

**A peer-reviewed version of this preprint was published in PeerJ on 12 October 2018.**

[View the peer-reviewed version](https://doi.org/10.7717/peerj.5801) (peerj.com/articles/5801), which is the preferred citable publication unless you specifically need to cite this preprint.

Kurenbach B, Hill AM, Godsoe W, van Hamelsveld S, Heinemann JA. 2018. Agrichemicals and antibiotics in combination increase antibiotic resistance evolution. PeerJ 6:e5801 <https://doi.org/10.7717/peerj.5801>

# Agrichemicals boost the effects of antibiotics on antibiotic resistance evolution

Brigitta Kurenbach<sup>1</sup>, Amy M Hill<sup>1</sup>, William Godsoe<sup>2</sup>, Sophie van Hamelsveld<sup>1</sup>, Jack A Heinemann<sup>Corresp. 1</sup>

<sup>1</sup> School of Biological Sciences and Centre for Integrated Research in Biosafety and Centre for Integrative Ecology, University of Canterbury, Christchurch, New Zealand

<sup>2</sup> Bio-Protection Centre, Lincoln University, Lincoln, New Zealand

Corresponding Author: Jack A Heinemann  
Email address: jack.heinemann@canterbury.ac.nz

Antibiotic resistance is medicine's climate change: caused by human activity, and resulting in more extreme outcomes. Resistance emerges in microbial populations when antibiotics act on phenotypic variance within the population. This can arise from either genotypic diversity (resulting from a mutation or horizontal gene transfer), or from 'adaptive' differences in gene expression due to environmental variation. Adaptive changes can increase fitness allowing bacteria to survive at higher concentrations of the antibiotic. They can also decrease fitness, potentially leading to selection for antibiotic resistance at lower concentrations. There are opportunities for other environmental stressors to promote antibiotic resistance in ways that are hard to predict using conventional assays. Exploiting our observation that commonly used herbicides can increase or decrease the minimum inhibitory concentration (MIC) of different antibiotics, we provide the first comprehensive test of the hypothesis that the rate of antibiotic resistance evolution under specified conditions can increase, regardless of whether a herbicide increases or decreases the antibiotic MIC. Short term evolution experiments were used for various herbicide and antibiotic combinations. We found conditions where acquired resistance arises more frequently regardless of whether the exogenous non-antibiotic agent increased or decreased antibiotic effectiveness. This "damned if you do/damned if you don't" outcome suggests that the emergence of antibiotic resistance is exacerbated by additional environmental factors that influence competition between bacteria. Our work demonstrates that bacteria may acquire antibiotic resistance in the environment at rates substantially faster than predicted from laboratory conditions.

1 **Classification: Biological sciences / evolution**

2

3 **Agrichemicals boost the effects of antibiotics on antibiotic resistance evolution**

4

5 Brigitta Kurenbach<sup>1,2†</sup>, Amy M. Hill<sup>1,2†</sup>, William Godsoe<sup>3</sup>, Sophie van Hamelsveld<sup>1,2</sup>, and Jack A.  
6 Heinemann<sup>1,2\*</sup>

7

8 <sup>1</sup>School of Biological Sciences, University of Canterbury, Christchurch 8140, NZ

9 <sup>2</sup>Centre for Integrated Research in Biosafety and Centre for Integrative Ecology, University of  
10 Canterbury, Christchurch, NZ

11 <sup>3</sup>Bio-Protection Centre, Lincoln University, Lincoln 7647, NZ

12 \*corresponding author: Jack A. Heinemann, School of Biological Sciences, University of  
13 Canterbury, Private Bag 4800, Christchurch 8140, NZ

14 Tel.: (+64) 3 364 2500

15 Email: jack.heinemann@canterbury.ac.nz

16 † BK and AMH contributed equally to this work.

17

18 **Short title: Herbicide evolved antibiotic resistance**

19

20 **Abstract**

21 Antibiotic resistance is medicine's climate change: caused by human activity, and resulting in  
22 more extreme outcomes. Resistance emerges in microbial populations when antibiotics act on  
23 phenotypic variance within the population. This can arise from either genotypic diversity  
24 (resulting from a mutation or horizontal gene transfer), or from 'adaptive' differences in gene  
25 expression due to environmental variation. Adaptive changes can increase fitness allowing  
26 bacteria to survive at higher concentrations of the antibiotic. They can also decrease fitness,

27 potentially leading to selection for antibiotic resistance at lower concentrations. There are  
28 opportunities for other environmental stressors to promote antibiotic resistance in ways that are  
29 hard to predict using conventional assays. Exploiting our observation that commonly used  
30 herbicides can increase or decrease the minimum inhibitory concentration (MIC) of different  
31 antibiotics, we provide the first comprehensive test of the hypothesis that the rate of antibiotic  
32 resistance evolution under specified conditions can increase, regardless of whether a herbicide  
33 increases or decreases the antibiotic MIC. Short term evolution experiments were used for  
34 various herbicide and antibiotic combinations. We found conditions where acquired resistance  
35 arises more frequently regardless of whether the exogenous non-antibiotic agent increased or  
36 decreased antibiotic effectiveness. This “damned if you do/damned if you don’t” outcome  
37 suggests that the emergence of antibiotic resistance is exacerbated by additional environmental  
38 factors that influence competition between bacteria. Our work demonstrates that bacteria may  
39 acquire antibiotic resistance in the environment at rates substantially faster than predicted from  
40 laboratory conditions.

41

## 42 **Significance**

43 Neither reducing the use of antibiotics nor discovery of new ones may be sufficient strategies to  
44 avoid the post-antibiotic era. This is because bacteria may be exposed to other non-antibiotic  
45 chemicals that predispose them to evolve resistance to antibiotics more quickly. Herbicides are  
46 examples of some of the most common non-antibiotic chemicals in frequent global use. In some  
47 combinations the herbicides we tested made bacteria phenotypically resistant to higher  
48 concentrations of antibiotics, while in other combinations bacteria became susceptible at lower  
49 antibiotic concentrations. In both cases the herbicides worked with antibiotics to accelerate  
50 genotypic resistance evolution. Unfortunately, antibiotic resistance may increase even if total  
51 antibiotic use is reduced, and new ones are invented, unless other environmental exposures are  
52 also controlled.

53

54 **Key words** antibiotic resistance, evolution, adaptive resistance, herbicide, minimum selective  
55 concentration

56

57 **Abbreviations** MIC, minimum inhibitory concentration; MSC, minimum selective

58 concentration; NOEL, no observable effect level

## 59 Introduction

60 As fundamental tools for infection control, antibiotics underpin diverse human systems ranging  
61 from hospital care to concentrated animal feeding operations through to crop and pollinator  
62 disease management. The loss of this tool due to antibiotic resistance will result in higher  
63 mortality and morbidity, but also deny access to many routine medical procedures for risk of  
64 subsequently untreatable infections (Teillant et al. 2015; Thomas et al. 2014). Antibiotic  
65 resistances also threaten agricultural productivity (Stockwell & Duffy 2013; Van Boeckel et al.  
66 2015). Despite over a half century of warning, neither science nor innovation has managed to  
67 invent us away from the threat of a post-antibiotic era.

