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Agrichemicals boost the effects of antibiotics on antibiotic resistance evolution

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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.

Classification: Biological sciences / evolution

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Short title: Herbicide evolved antibiotic resistance

Abstract

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.

Significance

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

Key words antibiotic resistance, evolution, adaptive resistance, herbicide, minimum selective 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 dependent 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^{500} (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 *Hin*dIII 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^R, Amp^S, Tet^S. 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^R, Str^S.

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. 10^5 cfu/mL for the former treatment and ca. 10^6 cfu/mL (*E.*
124 *coli*) and 10^5 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 10^3 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_{600} was determined every 6 min for 16 hrs and averaged over five replicates.
161 Cultures were started at densities of ca. 10^6 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 (≈ 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 µg/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 µg/mL Cip, were included
222 as controls.

223 The parental strain and 6 evolved isolates did not grow at 0.07 µg/mL, a concentration just above
224 the selection concentration. We observed MICs of 0.1 µg/mL Cip for 21 isolates and 0.2 µg/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 µg/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 µg/mL and for Tet^{high} it was 125 µg/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 µg/mL, but growth
245 of Tet^{high} was not affected for tetracycline concentrations up to 50 µg/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 ≥0.1 µg/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 µg/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 µg/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 ≥0.5 µg/mL, compared to ≥0.1 µg/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 (≈ 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.

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

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

The observed fitness differential was due to the faster reproductive rate of the Str^{high} and Tet^{high} strains in environments with sub-lethal concentrations of antibiotic for both strains in each pair. This was shown by measuring the growth rate of the strains in monoculture rather than in competition (Supplementary Fig. S1). No differences were observed for the monoculture growth rate (r) of matched isogenic pairs in LB medium, LB+antibiotic, or LB+herbicide ($p>0.1$ for all combinations; Supplementary Fig. S1A and C). Only in the combined LB+herbicide+antibiotic treatment did strains with higher MICs have shorter generation times in monoculture than their lower MIC counterparts ($p=1.6x10^{-5}$ (Kamba+Str) and $p=4.7x10^{-10}$ (Roundup+Tet)).

Significant differences in the carrying capacity of the environments for all treatment combinations were observed for the matched strains exposed to Kamba+Str measured in monoculture. The high MIC strain achieved higher final optical densities ($p<2x10^{-16}$; Supplementary Fig. S1B). In contrast, the Str^{low} strain population grew to higher final optical densities in LB, LB+Tet, and LB+Roundup treatments. In cultures with Roundup+Tet, Tet^{high} and Tet^{low} had similar densities ($p=0.24$, Supplementary Fig. S1D).

Discussion

In this study we report that when bacteria are simultaneously exposed to herbicides and antibiotics, mutants with higher levels of resistance can evolve. In some cases, resistance evolved 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 physiotypes. 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 x 10^9
374 kilograms in 2012 with up to 2 x 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 x 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 extend 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.

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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 Casadesus 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

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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.

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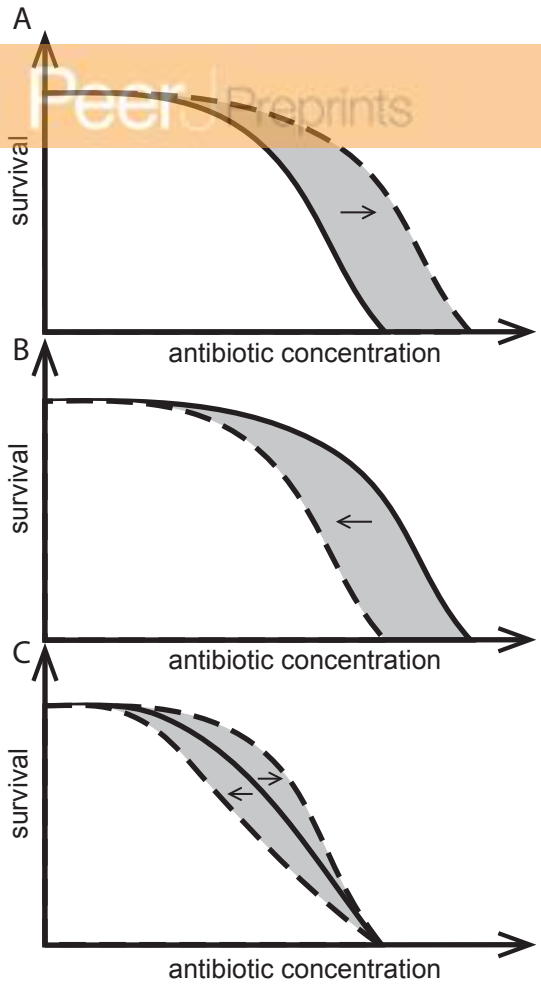

