Agrichemicals and antibiotics in combination increase antibiotic resistance evolution

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Antibiotic resistance in our pathogens 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 differences in gene expression due to environmental variation, referred to as adaptive resistance. Adaptive changes can increase fitness allowing bacteria to survive at higher concentrations of antibiotics. 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 previous 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 is attributed to the effect of the herbicide on either MIC or the minimum selective concentration (MSC) of a paired antibiotic. The MSC is the lowest concentration of antibiotic at which the fitness of individuals varies because of the antibiotic, and is lower than MIC. Our results suggest that additional environmental factors influencing competition between bacteria could enhance the ability of antibiotics to select antibiotic resistance. 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**

3 **Agrichemicals and antibiotics in combination increase antibiotic resistance evolution**

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

20 **Abstract**

21 Antibiotic resistance in our pathogens is medicine's climate change: caused by human activity,

22 and resulting in more extreme outcomes. Resistance emerges in microbial populations when

23 antibiotics act on phenotypic variance within the population. This can arise from either genotypic

24 diversity (resulting from a mutation or horizontal gene transfer), or from differences in gene

25 expression due to environmental variation, referred to as adaptive resistance. Adaptive changes

26 can increase fitness allowing bacteria to survive at higher concentrations of antibiotics. They can

27 also decrease fitness, potentially leading to selection for antibiotic resistance at lower

28   concentrations. There are opportunities for other environmental stressors to promote antibiotic

29   resistance in ways that are hard to predict using conventional assays. Exploiting our previous

30   observation that commonly used herbicides can increase or decrease the minimum inhibitory

31   concentration (MIC) of different antibiotics, we provide the first comprehensive test of the

32   hypothesis that the rate of antibiotic resistance evolution under specified conditions can increase,

33   regardless of whether a herbicide increases or decreases the antibiotic MIC. Short term evolution

34   experiments were used for various herbicide and antibiotic combinations. We found conditions

35   where acquired resistance arises more frequently regardless of whether the exogenous non-

36   antibiotic agent increased or decreased antibiotic effectiveness. This is attributed to the effect of

37   the herbicide on either MIC or the minimum selective concentration (MSC) of a paired

38   antibiotic. The MSC is the lowest concentration of antibiotic at which the fitness of individuals

39   varies because of the antibiotic, and is lower than MIC. Our results suggest that additional

40   environmental factors influencing competition between bacteria could enhance the ability of

41   antibiotics to select antibiotic resistance. Our work demonstrates that bacteria may acquire

42   antibiotic resistance in the environment at rates substantially faster than predicted from

43   laboratory conditions.

44

45   **Significance**

46   Neither reducing the use of antibiotics nor discovery of new ones may be sufficient strategies to

47   avoid the post-antibiotic era. This is because bacteria may be exposed to other non-antibiotic

48   chemicals that predispose them to evolve resistance to antibiotics more quickly. Herbicides are

49   examples of some of the most common non-antibiotic chemicals in frequent global use. We

50   previously showed that in some combinations the herbicides we tested made bacteria

51   phenotypically resistant to higher concentrations of antibiotics, while in other combinations

52   bacteria became susceptible at lower antibiotic concentrations. Here we demonstrate that in both

53   cases the herbicides worked with antibiotics to accelerate genotypic resistance evolution.

54   Unfortunately, antibiotic resistance may increase even if total antibiotic use is reduced, and new

55   ones are invented, unless other environmental exposures are also controlled.

56

57

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

59   concentration; NOEL, no observable effect level

Introduction

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

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

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

Innate resistance can also be dependent upon genes expressed or repressed conditionally, resulting in increased efflux or decreased influx of antibiotics and overall lower intracellular antibiotic concentrations (Fernandez & Hancock 2012). Such genes or expression induction thresholds may differ between species, and individuals within a species may phenotypically differ depending on whether or not they expressed those genes before being inhibited by the antibiotic. This resistance through changes in gene expression is also known as an *adaptive*

90   (change in phenotype) response (Fernandez et al. 2011) which acclimates bacteria to the

91   environment. It can be triggered by antibiotics and other chemical toxins or environmental cues

92   (Blair et al. 2015; Palmer & Kishony 2013; Sanchez-Romero & Casadesus 2014).

93   We have previously shown that concurrent exposure of *Escherichia coli* or *Salmonella enterica*

94   sv. Typhimurium to various commercial herbicides and antibiotics from different classes can

95   lead to adaptive increases (Fig. 1A) or decreases (Fig. 1B) in their susceptibility to the

96   antibiotics. In some cases, herbicide exposures changed the survival at below-MIC antibiotic

97   concentrations of antibiotic, while MIC itself was not affected (Fig. 1C). Adaptive responses

98   were specific for the combination of bacteria, antibiotic and herbicide used. Effects were shown

99   for commercial formulations (Kurenbach et al. 2015), for label listed active ingredients, and for

100   common adjuvants (Kurenbach et al. 2017).