68 One stewardship strategy is using them less so that we might use them for longer (CDC 2013;  
69 Collingnon et al. 2016). If bacteria almost never encounter antibiotics at concentrations high  
70 enough to harm them, there would be little opportunity for resistant variants to emerge and  
71 establish. Based on this, it has been suggested that judicious use of antibiotics that keeps most  
72 antibiotic exposures to less than the minimum inhibitory concentration (MIC) should preserve  
73 antibiotic susceptibility in bacteria (Andersson & Hughes 2014). In practice, the lowest  
74 concentration of antibiotic leading to the evolution of resistance in a given environment, the so  
75 called minimum selective concentration (MSC), can be much lower than the MIC (Fig. 1)  
76 (Andersson & Hughes 2014; Baquero et al. 1998b; Hermsen et al. 2012).

77 Variation in antibiotic responses can be caused by either genetic or physiological differences  
78 between individual bacteria. The toxic effect of an antibiotic may occur at different  
79 concentrations for different individuals because some have *acquired* genes or alleles through  
80 mutation or horizontal gene transfer (i.e. change in genotype). Also, organisms can have *innate*  
81 differences between them, e.g. due to differences in permeability.

82 Innate resistance can also depend upon genes expressed or repressed conditionally, resulting  
83 in increased efflux or decreased influx of antibiotics and overall lower intracellular antibiotic  
84 concentrations (Fernandez & Hancock 2012). Such genes or expression induction thresholds may  
85 differ between species, and individuals within a species may phenotypically differ depending on  
86 whether or not they expressed those genes before being inhibited by the antibiotic. This  
87 resistance through changes in gene expression is also known as an *adaptive* (change in  
88 phenotype) response. It can be triggered by antibiotics and other chemical toxins or

89 environmental cues (Blair et al. 2015; Palmer & Kishony 2013; Sanchez-Romero & Casadesus  
90 2014).

91 Populations of adaptively resistant bacteria can in time produce variants with acquired resistance  
92 to even higher concentrations of antibiotic (Cohen et al. 1989; Gustafson et al. 1999; Shen et al.  
93 2011). This raises the possibility that environmental stimuli that cause phenotypic antibiotic  
94 resistance variation between individuals could be hotspots for evolution of acquired antibiotic  
95 resistance.

96 In earlier studies, we found that herbicides could induce adaptive changes in how bacteria  
97 respond to antibiotics (Kurenbach et al. 2017; Kurenbach et al. 2015). Some herbicides and  
98 antibiotic combinations increased the antibiotic MIC (Fig. 1A) and some lowered it (Fig. 1B),  
99 while others did not change the MIC but did alter survival at below-MIC concentrations (Fig.  
100 1C). Here we exploit those observations to test the novel hypothesis that both increases *and*  
101 decreases in MIC caused by exposure to herbicides can lead to an increase in the rate of acquired  
102 resistance evolution in populations of *Escherichia coli* and *Salmonella enterica* serovar  
103 Typhimurium.

## 104 **Materials and Methods**

### 105 **Media**

106 Strains and plasmids are listed in Table 1. Bacteria were grown in standard rich growth medium,  
107 LB (Lennox) (Invitrogen) at 37°C and supplemented with ampicillin (Amp, AppliChem),  
108 chloramphenicol (Cam, Sigma) ciprofloxacin (Cip, Pentex), streptomycin (Str, Sigma),  
109 tetracycline (Tet, Sigma), or nalidixic acid (Nal, Sigma) as appropriate. Liquid cultures were  
110 grown with aeration (180 rpm), and plates were incubated in plastic bags to avoid drying out.  
111 Commercial herbicide formulations were Kamba<sup>500</sup> (Nufarm, NZ) containing 500 g/L dimethyl  
112 salt of dicamba, and Roundup Weedkiller (Monsanto, Australia) containing 360 g/L  
113 isopropylamine salt of glyphosate. Herbicide concentrations are reported in parts per million acid  
114 equivalent (ppm ae) to allow for comparison with other formulations. Antibiotic and herbicide  
115 concentrations used are specified in the main text or in figure legends.

### 116 **Plasmid constructs**

117 Plasmid pAH14 was created by removing the *Hind*III and *Bam*HI fragment of pBR322 and  
118 inserting *cat* from pACYC184 at the *Pst*I site within *bla*. The resulting traits for this plasmid  
119 were Cam<sup>R</sup>, Amp<sup>S</sup>, Tet<sup>S</sup>. pAH11 was created by insertion of *cat* from pACYC184 into the *Eco*RI  
120 and *Not*I sites of RSF1010, resulting in plasmid-determined traits Cam<sup>R</sup>, Str<sup>S</sup>.

### 121 **Culturing conditions**

122 *E. coli* or *S. enterica* were grown in 10 mL liquid LB medium containing Cip, herbicide, both,  
123 or neither. Initial densities were ca. 10<sup>5</sup> cfu/mL for the former treatment and ca. 10<sup>6</sup> cfu/mL (*E.*  
124 *coli*) and 10<sup>5</sup> cfu/mL (*S. enterica*) for the latter three. Cultures were grown for 24 hrs. For the  
125 experiments described in the section “Acquired resistance frequencies increase due to herbicide-  
126 induced increases in MIC” the experiment was abandoned if cultures containing Cip in the  
127 absence of herbicide were visibly turbid at this point. Here, the chosen Cip concentration was  
128 above MIC, and growth was therefore interpreted as the presence of a resistant mutant in the  
129 starting culture. Herbicide and antibiotic concentrations used are detailed in Table 2 and Table 3.  
130 Cultures were then diluted as above in the same conditions and incubated again for 24 hrs. A 10-  
131 fold dilution in LB without herbicides or antibiotics followed to ensure observed effects were not  
132 due to herbicide induced adaptive responses. Titers (cfu/mL) were determined at the end of each  
133 incubation step on both LB and on Cip at an initially non-permissive concentration (ca. 2x MIC)  
134 (Dan et al. 2016). The frequency of Cip resistant mutants and the number of generations was  
135 calculated.

### 136 **Determination of resistance levels**

137 Individual colonies of strains grown on solid LB medium were used to inoculate 100 µL of liquid  
138 LB in a 96 well plate. The plate was incubated with aeration to saturation before ca. 4 µL  
139 samples were stenciled onto LB plates containing the appropriate antibiotics. Plates were  
140 incubated at 37°C for 18 hrs. Strains were scored positively for growth if growth was tangible.

### 141 **Competition experiments**

142 Isogenic strains of *E. coli* differing only in the MIC phenotype (i.e. high vs. low) and an  
143 additional selection marker encoded on a low copy number plasmid were co-incubated in liquid  
144 LB medium containing herbicide, antibiotic, both, or neither and grown to saturation before  
145 dilution by a factor of 10<sup>3</sup> in the same conditions. The antibiotic concentration chosen was below



146 NOEL for both strains. The titer of each culture was determined by plating on non-selective  
147 medium after each incubation step. After 5 rounds of incubation, the ratio of strains was  
148 determined by selecting for the second – competition irrelevant – marker.

