defences to protist enemies correlated positively with fitness increases to antibiotics. We suggest two explanations for this pattern. First, increased fitness to antibiotics could have increased bacterial population densities and mutation supply rates, which could have promoted defence evolution against protists: anti-predatory defences have been shown to evolve more readily in larger bacterial populations [35–37]. Second, it is possible that mechanisms linked to both increased fitness to antibiotics and increased levels of defence to protist enemies were positively correlated (pleiotropy). In support for this, we found that evolved bacteria formed most cell aggregates in the high antibiotic environment, which is consistent with previous studies reporting linear increase in biofilm formation with increasing sublethal gentamicin concentrations (0–0.4 $\mu g\ ml^{-1}$) using *P. aeruginosa*, a closely related species [24,27]. Ciliate predators often select for bacterial biofilm lifestyle because large cell aggregates cannot be easily consumed by protists [21,22,35]. Interestingly, it has also been observed that biofilm formation can directly increase bacterial resistance to antibiotics [24,27]. While our data cannot separate the causal driver of bacterial cell aggregation, this trait probably increased bacterial fitness in the presence of both antibiotics and protists. These results are consistent with previous studies reporting a positive correlation between abiotic and biotic stress resistances [38,39], and suggest that trade-offs might not always limit adaptation in complex selective environments.

Bacteria evolved weakest defences against phage, and in contrast to protists, the level of phage defence evolved highest in the low antibiotic environment. It is possible that phage defence evolution correlated negatively with bacterial fitness increase to antibiotics. For example, deficiency in *Escherichia coli* lipopolysaccharide formation has been shown to lead to phage resistance, but susceptibility to antibiotics [40]. In support for this, we found that high levels of phage defence correlated with small fitness increases to antibiotics (trade-off). Bacteria might thus have been able to evolve high defence to phages only in conditions where the selection by antibiotics was weak (low antibiotic concentration). In addition, we found that high phage defence correlated with weak protist defence. This suggests a trade-off in simultaneously evolving defences to phage and protist enemies [18,21,41]. Phage selection could have thus indirectly limited bacterial fitness increase to antibiotics by constraining evolution of protist defence, which correlated positively with bacterial ability to grow in the presence of antibiotics. Unfortunately, it is not possible to distinguish these hypotheses with the current data.

Resistance traits are often connected to pleiotropic growth costs in the absence of the given selective agents [6,18,34]. We found that bacterial growth cost was greatest in the environment with the lowest degree of adaptation (low antibiotic environment), and smallest in the environment with the highest degree of adaptation (high antibiotic environment). This result is surprising because evolution of generalism is often expected to lead to the highest growth costs [42]. However, cost-free generalism has been observed to evolve in response to selection by fluctuating temperatures [38] or resource

availability [43]. There are multiple possible explanations for these results. First, bacteria could have had more changes to acquire compensatory mutations in the high antibiotic environment where they reached both relatively highest population densities and mutation supply rates. Second, different antibiotic resistance mechanisms could explain different degrees of pleiotropic growth costs; decreased cell permeability is often connected to clearly reduced growth in the absence of antibiotics [44]. Third, it has also been shown that bacterial hypermutator phenotypes bear small pleiotropic costs [45] and that hypermutators can be favoured under phage selection [46]. While further experiments are needed to test these hypotheses, we conclude that the cost of adaptation might not always limit fitness increases to multiple stresses as observed in this study.

Lastly, we found that bacterial evolutionary changes cascaded through the food web, having substantial impacts on the ecological properties of enemy communities. Increasing antibiotic concentration and rate of bacterial adaptation led to an overall decrease in predator densities, reduced enemy community diversity and increased temporal fluctuations of bacterial and enemy populations. Even though autocorrelation structure and variability also affected these ecological properties, their effects were considerably smaller compared with effects of the mean antibiotic concentration (electronic supplementary material, table S4). As a result, bacterial evolution was the most likely driver for all these changes. For example, prey defence evolution is predicted to cause destabilization of predator–prey dynamics [47], while evolution of generalist defences has been suggested to constrain the coexistence of multiple predators [48]. Interestingly, environmental variability and autocorrelation had only small effects on the stability of populations, which suggests that evolutionary dynamics can have a larger effect on population fluctuations than external environmental forcing. In the future, it would be interesting to study if these observed ecological changes could feed back into the selection process.

It has been recently pointed out that leaking of antibiotics can enrich antibiotic resistance genes in environments [1,49]. Here, we show that increased fitness to antibiotics can correlate with adaptation to phage and protist enemies, having indirect cascading effects on the ecology of bacterial consumers that are not directly affected by antibiotics. We thus suggest that even relatively low, sublethal antibiotic concentrations could have far-reaching and unexpected community-wide consequences for the trophic dynamics and functioning of natural microbial ecosystems. In the future, patterns observed in our simplified microbial laboratory systems should be validated in more complex natural communities under selection by different antibiotics, whose effects might depend critically on their mode of action [10].