101   Populations of adaptively resistant bacteria can in time produce variants with acquired resistance

102   to even higher concentrations of antibiotic (Cohen et al. 1989; Gustafson et al. 1999; Shen et al.

103   2011). This raises the possibility that environmental stimuli that cause phenotypic antibiotic

104   resistance variation between individuals could be hotspots for evolution of acquired antibiotic

105   resistance.

106   Here we exploit our previous observations to test the novel hypothesis that both adaptive

107   increases *and* decreases in antibiotic susceptibility caused by exposure to herbicide formulations

108   can lead to an increase in the rate of acquired resistance evolution in populations of *E. coli* and *S.*

109   *typhimurium*. For this purpose we conducted short term evolution experiments using either the

110   natural variability within a monoculture of *E. coli* or *S. typhimurium*, or isogenic strains only

111   differing in their level of antibiotic resistance, which represents natural variability.

112   **Materials and Methods**

113   **Media**

114   Strains and plasmids are listed in Table 1. Bacteria were grown in standard rich growth medium,

115   LB (Lennox) (Invitrogen) at 37°C and supplemented with ampicillin (Amp, AppliChem),

116   chloramphenicol (Cam, Sigma) ciprofloxacin (Cip, Pentex), streptomycin (Str, Sigma),

117   tetracycline (Tet, Sigma), or nalidixic acid (Nal, Sigma) as appropriate. Liquid cultures were

118   grown with aeration (180 rpm), and plates were incubated in plastic bags to avoid drying out.

119 Commercial herbicide formulations used were Kamba[500] (Nufarm, NZ) containing 500 g/L

120 dimethyl salt of dicamba, and Roundup Weedkiller (Monsanto, Australia) containing 360 g/L

121 isopropylamine salt of glyphosate. Herbicide concentrations are reported in parts per million acid

122 equivalent (ppm ae) of the active ingredient to allow for comparison with other formulations.

123 Antibiotic and herbicide concentrations used are specified in the main text or in figure legends.

124 Plasmid constructs

125 pBR322 (Table 1) was used as the base to create a pair of plasmids that only differed in

126 antibiotic resistance determinants. Plasmid pAH14 was created by deleting a section of the gene

127 for Tet[R] by removing the *Hin*dIII and *Bam*HI fragment of pBR322 and inserting *cat* from

128 pACYC184 at the *Pst*I site within *bla*. The resulting plasmid conferred resistance to

129 chloramphenicol, but not to ampicillin or tetracycline (Cam[R], Amp[S], Tet[S]). RSF1010 (Table 1)

130 was the base for pAH11, which was created by insertion of *cat* from pACYC184 into the *Eco*RI

131 and *Not*I sites of RSF1010, resulting in a plasmid conferring chloramphenicol but not

132 streptomycin resistance (Cam[R], Str[S]).

133 Culturing conditions

134 For the experiments described in the section "Evolution of acquired antibiotic resistance in

135 cultures with herbicide-induced increases in MIC" *E. coli* or *S. typhimurium* were grown for 24

136 hrs in 10 mL liquid LB medium containing Cip, herbicide, both, or neither. Initial densities were

137 ca. 10^5 cfu/mL for the former treatment and ca. 10^6 cfu/mL (*E. coli*) and 10^5 cfu/mL (*S.*

138 *typhimurium*) for the latter three. The experiment was abandoned at this point if cultures

139 containing Cip but not herbicide were visibly turbid. This was because in this experiment the

140 chosen Cip concentration was above MIC in cultures not exposed to herbicide, and growth was

141 therefore interpreted as due to a resistant mutant in the starting culture. Herbicide and antibiotic

142 concentrations used are detailed in Table 2 and Table 3. Cultures were then diluted as above in

143 the same conditions and incubated again for 24 hrs. A 10-fold dilution in LB without herbicides

144 or antibiotics followed to ensure observed effects were not due to herbicide induced adaptive

145 responses. Titers (cfu/mL) were determined at the end of each incubation step on both LB and on

146 Cip at an initially non-permissive concentration (ca. 2x MIC) (Dan et al. 2016). The frequency of

147 Cip resistant mutants and the number of generations was calculated.

148 Experiments described in the section "Herbicide-induced changes in MSC can occur without a
149 change in MIC" were conducted similarly. However, in this case the culture supplemented with
150 only Cip was able to grow because the chosen Cip concentration was below MIC.

151 **Determination of resistance levels**

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

156 **Mixed culture experiments**

157 Isogenic strains of *E. coli* differing only in the MIC phenotype (i.e. high vs. low) and an
158 additional selection marker encoded on a low copy number plasmid were co-incubated in liquid
159 LB medium containing herbicide, antibiotic, both, or neither and grown to saturation before
160 dilution by a factor of 10^3 in the same conditions. The antibiotic concentration chosen was below
161 NOEL for both strains. NOEL is defined here as the highest antibiotic concentration for which
162 no effect on survival or selection were observed. The titer of each culture was determined by
163 plating on non-selective medium after each incubation step. After 5 rounds of incubation, the
164 ratio of strains was determined by selecting for the second – competition irrelevant – marker.