149 Natural selection was defined as the difference in the exponential growth rate of the two strains  
150 (van den Bosch et al. 2014). Under this interpretation, the change in the proportion of resistant  
151 individuals per unit of time is the logistic curve (Mallet 2012) with the explicit solution  
152  $p=e^{st}/(c+e^{st})$ , where  $t$ =time,  $p$ =proportion of resistant individuals,  $s$ =strength of selection,  
153  $c$ =constant describing the initial proportion of resistant individuals,  $c=(1/p_0)-1$ . The constant  $p_0$  is  
154 the proportion of resistant individuals at  $t_0$  (the start of the experiment).  $t$  is defined as the  
155 number of generations, using the generation time for the more resistant strain. By rearranging  
156 this formula, the strength of selection is  $s= \ln(pc/(1-p))/t$ .

### 157 **Growth curves**

158 Growth curves were established at 37°C in liquid LB medium supplemented with herbicide,  
159 antibiotic, both, or neither using a FLUOstar Omega microplate reader (BMG LABTECH,  
160 Germany). The OD<sub>600</sub> was determined every 6 min for 16 hrs and averaged over five replicates.  
161 Cultures were started at densities of ca. 10<sup>6</sup> cfu/mL. Antibiotic and herbicide concentrations used  
162 are detailed in the legend of Suppl. Fig. S1.

### 163 **Statistical analysis**

164 R was used for all statistical analyses (R Core Team 2013). For mutant frequency experiments  
165 (changes to MIC), an ANOVA was used to analyze the randomized complete block design, using  
166 each independent experiment as a block with presence/absence of antibiotic/herbicide as levels.  
167 Residuals were used to test for normality and equality of variances and log transformed data  
168 where appropriate. Tukey's HSD test was used to determine which treatments were significantly  
169 different from each other.

170 For mixed culture experiments (changes to MSC), two sets of analyses were performed. First, we  
171 determined whether adding antibiotics increases the strength of selection at different herbicide  
172 concentrations. Second, we determined whether adding herbicides increases the strength of  
173 selection at different antibiotic concentrations. Each set of questions was tested using contrasts  
174 performed with the glht function in the multcomp package (Hothorn et al. 2008) with a two-sided

175 alternative and sequential Bonferroni procedure. These contrasts were fit to an ANOVA model  
176 treating each combination of herbicide and antibiotic as a treatment category. Residual plots  
177 confirmed that the assumptions of normality and equality of variance were met.

### 178 **Statistical analysis for growth curves**

179 To estimate carrying capacity ( $k$ ), a logistic growth model on the raw data using non-linear least  
180 squares (nls) was fit in R. Differences in population growth between strains in different  
181 treatments were estimated by estimating  $r$ , the intrinsic growth rate, of each strain. Data sets were  
182 log transformed and the slope of the growth curve between  $t=48$  mins and  $t=150$  mins was  
183 measured. A visual inspection of the plots revealed that before  $t=48$  mins graphs were not linear.  
184 After  $t=150$  mins, growth slowed as cells were entering stationary phase.

185 We tested for differences between the two strains in both  $r$  and  $k$  by calculating contrast in an  
186 ANOVA (aov). Residual plots were used to test for violations of assumptions for ANOVA.  
187 Assumptions of normality and equal variances were met in all data sets. In two data sets there  
188 were a small number of outliers. These had small influences on parameter estimates and were  
189 hence not removed. Contrasts between treatments were calculated using the `glht` package in R  
190 using the sequential Bonferroni correction.

191

## 192 **Results**

### 193 **Acquired resistance frequencies increase due to herbicide-induced increases in MIC**

194 Exposing either *E. coli* or *S. enterica* to the herbicide formulation Roundup increased the MIC of  
195 the fluoroquinolone antibiotic ciprofloxacin, as did exposing *S. enterica* to the herbicide Kamba.  
196 This was due to adaptive changes induced by herbicide exposure (Kurenbach et al. 2015). These  
197 combinations were used to test the hypothesis that the opportunity afforded by reproduction of  
198 bacteria exhibiting adaptive resistance to normally lethal concentrations of antibiotic was  
199 sufficient for the population to evolve higher frequencies of resistant genotypes.

200 The rate of acquired resistance mutations in populations of *E. coli* or *S. enterica* (all strains  
201 described in Table 1) was measured over the course of about 25 generations in a standard rich  
202 growth medium, liquid LB, or LB medium containing the appropriate herbicide (below the No  
203 Observable Effect Level, NOEL) with or without ciprofloxacin supplementation (Table 2). The

204 ciprofloxacin concentration was the same in all cultures to which it was added but below MIC  
205 for bacteria in cultures simultaneously exposed to herbicide. The bacteria were then transferred  
206 to solid medium supplemented with high levels of ciprofloxacin ( $\approx 2x$  MIC) and no herbicide,  
207 which was permissive only to the growth of variants with acquired resistance.

208 Genetic variants able to grow on high concentrations of ciprofloxacin after 25 generations in LB  
209 medium, with or without herbicide supplementation, arose at the same frequency. This indicated  
210 that the herbicides were not mutagens at these concentrations. In a separate standard test of  
211 mutagenicity (Funchain et al. 2001), bacteria were exposed to herbicides and plated on the  
212 antibiotic rifampicin. No difference in resistance frequencies were observed (data not shown).

213 Consistent with our prediction that herbicide-induced adaptive resistance allowed rare  
214 spontaneously arising ciprofloxacin resistant mutants to increase a culture's MIC, populations of  
215 bacteria with continuous exposure to herbicide and antibiotic had significantly higher numbers of  
216 ciprofloxacin resistant variants. The frequency ranged from  $10^2$  times higher for the combination  
217 of Cip+Kamba+*S. enterica* to  $10^5$  times higher for Cip+Roundup+*E. coli* (Table 2).

218 Cip resistant colonies were isolated at the end of each experiment from all treatments. We  
219 determined MICs for 56 *S. enterica* isolates (isolated on  $0.05 \mu\text{g/mL}$  Cip): 27 isolates from  
220 Kamba+Cip and 29 isolates from Roundup+Cip treatments. The parental strain and 2 isolates  
221 from each LB, Kamba, and Roundup treatments, also isolated on  $0.05 \mu\text{g/mL}$  Cip, were included  
222 as controls.

223 The parental strain and 6 evolved isolates did not grow at  $0.07 \mu\text{g/mL}$ , a concentration just above  
224 the selection concentration. We observed MICs of  $0.1 \mu\text{g/mL}$  Cip for 21 isolates and  $0.2 \mu\text{g/mL}$   
225 Cip for 28 isolates. Only 7 isolates displayed higher MICs (2 from Kamba+Cip, 4 from Roundup  
226 and Cip, and 1 from LB), the highest being  $1.25 \mu\text{g/mL}$  Cip reached by one isolate isolated from  
227 a Roundup+Cip culture. Importantly, we observed no correlation between level of resistance and  
228 original treatment, which indicates that there are no qualitative differences between the  
229 ciprofloxacin resistant variants arising in the different treatments.

230

231 **Herbicide-induced adaptive changes in MIC also change MSC**

232 Antibiotic resistant bacteria are becoming a fixed part of many environments despite the  
233 concentration of antibiotics often being very low (Hermsen et al. 2012). We hypothesized that  
234 exposure to some herbicides can shift the MSC to lower antibiotic concentrations leading to  
235 competition between individuals with different physiological responses and thus providing an  
236 environment in which genotypically resistant bacteria evolve.