Figure 2(on next page)

Competition between strains of different potential to resist tetracycline.

Competition between tetracycline resistant (AH201, Tet^{high}) and susceptible (AH214, Tet^{low}) 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/√n). * indicate the lowest tetracycline concentrations for each treatment where selection coefficients were significantly different from the -Tet/-herbicide treatment.

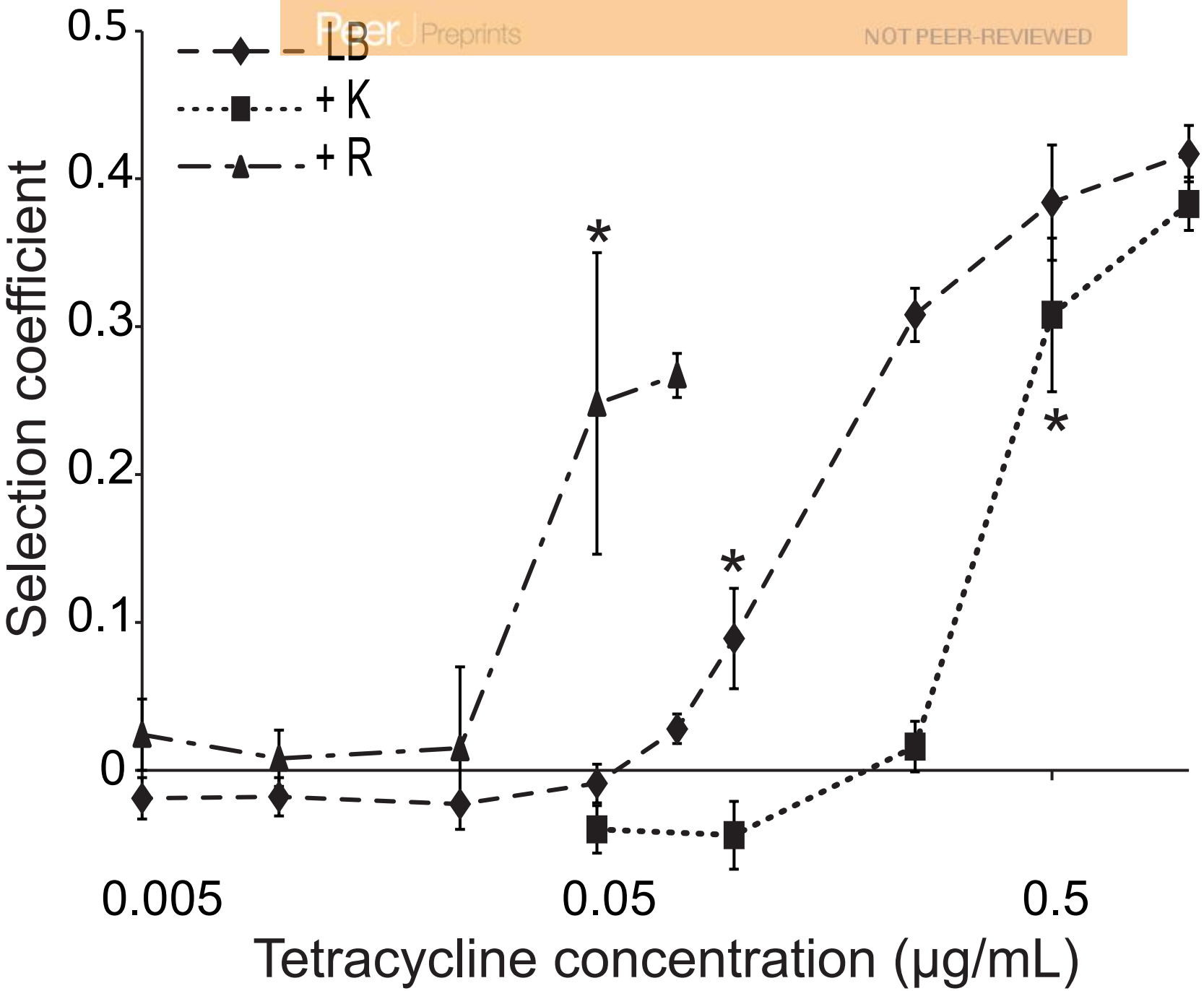

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Figure 3(on next page)

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/√n). (A): Kamba+Str, using strains AH204 (Str^{high}, MIC_{Str}: 200µg/mL) and AH211 (Str^{low}, MIC_{Str}: 1µg/mL); (B): Roundup+Tet, using strains AH201 (Tet^{high}, MIC_{Tet}: 125µg/mL) and AH214 (Tet^{low}, MIC_{Tet}: 1.5µg/mL). Concentrations of antibiotics were 0.25µg/mL for Str and 0.05µg/mL for Tet. Asterisks indicate contrasts where addition of antibiotic significantly changed the strength of selection, with *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