Data accessibility. The raw data for this study can be found in the Dryad data repository: doi:10.5061/dryad.fp85d.

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References

1. Andersson DI, Hughes D. 2014 Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **12**, 465–478. (doi:10.1038/nrmicro3270)
2. Bryskier A (ed.). 2005 *Antimicrobial agents: antibacterials and antifungals*. Washington, DC: ASM Press.
3. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI. 2011 Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* **7**, e1002158. (doi:10.1371/journal.ppat.1002158)
4. Davies J, Davies D. 2010 Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* **74**, 417–433. (doi:10.1128/MMBR.00016-10)
5. Lindsey HA, Gallie J, Taylor S, Kerr B. 2013 Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature* **494**, 463–467. (doi:10.1038/nature11879)
6. Perron GG, Gonzalez A, Buckling A. 2008 The rate of environmental change drives adaptation to an antibiotic sink. *J. Evol. Biol.* **21**, 1724–1731. (doi:10.1111/j.1420-9101.2008.01596.x)
7. Coutu S, Rossi L, Barry DA, Rudaz S, Vernaz N. 2013 Temporal variability of antibiotics fluxes in wastewater and contribution from hospitals. *PLoS ONE* **8**, e53592. (doi:10.1371/journal.pone.0053592)
8. Kümmerer K (ed.). 2008 *Pharmaceuticals in the environment: sources, fate, effects and risks*. Berlin, Germany: Springer.
9. Marti E, Variatza E, Balcazar JL. 2014 The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends Microbiol.* **22**, 36–41. (doi:10.1016/j.tim.2013.11.001)
10. De Visser M *et al*. 1999 Diminishing returns from mutation supply rate in asexual populations. *Science* **283**, 404–406. (doi:10.1126/science.283.5400.404)
11. Lawton JH. 1988 More time means more variation. *Nature* **334**, 563. (doi:10.1038/334563a0)
12. Menges ES. 1992 Stochastic modeling of extinction in plant populations. In *Conservation biology* (eds PL Fiedler, SK Jain), pp. 253–275. New York, NY: Chapman & Hall.
13. Gonzalez A, Holt RD. 2002 The inflationary effects of environmental fluctuations in source–sink systems. *Proc. Natl Acad. Sci. USA* **99**, 14 872–14 877. (doi:10.1073/pnas.232589299)
14. Holt RD, Barfield M, Gomulkiewicz R. 2004 Temporal variation can facilitate niche evolution in harsh sink environments. *Am. Nat.* **164**, 187–200. (doi:10.1086/422343)
15. Foley P. 1994 Predicting extinction times from environmental stochasticity and carrying capacity. *Conserv. Biol.* **8**, 124–137. (doi:10.1046/j.1523-1739.1994.08010124.x)
16. Bell G, Gonzalez A. 2009 Evolutionary rescue can prevent extinction following environmental change. *Ecol. Lett.* **12**, 942–948. (doi:10.1111/j.1461-0248.2009.01350.x)
17. Pernthaler J. 2005 Predation on prokaryotes in the water column and its ecological implications. *Nat. Rev. Microbiol.* **3**, 537–546. (doi:10.1038/nrmicro1180)
18. Friman V-P, Buckling A. 2013 Effects of predation on real-time host–parasite coevolutionary dynamics. *Ecol. Lett.* **16**, 39–46. (doi:10.1111/ele.12010)
19. Meyer JR, Kassen R. 2007 The effects of competition and predation on diversification in a model adaptive radiation. *Nature* **446**, 432–435. (doi:10.1038/nature05599)
20. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010 Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* **35**, 322–332. (doi:10.1016/j.ijantimicag.2009.12.011)
21. Friman V-P, Buckling A. 2014 Phages can constrain protist predation-driven attenuation of *Pseudomonas aeruginosa* virulence in multienemy communities. *ISME J.* **8**, 1820–1830. (doi:10.1038/ismej.2014.40)