237 Mixed cultures of *E. coli* were created to represent pre-existing antibiotic resistance  
238 heterogeneity within natural environments. The phenotypic differences were created using  
239 bacteria carrying isogenic plasmids with different alleles of *tetA* ( $Tet^{high}$ ,  $Tet^{low}$ , Table 1). The  
240 MIC for tetracycline of  $Tet^{low}$  was 1.5  $\mu\text{g}/\text{mL}$  and for  $Tet^{high}$  it was 125  $\mu\text{g}/\text{mL}$ .

241  $Tet^{high}$  and  $Tet^{low}$  were competed in liquid LB, LB+Tet, and LB+Tet+Roundup media for about  
242 42 generations. This herbicide lowers the antibiotic MIC (Fig. 1B). The same concentration of  
243 Roundup was used in combination with various concentrations of tetracycline, all of which were  
244 below the MIC of  $Tet^{low}$ . Roundup reduced tetracycline MIC for  $Tet^{low}$  to 0.5  $\mu\text{g}/\text{mL}$ , but growth  
245 of  $Tet^{high}$  was not affected for tetracycline concentrations up to 50  $\mu\text{g}/\text{mL}$ , far above levels used in  
246 this experiment.

247 The MSC, i.e. the concentration of tetracycline at which one strain started to dominate the  
248 culture, was determined from the calculated selection coefficients. A positive coefficient  
249 indicated selection for the more resistant strain. The selection coefficient was significantly  
250 different for competitions in environments supplemented with  $\geq 0.1$   $\mu\text{g}/\text{mL}$  tetracycline compared  
251 to competitions in LB medium (Fig. 2). Mixed cultures in LB+Tet+Roundup had significantly  
252 different selection coefficients compared to the LB competitions beginning at a tetracycline  
253 concentration of 0.05  $\mu\text{g}/\text{mL}$ .

254 A reverse experiment was performed using Kamba instead of Roundup. This herbicide increases  
255 the MIC of  $Tet^{low}$  from 1.5 to 3  $\mu\text{g}/\text{mL}$ . When Kamba was part of the environment, the selection  
256 coefficient was significantly different from the competition in LB medium at a tetracycline  
257 concentration  $\geq 0.5$   $\mu\text{g}/\text{mL}$ , compared to  $\geq 0.1$   $\mu\text{g}/\text{mL}$  in its absence (Fig. 2). In both cases, MIC  
258 for the  $Tet^{low}$  strain and MSC moved in the same direction.

259

**260 Herbicide-induced changes in MSC can occur without a change in MIC**

261 *E. coli* cultures in medium supplemented with Kamba grew faster at some sub-lethal  
262 concentrations of ciprofloxacin, but Kamba did not change the MIC (Fig. 1C) (Kurenbach et al.  
263 2015). To determine if exogenous chemicals that alter fitness at sub-lethal antibiotic  
264 concentrations - but do not change the MIC - have an impact on the evolution of resistance, we  
265 measured the frequency at which acquired ciprofloxacin resistance arose during culture in the  
266 combination of Cip+Kamba+*E. coli*.

267 The mutation rate of an *E. coli* monoculture was measured over the course of about 25  
268 generations in a standard rich growth medium, liquid LB, and in LB supplemented with Cip,  
269 Kamba, or Cip+Kamba. The experiment was similar to those described in the section “Acquired  
270 resistance frequencies increase due to herbicide-induced increases in MIC”, but here the  
271 ciprofloxacin concentration was below MIC. The bacteria were then transferred to solid medium  
272 supplemented with high levels of ciprofloxacin ( $\approx 2x$  MIC) and no herbicide, which was  
273 permissive only to the growth of variants with acquired resistance.

274 The frequency of more resistant variants was the same for cultures grown in LB, LB+Kamba or  
275 LB+Cip+Kamba (for both treatment combinations  $p > 0.2$ ; Table 3). Therefore the ciprofloxacin  
276 MSC was not reached under any of these conditions.

277 The frequency of more resistant variants recovered from the LB+Cip culture condition was about  
278  $10^5$  times higher than the other three culture conditions ( $p < 10^{-4}$  for all treatment combinations),  
279 indicating that the ciprofloxacin concentration met or exceeded the MSC. The addition of Kamba  
280 neutralized the selective effect of ciprofloxacin by shifting the MSC to a higher level without  
281 changing the MIC.

282

**283 Herbicide-induced changes to MSC are herbicide concentration-dependent**

284 Pairs of isogenic strains of *E. coli* (Table 1) with either different streptomycin ( $Str^{high}$ ,  $Str^{low}$ ) or  
285 tetracycline ( $Tet^{high}$ ,  $Tet^{low}$ ) MICs were competed for about 40 generations. The frequency of  
286 each strain after competition was measured. The concentration of the appropriate antibiotic did  
287 not vary between cultures, but the concentration of herbicide did. Combinations were chosen that  
288 lead to a decrease in MIC with the chosen herbicide, namely Str+Kamba, and Tet+Roundup.

289 There was no differential in the fitness of paired strains when they were co-cultured in standard  
290 rich medium (Fig. 3). In cultures containing antibiotic but not supplemented with herbicide  
291 neither strain had a fitness advantage, indicating that the antibiotic concentration was below  
292 MSC. Likewise, the herbicide alone did not affect fitness except for a statistically non-significant  
293 effect at the highest tested concentration of Roundup.

294 In each test, the combination of antibiotic and herbicide reduced MSC. The more resistant strain  
295 invariably increased relative to the isogenic competitor in a herbicide dose-dependent manner  
296 when both antibiotic and herbicide were in the environment. Selection coefficients were  
297 statistically significantly different from LB medium at 183 parts per million acid equivalent  
298 (ppm ae) Kamba and 311 ppm ae Roundup.

299 The observed fitness differential was due to the faster reproductive rate of the Str<sup>high</sup> and Tet<sup>high</sup>  
300 strains in environments with sub-lethal concentrations of antibiotic for both strains in each pair.  
301 This was shown by measuring the growth rate of the strains in monoculture rather than in  
302 competition (Supplementary Fig. S1). No differences were observed for the monoculture growth  
303 rate ( $r$ ) of matched isogenic pairs in LB medium, LB+antibiotic, or LB+herbicide ( $p > 0.1$  for all  
304 combinations; Supplementary Fig. S1A and C). Only in the combined LB+herbicide+antibiotic  
305 treatment did strains with higher MICs have shorter generation times in monoculture than their  
306 lower MIC counterparts ( $p = 1.6 \times 10^{-5}$  (Kamba+Str) and  $p = 4.7 \times 10^{-10}$  (Roundup+Tet)).

307 Significant differences in the carrying capacity of the environments for all treatment  
308 combinations were observed for the matched strains exposed to Kamba+Str measured in  
309 monoculture. The high MIC strain achieved higher final optical densities ( $p < 2 \times 10^{-16}$ ;  
310 Supplementary Fig. S1B). In contrast, the Str<sup>low</sup> strain population grew to higher final optical  
311 densities in LB, LB+Tet, and LB+Roundup treatments. In cultures with Roundup+Tet, Tet<sup>high</sup>  
312 and Tet<sup>low</sup> had similar densities ( $p = 0.24$ , Supplementary Fig. S1D).