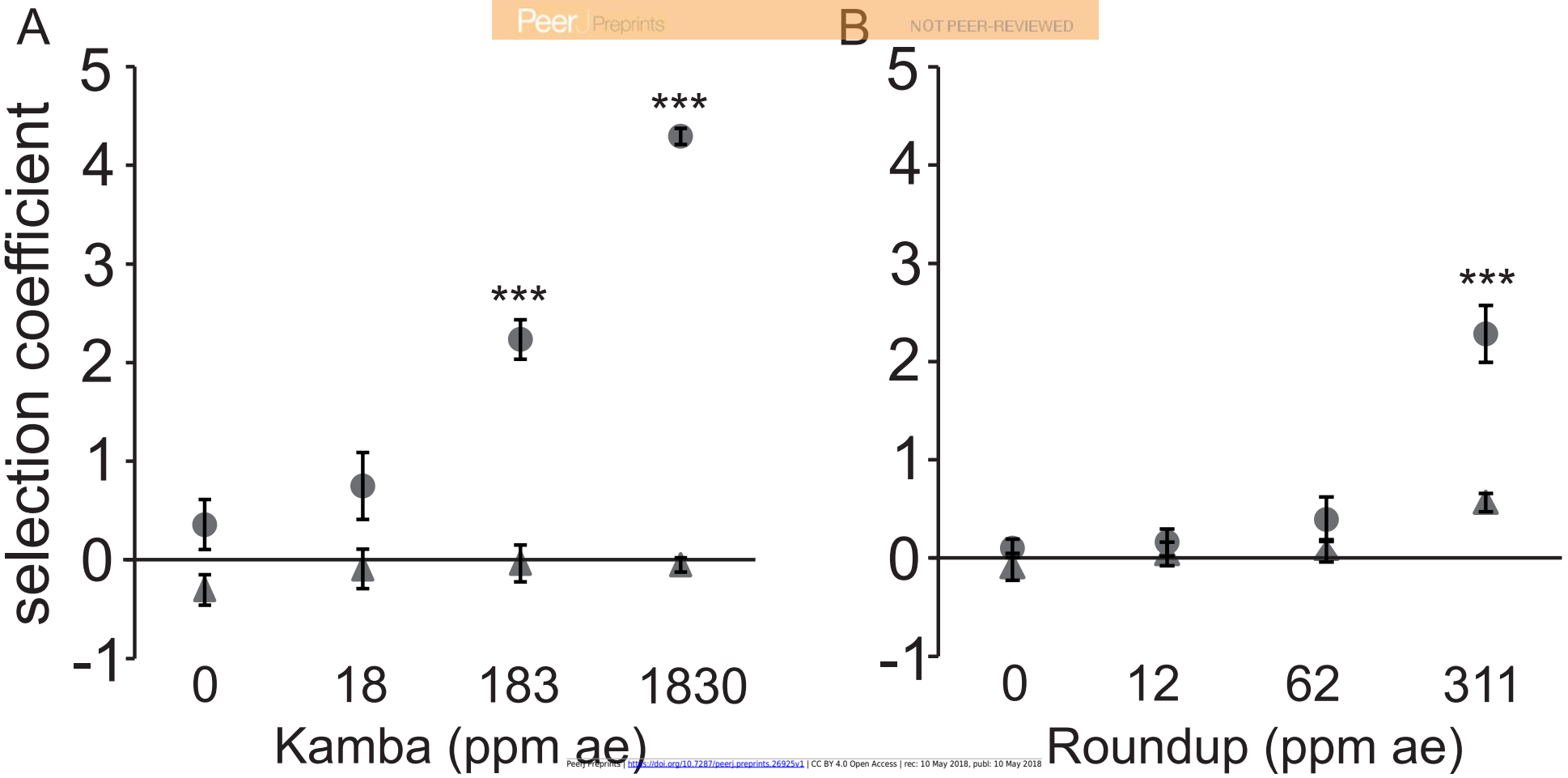

Table 1 (on next page)

Bacteria and plasmids

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Bacteria	Genotype/comments	Relevant antibiotic resistance level	Reference/ source
E. coli			
BW25113	wild-type. F^-, λ^-, Δ*(araD-araB)567*, *ΔlacZ4787(::rrnB-3), rph-1,* Δ*(rhaD-rhaB)568, hsdR514*		(41)
SB21	*hsdS*, *leuB6*, *thr*		(42)
AH201 (Tet^{high})	SB21 (pBR322)	Tet: 125 µg/mL	This study
AH214(Tet^{low})	SB21 (pAH14)	Tet: 1.5 µg/mL	This study
JB436	SB21 Nal^R	Nal: 60 µg/mL	(43)
AH204 (Str^{high})	JB436 (RSF1010)	Str: 250 µg/mL	This study
AH211 (Str^{low})	SB21 (pAH11)	Str: 1 µg/mL	This study
S. enterica sv Typhimurium			
SL3770	LT2, pyr^+, *rfa*		(44)
Plasmids			
pBR322	Amp^R, Tet^R	Amp: 100 µg/mL, Tet: 125 µg/mL	(45)
pAH14	pBR322 derivative, Cam^R, Amp^S, Tet^S	Cam:20 µg/mL	This study
RSF1010	Str^R	Str: 250 µg/mL	(46)
pAH11	RSF1010 derivative, Cam^R, Str^S	Cam: 20 µg/mL, Str: 1 µg/mL	This study

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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/√n). Cip concentration used for final plating: 0.05 µg/mL for *S. enterica* and 0.06 µg/mL for *E. coli*. 1,250 ppm ae Roundup or 1830 ppm ae Kamba were used. [a]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. [b]*S. enterica* experiments were conducted concurrently, using the same LB controls for both assays. *E. coli* was strain BW25113. [c]$P < 0.01$ [d]$P < 0.001$

1

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

2

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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.

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Treatment	Resistant variants / generation	SEM	p-value[a]
LB	7.5×10^{-9}	3.9×10^{-9}	na
Kamba	10^{-7}	6.3×10^{-8}	0.22
Cip	1.2×10^{-3}	6.5×10^{-4}	2.4×10^{-6}
Kamba+Cip	5×10^{-7}	4.5×10^{-7}	0.27

2