22. Friman V-P, Laakso J. 2011 Pulsed-resource dynamics constrain the evolution of predator–prey interactions. *Am. Nat.* **177**, 334–345. (doi:10.1086/658364)
23. Lenormand T. 2002 Gene flow and the limits to natural selection. *Trends Ecol. Evol.* **17**, 183–189. (doi:10.1016/S0169-5347(02)02497-7)
24. Jones C, Allsopp L, Horlick J, Kulasekara H, Filloux A. 2013 Subinhibitory concentration of kanamycin induces the *Pseudomonas aeruginosa* type VI secretion system. *PLoS ONE* **8**, e81132. (doi:10.1371/journal.pone.0081132)
25. Buckling A, Rainey PB. 2002 Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. R. Soc. Lond. B* **269**, 931–936. (doi:10.1098/rspb.2001.1945)
26. Davies J, Wright GD. 1997 Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol.* **5**, 234–240. (doi:10.1016/S0966-842X(97)01033-0)
27. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. 2005 Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**, 1171–1175. (doi:10.1038/nature03912)
28. Cohen JE, Newman CM, Cohen AE, Petchey OL, Gonzalez A. 1999 Spectral mimicry: a method of synthesizing matching time series with different Fourier spectra. *Circuits Syst. Signal Process.* **18**, 431–442. (doi:10.1007/BF01200792)
29. Ellis B, Haaland P, Hahne F, Meur NL, Gopalakrishnan N. 2009 flowCore: basic structures for flow cytometry data. R package version 1.0. See http://watson.nci.nih.gov/bioc_mirror/packages/2.13/bioc/html/flowCore.html.
30. Carpenter AE *et al*. 2006 CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **7**, R100. (doi:10.1186/gb-2006-7-10-r100)
31. Shannon CE, Weaver W. 1962 *The mathematical theory of communication*. Urbana, IL: University of Illinois Press.
32. Mingeot-Leclercq M-P, Glupczynski Y, Tulkens PM. 1999 Aminoglycosides: activity and resistance. *Antimicrob. Agents Chemother.* **43**, 727–737.
33. Kucers A, Grayson ML, Crowe SM. 1997 *The use of antibiotics: a clinical review of antibacterial, antifungal, and antiviral drugs*. Oxford, UK: Butterworth-Heinemann.
34. Perron GG, Gonzalez A, Buckling A. 2007 Source–sink dynamics shape the evolution of antibiotic resistance and its pleiotropic fitness cost. *Proc. R. Soc. B* **274**, 2351–2356. (doi:10.1098/rspb.2007.0640)
35. Friman V-P, Diggle SP, Buckling A. 2013 Protist predation can favour cooperation within bacterial species. *Biol. Lett.* **9**, 20130548. (doi:10.1098/rsbl.2013.0548)
36. Friman V-P, Hiltunen T, Laakso J, Kaitala V. 2008 Availability of prey resources drives evolution of predator–prey interaction. *Proc. R. Soc. B* **275**, 1625–1633. (doi:10.1098/rspb.2008.0174)
37. Hall AR, Meyer JR, Kassen R. 2008 Selection for predator resistance varies with resource supply in a model adaptive radiation. *Evol. Ecol. Res.* **10**, 735–746.
38. Ketola T, Mikonranta L, Zhang J, Saarinen K, Örmälä A-M, Friman V-P, Mappes J, Laakso J. 2013 Fluctuating temperature leads to evolution of thermal generalism and preadaptation to novel environments. *Evolution* **67**, 2936–2944.
39. Kissoudis C, van de Wiel C, Visser RGF, Van Der Linden G. 2014 Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Front. Plant Sci.* **5**, 207. (doi:10.3389/fpls.2014.00207)
40. Tamaki S, Sato T, Matsuhashi M. 1971 Role of lipopolysaccharides in antibiotic resistance and bacteriophage adsorption of *Escherichia coli* K-12. *J. Bacteriol.* **105**, 968–975.
41. Siepielski AM, Benkman CW. 2008 A seed predator drives the evolution of a seed dispersal mutualism. *Proc. R. Soc. B* **275**, 1917–1925. (doi:10.1098/rspb.2008.0451)
42. Kassen R. 2002 The experimental evolution of specialists, generalists, and the maintenance of diversity. *J. Evol. Biol.* **15**, 173–190. (doi:10.1046/j.1420-9101.2002.00377.x)
43. Buckling A, Brockhurst MA, Travisano M, Rainey PB. 2007 Experimental adaptation to high and low quality environments under different scales of temporal variation. *J. Evol. Biol.* **20**, 296–300. (doi:10.1111/j.1420-9101.2006.01195.x)
44. Proctor RA, Von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G. 2006 Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* **4**, 295–305. (doi:10.1038/nrmicro1384)
45. Perron GG, Hall AR, Buckling A. 2010 Hypermutability and compensatory adaptation in antibiotic-resistant bacteria. *Am. Nat.* **176**, 303–311. (doi:10.1086/655217)
46. Pal C, Maciá MD, Oliver A, Schachar I, Buckling A. 2007 Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature* **450**, 1079–1081. (doi:10.1038/nature06350)
47. Abrams PA. 2000 The evolution of predator-prey interactions: theory and evidence. *Annu. Rev. Ecol. Syst.* **31**, 79–105. (doi:10.1146/annurev.ecolsys.31.1.79)
48. Matsuda H, Hori M, Abrams PA. 1996 Effects of predator-specific defence on biodiversity and community complexity in two-trophic-level communities. *Evol. Ecol.* **10**, 13–28. (doi:10.1007/BF01239343)
49. Czekalski N, Díez EG, Bürgmann H. 2014 Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *ISME J.* **8**, 1381–1390. (doi:10.1038/ismej.2014.8)