313

## 314 Discussion

315 In this study we report that when bacteria are simultaneously exposed to herbicides and  
316 antibiotics, mutants with higher levels of resistance can evolve. In some cases, resistance evolved  
317 100,000 times faster.

318 Herbicides can increase the MIC of some antibiotics. At what otherwise would be a lethal  
319 concentration of the antibiotic, the bacteria can continue to reproduce. Each reproductive event  
320 has a low but steady potential of producing a variant daughter with a higher MIC. We found that  
321 these strains have a fitness advantage and accumulate differentially to their low MIC cousins.

322 Herbicides can also decrease the MIC of some antibiotics. At what otherwise would be a  
323 concentration of antibiotic below the MSC, too low to have an effect on the fitness of two  
324 bacteria differing in their MICs, we found that the bacteria with the higher MICs replaced the  
325 bacteria with lower MICs. The shift in MSC seemed to be of a similar magnitude as the shift in  
326 MIC observed for the lower MIC strain.

327 Finally, herbicides can also alter survival potential at some antibiotic concentrations but not  
328 change the concentration to which the entire population is innately susceptible. In the case  
329 presented here, MSC was shifted by the herbicides, but MIC was not. The herbicide mitigated  
330 the selective pressure caused by the antibiotic, and no genotypes with higher resistance levels  
331 established in the population. Although not tested, it is likely that in the reverse case, when MSC  
332 is lowered by a substance, antibiotic resistance may arise at higher frequencies.

333 Our research shows that manufactured chemical products such as the herbicides can have a  
334 complex effect on the evolution of antibiotic resistance. They did not replace antibiotics, but  
335 could accelerate resistance evolution. Herbicides that reduce the MSC will be more effective at  
336 stimulating resistance evolution at the lower ends of the antibiotic concentration gradient, while  
337 herbicides that increase the MIC will be more effective at stimulating resistance evolution at the  
338 higher ends.

339 Infections by antibiotic resistant bacteria are increasingly common, with the human and  
340 economic costs also increasing (Friedman et al. 2016). As a consequence of the rise of resistant  
341 pathogens, combination treatments using a non-antibiotic to increase an antibiotic's effectiveness  
342 have been suggested to help preserve the usefulness of antibiotics (Allen et al. 2014; Schneider et  
343 al. 2017; Wright 2016). Indeed, various combinations are in clinical use (Worthington &  
344 Melander 2013). While this might improve or enable treatment of an infection, our results  
345 indicate that decreasing bacteria's survival by making an antibiotic more potent may increase the  
346 development of resistance by creating more environments where adaptive pressure is present.

347 Herbicides and different ingredients in product formulations can have antimicrobial activities,  
348 with some being more potent antimicrobial agents than are others (Kurenbach et al. 2017). For  
349 example, Roundup was more toxic than Kamba to the bacteria that we used. The low level  
350 toxicity is likely how they induce adaptive resistance, a source of phenotypic heterogeneity  
351 (Kurenbach et al. 2015). In addition, biocidal agents that are mutagens may introduce genotypic  
352 heterogeneity. At the concentrations used in this work, the herbicides did not appear to be  
353 mutagenic.

354 Antibiotic and herbicide gradients may be viewed as two environmental dimensions  
355 concentrating competition between bacteria of different genotypes and phenotypes. The  
356 particular concentrations of antibiotic and herbicide at any point of intersection of the gradients  
357 above a threshold level increases the frequency of the most fit strains and species, amplifying  
358 any linked or associated traits in the more fit competitor. While antibiotics can do this without  
359 herbicides (Andersson & Hughes 2014; Baquero et al. 1998a; Denamur & Matic 2006), the  
360 herbicides used in this study increase the range of concentrations under which the antibiotic  
361 affects the evolution of resistance.

362 The concentrations of herbicides and antibiotics we used were below recommended application  
363 levels and hence within relevant environmental ranges, suggesting that what we observe has the  
364 potential to occur in many places where the two biocides are found together. Simultaneous  
365 herbicide and antibiotic exposures are common. Herbicides are used in agriculture, where spray  
366 drift or walking through treated fields exposes farm livestock and pets, which may be on  
367 therapeutic or prophylactic antibiotics. Most ingested antibiotic is not metabolized and thus  
368 excreted (Chee-Sanford et al. 2009), becoming mixed with soil as crop fertilizer which *in situ*  
369 may be subsequently sprayed with herbicide. Microbes from these mixes may be carried by  
370 blow- and house-flies (Zurek & Ghosh 2014). Likewise honeybees may be exposed to herbicide  
371 spray or residues as they forage and return to an antibiotic-treated hive. Additionally, herbicides  
372 are used in urban environments for purposes like gardening and lawn care, including parks and  
373 roadsides (Atwood & Paisley-Jones 2017). Worldwide, herbicide use was approximately  $1 \times 10^9$   
374 kilograms in 2012 with up to  $2 \times 10^8$  kilograms of the active herbicidal ingredients glyphosate, 2,  
375 4-D and dicamba used in the US in 2012 (Atwood & Paisley-Jones 2017).



376 Other chemicals also have been shown to cause adaptive resistance and to increase resistance  
377 frequencies (Egeghy et al. 2012; Gustafson et al. 1999; Levy 2001). Non-antibiotic prescription  
378 medicines and food emulsifiers select antibiotic resistant gut bacteria (Kurenbach et al. 2017;  
379 Maier et al. 2018). Approximately 8 million manufactured chemical substances are currently in  
380 commerce (Egeghy et al. 2012; Shen et al. 2011). According to the US Environmental Protection  
381 Agency, annual production of each of the top 3,000 chemicals is greater than  $6 \times 10^{11}$   
382 kilograms/year (EPA 2008). They are not regulated for effects on antibiotic resistance and not  
383 tested for such effects.

384 The susceptibility of bacteria to antibiotics must be seen as a non-renewable resource, one that  
385 requires careful stewardship worldwide (Amabile-Cuevas 2016; Heinemann & Kurenbach 2017).  
386 Evidence that antibiotic resistance evolution is influenced by exposure of bacteria to a wide  
387 range of substances may require us to make changes in how we manage both antibiotics and  
388 other manufactured and widely distributed chemical products. This is because many facets of the  
389 extrinsic environment induce adaptive changes, a complexity frequently ignored in standard  
390 studies of resistance. As our results show, complex effects of exposures to non-therapeutic  
391 chemicals may undermine strategies to preserve the effectiveness of antibiotics through altering  
392 just their use. To our knowledge, there has been no attempt to systematically test common  
393 chemicals to which pathogenic bacteria are chronically exposed for effects on antibiotic  
394 resistance.

395

## 396 **Conclusions**

397 Neither reducing the use of antibiotics nor discovery of new ones may prevent the post-antibiotic  
398 era. This is because bacteria may be exposed to other non-antibiotic chemicals that predispose  
399 them to evolve resistance to antibiotics more quickly. Herbicides are examples of some of the  
400 most common non-antibiotic chemicals in frequent global use. More research is necessary to see  
401 to what extent other different manufactured chemicals may contribute to this effect. Moreover,  
402 depending on how the manufactured chemicals are used, or how they move through the waste  
403 stream, there may be combinatorial effects caused by mixtures of different products. Future work  
404 should take into account likely combinations as well as different ways that microbes could be  
405 exposed to chemical products.

406

407 **Acknowledgements**

408 The authors thank Mark Silby for helpful comments. AMH received the New Zealand Federation  
409 of Graduate Women and UC for support. This project received funding from the Brian Mason  
410 Trust (JAH) and donations to the UC Foundation (JAH) including from, inter alia, donors Third  
411 World Network (Malaysia) and the Sustainable Food Trust (UK).

412 **References**

- 413 Allen HK, Trachsel J, Looft T, and Casey TA. 2014. Finding alternatives to antibiotics. *Annals*  
414 *of the New York Academy of Sciences* 1323:91-100. 10.1111/nyas.12468
- 415 Amabile-Cuevas CF. 2016. Society must seize control of the antibiotics crisis. *Nature*  
416 533:439. 10.1038/533439a
- 417 Andersson DI, and Hughes D. 2014. Microbiological effects of sublethal levels of antibiotics.  
418 *Nature Reviews Microbiology* 12:465-478. 10.1038/nrmicro3270
- 419 Atwood D, and Paisley-Jones C. 2017. Pesticide industry sales and usage 2008-2012 market  
420 estimates. United States Environmental Protection Agency.
- 421 Baquero F, Negri MC, Morosini MI, and Blazquez J. 1998a. Antibiotic-selective  
422 environments. *Clinical Infectious Diseases* 27 Suppl 1:S5-11.
- 423 Baquero F, Negri MC, Morosini MI, and Blazquez J. 1998b. Selection of very small  
424 differences in bacterial evolution. *International Microbiology* 1:295-300.
- 425 Blair JM, Webber MA, Baylay AJ, Ogbolu DO, and Piddock LJ. 2015. Molecular mechanisms  
426 of antibiotic resistance. *Nature Reviews Microbiology* 13:42-51.  
427 10.1038/nrmicro3380
- 428 CDC. 2013. Antibiotic resistance threats in the United States, 2013. Atlanta, GA, USA:  
429 Centers for Disease Control and Prevention.
- 430 Chee-Sanford JC, Mackie RI, Koike S, Krapac IG, Lin YF, Yannarell AC, Maxwell S, and  
431 Aminov RI. 2009. Fate and transport of antibiotic residues and antibiotic resistance  
432 genes following land application of manure waste. *Journal of Environmental Quality*  
433 38:1086-1108. 10.2134/jeq2008.0128
- 434 Cohen SP, McMurry LM, Hooper DC, Wolfson JS, and Levy SB. 1989. Cross-resistance to  
435 fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by  
436 tetracycline or chloramphenicol: decreased drug accumulation associated with  
437 membrane changes in addition to OmpF reduction. *Antimicrobial Agents and*  
438 *Chemotherapy* 33:1318-1325.
- 439 Collingnon PC, Conly JM, Andremont A, and McEwen SA. 2016. World Health Organization  
440 ranking of antimicrobials according to their importance in human medicine: a  
441 critical step for developing risk management strategies to control antimicrobial  
442 resistance from food and animal production. *Clinical Infectious Diseases* 63:1087-  
443 1093.
- 444 Dan S, Shah A, Justo JA, Bookstaver PB, Kohn J, Albrecht H, and Al-Hasan MN. 2016.  
445 Prediction of Fluoroquinolone Resistance in Gram-Negative Bacteria Causing  
446 Bloodstream Infections. *Antimicrobial Agents and Chemotherapy* 60:2265-2272.  
447 10.1128/AAC.02728-15
- 448 Denamur E, and Matic I. 2006. Evolution of mutation rates in bacteria. *Molecular*  
449 *Microbiology* 60:820-827. 10.1111/j.1365-2958.2006.05150.x

- 450 Egeghy PP, Judson R, Gangwal S, Mosher S, Smith D, Vail J, and Cohen Ubal EA. 2012. The  
451 exposure data landscape for manufactured chemicals. *Science of The Total*  
452 *Environment* 414:159-166.
- 453 EPA. 2008. 2006 Inventory Update Reporting: Data Summary. In: Agency UEP, editor: US  
454 Environmental Protection Agency.
- 455 Fernandez L, and Hancock RE. 2012. Adaptive and mutational resistance: role of porins and  
456 efflux pumps in drug resistance. *Clinical Microbiology Reviews* 25:661-681.  
457 10.1128/CMR.00043-12
- 458 Friedman ND, Temkin E, and Carmeli Y. 2016. The negative impact of antibiotic resistance.  
459 *Clinical Microbiology and Infection* 22:416-422. 10.1016/j.cmi.2015.12.002
- 460 Funchain P, Yeung A, Stewart J, Clendenin WM, and Miller JH. 2001. Amplification of  
461 mutator cells in a population as a result of horizontal gene transfer. *Journal of*  
462 *Bacteriology* 183:3737-3741.
- 463 Gustafson JE, Candelaria PV, Fisher SA, Goodridge JP, Lichocik TM, McWilliams TM, Price  
464 CT, O'Brien FG, and Grubb WB. 1999. Growth in the presence of salicylate increases  
465 fluoroquinolone resistance in *Staphylococcus aureus*. *Antimicrobial Agents and*  
466 *Chemotherapy* 43:990-992.
- 467 Heinemann JA, and Kurenbach B. 2017. Post-Antibiotic Era is Business as Usual. *APUA*  
468 *Newsletter* 35:7-9.
- 469 Hermsen R, Deris JB, and Hwa T. 2012. On the rapidity of antibiotic resistance evolution  
470 facilitated by a concentration gradient. *Proceedings of the National Academy of*  
471 *Sciences* 109:10775-10780. 10.1073/pnas.1117716109
- 472 Hothorn T, Bretz F, and Westfall P. 2008. Simultaneous inference in general parametric  
473 models. *Biometrical Journal* 50:346-363. 10.1002/bimj.200810425
- 474 Kurenbach B, Gibson PS, Hill AM, Bitzer AS, Silby MW, Godsoe W, and Heinemann JA. 2017.  
475 Herbicide ingredients change *Salmonella enterica* sv. Typhimurium and *Escherichia*  
476 *coli* antibiotic responses. *Microbiology* 163:1791-1801. 10.1099/mic.0.000573
- 477 Kurenbach B, Marjoshi D, Amabile-Cuevas CF, Ferguson GC, Godsoe W, Gibson P, and  
478 Heinemann JA. 2015. Sublethal exposure to commercial formulations of the  
479 herbicides dicamba, 2,4-dichlorophenoxyacetic acid, and glyphosate cause changes  
480 in antibiotic susceptibility in *Escherichia coli* and *Salmonella enterica* serovar  
481 Typhimurium. *mBio* 6. 10.1128/mBio.00009-15
- 482 Levy SB. 2001. Antibacterial household products: cause for concern. *Emerging Infectious*  
483 *Diseases* 7:512-515. 10.3201/eid0707.010705
- 484 Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, Brochado AR, Fernandez  
485 KC, Dose H, Mori H, Patil KR, Bork P, and Typas A. 2018. Extensive impact of non-  
486 antibiotic drugs on human gut bacteria. *Nature* 555:623-628. 10.1038/nature25979
- 487 Mallet J. 2012. The struggle for existence: how the notion of carrying capacity,  $K$ , obscures  
488 the links between demography, Darwinian evolution, and speciation. *Evolutionary*  
489 *Ecology Research* 14:627-665.

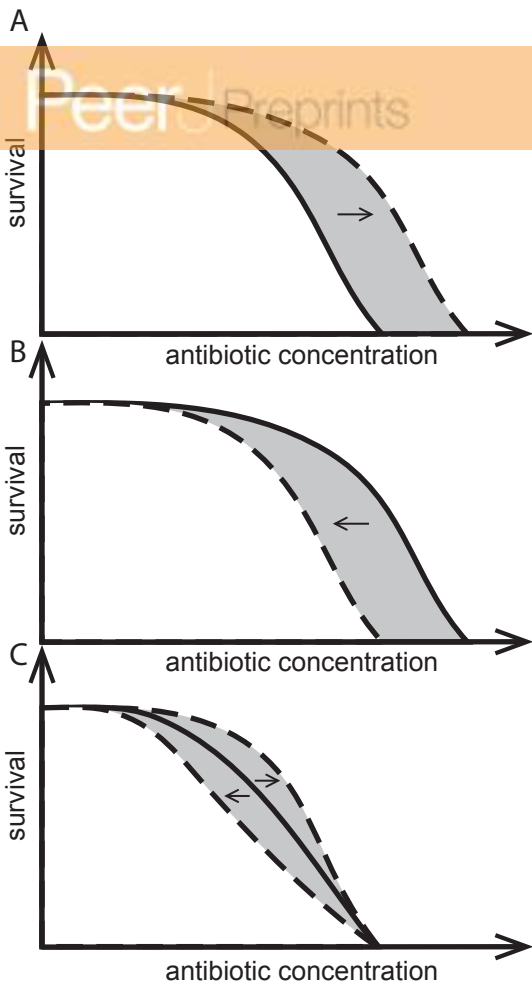
- 490 Palmer AC, and Kishony R. 2013. Understanding, predicting and manipulating the  
491 genotypic evolution of antibiotic resistance. *Nature Reviews Genetics* 14:243-248.  
492 10.1038/nrg3351
- 493 R Core Team. 2013. R: a language and environment for statistical computing. Vienna,  
494 Austria: R Foundation for Statistical Computing.
- 495 Sanchez-Romero MA, and Casades J. 2014. Contribution of phenotypic heterogeneity to  
496 adaptive antibiotic resistance. *Proceedings of the National Academy of Sciences*  
497 111:355-360. 10.1073/pnas.1316084111
- 498 Schneider EK, Reyes-Ortega F, Velkov T, and Li J. 2017. Antibiotic-non-antibiotic  
499 combinations for combating extremely drug-resistant Gram-negative 'superbugs'.  
500 *Essays in Biochemistry* 61:115-125. 10.1042/EBC20160058
- 501 Shen Z, Pu XY, and Zhang Q. 2011. Salicylate functions as an efflux pump inducer and  
502 promotes the emergence of fluoroquinolone-resistant *Campylobacter jejuni*  
503 mutants. *Applied and Environmental Microbiology* 77:7128-7133.  
504 10.1128/AEM.00763-11
- 505 Stockwell VO, and Duffy B. 2013. Use of antibiotics in plant agriculture. *Scientific and*  
506 *Technical Review of the Office International des Epizooties* 31:199-210.
- 507 Teillant A, Gandra S, Barter D, Morgan DJ, and Laxminarayan R. 2015. Potential burden of  
508 antibiotic resistance on surgery and cancer chemotherapy antibiotic prophylaxis in  
509 the USA: a literature review and modelling study. *Lancet Infectious Diseases*  
510 15:1429-1437.
- 511 Thomas MG, Smith AJ, and Tilyard M. 2014. Rising antimicrobial resistance: a strong reason  
512 to reduce excessive antimicrobial consumption in New Zealand. *New Zealand*  
513 *Medical Journal* 127:72-84.
- 514 Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, and  
515 Laxminarayan R. 2015. Global trends in antimicrobial use in food animals.  
516 *Proceedings of the National Academy of Sciences* 112:5649-5654.  
517 10.1073/pnas.1503141112
- 518 van den Bosch F, Oliver R, van den Berg F, and Paveley N. 2014. Governing principles can  
519 guide fungicide-resistance management tactics. *Annual Reviews Phytopathology*  
520 52:175-195. 10.1146/annurev-phyto-102313-050158
- 521 Worthington RJ, and Melander C. 2013. Combination approaches to combat multidrug-  
522 resistant bacteria. *Trends in Biotechnology* 31:177-184.  
523 10.1016/j.tibtech.2012.12.006
- 524 Wright GD. 2016. Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. *Trends in*  
525 *Microbiology* 24:862-871. 10.1016/j.tim.2016.06.009
- 526 Zurek L, and Ghosh A. 2014. Insects represent a link between food animal farms and the  
527 urban environment for antibiotic resistance traits. *Applied Environmental*  
528 *Microbiology* 80:3562-3567. 10.1128/AEM.00600-14
- 529



**Figure 1**(on next page)

Effects of herbicides on bacterial responses to antibiotics.

Herbicides can change responses to antibiotics. Solid curves indicate survival as a function of only antibiotic concentration, intersecting the x-axes at the MIC. Dashed curves show herbicides (A) increasing the MIC, (B) decreasing the MIC, and (C) having no effect on MIC but altering population survival at antibiotic concentrations below-MIC. The hypothetical MSC is the point of divergence of solid and dashed curves and the area in gray illustrates all antibiotic concentrations where the herbicide changes the response to antibiotics.

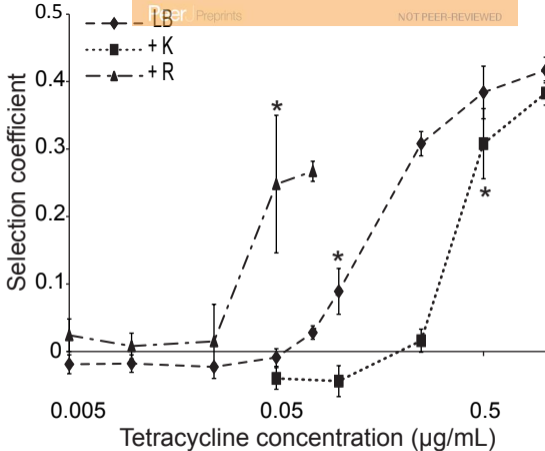




**Figure 2** (on next page)

Competition between strains of different potential to resist tetracycline.

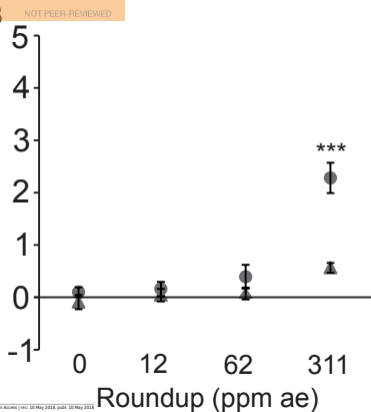
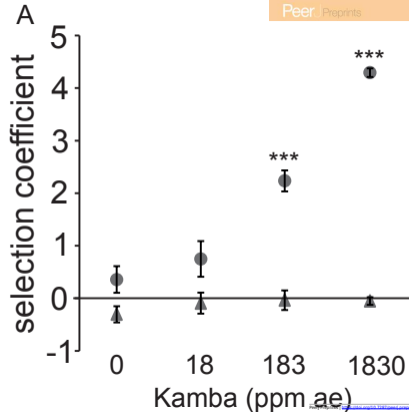
Competition between tetracycline resistant (AH201, Tet<sup>high</sup>) and susceptible (AH214, Tet<sup>low</sup>) strains in different concentrations of tetracycline with or without Kamba (K) or Roundup (R). Kamba was used at 1830 ppm ae, Roundup at 311 ppm ae. Selection coefficients are presented as a function of tetracycline concentration, with positive values indicating a selection for the more resistant strain. Values are averages of three independent experiments; error bars are SEM (standard deviation/ $\sqrt{n}$ ). \* indicate the lowest tetracycline concentrations for each treatment where selection coefficients were significantly different from the -Tet/-herbicide treatment.



**Figure 3**(on next page)

Dose-response to two herbicides with (●) or without (▲) antibiotic (used at a single concentration).

A positive selection coefficient indicates selection for the strain with a higher MIC. Results are averages of 3 independent experiments. Error bars are SEM (standard deviation/ $\sqrt{n}$ ). (A): Kamba+Str, using strains AH204 (Str<sup>high</sup>, MIC<sub>Str</sub>: 200 $\mu$ g/mL) and AH211 (Str<sup>low</sup>, MIC<sub>Str</sub>: 1 $\mu$ g/mL); (B): Roundup+Tet, using strains AH201 (Tet<sup>high</sup>, MIC<sub>Tet</sub>: 125 $\mu$ g/mL) and AH214 (Tet<sup>low</sup>, MIC<sub>Tet</sub>: 1.5 $\mu$ g/mL). Concentrations of antibiotics were 0.25 $\mu$ g/mL for Str and 0.05 $\mu$ g/mL for Tet. Asterisks indicate contrasts where addition of antibiotic significantly changed the strength of selection, with \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .



**Table 1** (on next page)

Bacteria and plasmids

Bacteria	Genotype/comments	Relevant antibiotic resistance level	Reference/source
<i>E. coli</i>			
BW25113	wild-type. F <sup>-</sup> , λ <sup>-</sup> , Δ( <i>araD-araB</i> )567, Δ <i>lacZ</i> 4787(:: <i>rrnB-3</i> ), <i>rph-1</i> , Δ( <i>rhaD-rhaB</i> )568, <i>hsdR514</i>		(41)
SB21	<i>hsdS</i> , <i>leuB6</i> , <i>thr</i>		(42)
AH201 (Tet <sup>high</sup> )	SB21 (pBR322)	Tet: 125 μg/mL	This study
AH214(Tet <sup>low</sup> )	SB21 (pAH14)	Tet: 1.5 μg/mL	This study
JB436	SB21 Nal <sup>R</sup>	Nal: 60 μg/mL	(43)
AH204 (Str <sup>high</sup> )	JB436 (RSF1010)	Str: 250 μg/mL	This study
AH211 (Str <sup>low</sup> )	SB21 (pAH11)	Str: 1 μg/mL	This study
<i>S. enterica</i> sv Typhimurium			
SL3770	LT2, <i>pyr</i> <sup>+</sup> , <i>rfa</i>		(44)
Plasmids			
pBR322	Amp <sup>R</sup> , Tet <sup>R</sup>	Amp: 100 μg/mL, Tet: 125 μg/mL	(45)
pAH14	pBR322 derivative, Cam <sup>R</sup> , Amp <sup>S</sup> , Tet <sup>S</sup>	Cam:20 μg/mL	This study
RSF1010	Str <sup>R</sup>	Str: 250 μg/mL	(46)
pAH11	RSF1010 derivative, Cam <sup>R</sup> , Str <sup>S</sup>	Cam: 20 μg/mL, Str: 1 μg/mL	This study

1

2

**Table 2** (on next page)

## Mutant frequency per generation

Frequencies of acquired resistance were determined with or without herbicide and Cip exposure. Results are averages of at least 4 independent experiments. Brackets show SEM (standard deviation/ $\sqrt{n}$ ). Cip concentration used for final plating: 0.05  $\mu\text{g/mL}$  for *S. enterica* and 0.06  $\mu\text{g/mL}$  for *E. coli*. 1,250 ppm ae Roundup or 1830 ppm ae Kamba were used.

<sup>a</sup>Herbicide+antibiotic mutant frequency is significantly different from LB but not from herbicide treatment, for all other combinations herbicide+antibiotic is significantly different from both LB and herbicide treatments. <sup>b</sup>*S. enterica* experiments were conducted concurrently, using the same LB controls for both assays. *E. coli* was strain BW25113. <sup>c</sup> $P < 0.01$  <sup>d</sup> $P < 0.001$

1

	LB	LB+ Herbicide	LB+ Herbicide +Cip
<i>S. enterica</i>			
Kamba	$3.57 \times 10^{-6}$ ( $1.27 \times 10^{-6}$ ) <sup>b</sup>	$2.01 \times 10^{-4}$ ( $1.95 \times 10^{-4}$ )	$1.30 \times 10^{-2}$ ( $1.29 \times 10^{-2}$ ) <sup>c</sup>
Roundup	$3.57 \times 10^{-6}$ ( $1.27 \times 10^{-6}$ ) <sup>b</sup>	$2.91 \times 10^{-5}$ ( $2.47 \times 10^{-5}$ )	$2.79 \times 10^{-2}$ ( $1.71 \times 10^{-2}$ ) <sup>ac</sup>
<i>E. coli</i>			
Roundup	$1.80 \times 10^{-9}$ ( $1.62 \times 10^{-9}$ )	$1.97 \times 10^{-10}$ ( $5.46 \times 10^{-11}$ )	$2.72 \times 10^{-5}$ ( $2.67 \times 10^{-5}$ ) <sup>d</sup>

2



**Table 3** (on next page)

Ciprofloxacin resistant variants of *E. coli* BW25113 per generation

a) p-value for comparison between the treatment condition and LB medium. Kamba was used at 1830 ppm ae, Cip was 0.025 µg/mL in liquid and 0.06 µg/mL in solid media.

1

Treatment	Resistant variants / generation	SEM	p-value <sup>a</sup>
LB	$7.5 \times 10^{-9}$	$3.9 \times 10^{-9}$	na
Kamba	$10^{-7}$	$6.3 \times 10^{-8}$	0.22
Cip	$1.2 \times 10^{-3}$	$6.5 \times 10^{-4}$	$2.4 \times 10^{-6}$
Kamba+Cip	$5 \times 10^{-7}$	$4.5 \times 10^{-7}$	0.27

2