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

173 **Growth curves**

174 Growth curves were established at 37°C in liquid LB medium supplemented with herbicide,
175 antibiotic, both, or neither using a FLUOstar Omega microplate reader (BMG LABTECH,
176 Germany). The OD_{600} was determined every 6 min for 16 hrs and averaged over five biological

177   replicates. Cultures were started at densities of ca. 10^6 cfu/mL. Antibiotic and herbicide

178   concentrations used are detailed in the legend of Suppl. Fig. S1.

179   Statistical analysis

180   R was used for all statistical analyses (R Core Team 2013). For experiments measuring changes

181   to MIC, an ANOVA was used to analyze the randomized complete block design, using each

182   independent experiment as a block with presence/absence of antibiotic/herbicide as levels.

183   Residuals were used to test for normality and equality of variances and log transformed data

184   where appropriate. Tukey's HSD test was used to determine which treatments were significantly

185   different from each other.

186   For mixed culture experiments (changes to MSC), two sets of analyses were performed. First, we

187   determined whether adding antibiotics increases the strength of selection at different herbicide

188   concentrations. Second, we determined whether adding herbicides increases the strength of

189   selection at different antibiotic concentrations. Each set of questions was tested using contrasts

190   performed with the glht function in the multcomp package (Hothorn et al. 2008) with a two-sided

191   alternative and sequential Bonferroni procedure. These contrasts were fit to an ANOVA model

192   treating each combination of herbicide and antibiotic as a treatment category. Residual plots

193   confirmed that the assumptions of normality and equality of variance were met. Because

194   residuals were approximately normally distributed we report the arithmetic mean (the maximum

195   likelihood estimator of population mean under the assumption of normality).

196   Statistical analysis for growth curves

197   To estimate carrying capacity (k), a logistic growth model on the raw data using non-linear least

198   squares (nls) was fit in R. Differences in population growth between strains in different

199   treatments were estimated by estimating r, the intrinsic growth rate, of each strain. Data sets were

200   log transformed and the slope of the growth curve between t=48 mins and t=150 mins was

201   measured. A visual inspection of the plots revealed that before t=48 mins graphs were not linear.

202   After t=150 mins, growth slowed as cells were entering stationary phase.

203   We tested for differences between the two strains in both r and k by calculating contrast in an

204   ANOVA (aov). Residual plots were used to test for violations of assumptions for ANOVA.

205   Assumptions of normality and equal variances were met in all data sets. In two data sets there

206 were a small number of outliers. These had small influences on parameter estimates and were

207 hence not removed. Contrasts between treatments were calculated using the glht package in R

208 using the sequential Bonferroni correction.

209

210 **Results**

211 As reported previously, simultaneous exposure to a variety of antibiotics and either various

212 commercial herbicide formulations (Kurenbach et al. 2015) or to either active ingredients and

213 some surfactants (Kurenbach et al. 2017), alters the survival of *E. coli* and *S. typhimurium*

214 compared to exposure to only the antibiotics. This was due to adaptive changes in exposed

215 bacteria. From these observations we hypothesized that particular combinations of antibiotics

216 and these formulations alter the MSC or MIC of the antibiotic in a way that favours genetically

217 resistant variants. To test this hypothesis, we conducted short-term evolution in antibiotics with

218 and without additional exposures to commercial herbicide formulations.

219 Because the effect of the herbicide exposure was specific to the combination of herbicide and

220 antibiotic, primarily two kinds of experiments were done. Firstly, in the case where the herbicide

221 raised the MIC of the antibiotic, we tested whether antibiotic survivors could give rise to

222 antibiotic resistant variants. In this case, only bacteria simultaneously exposed to both the

223 herbicide and antibiotic would survive when the antibiotic concentration exceeded the MIC of

224 the bacteria measured without the addition of the herbicide. Secondly, in the case where the

225 herbicide decreased the MIC of the antibiotic, we tested whether the herbicide decreased the

226 MSC of the antibiotic, leading to a greater rate of resistance evolution at lower concentrations of

227 antibiotic because of exposure to the herbicide formulation.

228

229 **Evolution of acquired antibiotic resistance in cultures with herbicide-induced increases in**

230 **MIC**

231 Exposing either *E. coli* or *S. typhimurium* to the herbicide formulation Roundup increased the

232 MIC of the fluoroquinolone antibiotic ciprofloxacin, as did exposing *S. typhimurium* to the

233 herbicide formulation Kamba (Kurenbach et al. 2015). These three combinations were used to

234 test the hypothesis that the survival of bacteria exposed to normally lethal concentrations of

235   antibiotic provides the opportunity for the population to evolve higher frequencies of resistant
236   genotypes.

237   The rate of acquired resistance mutations in populations of *E. coli* BW25113 or *S. typhimurium*
238   SL3770 (all strains described in Table 1) was measured over the course of about 25 generations
239   in a standard rich growth medium, liquid LB, or LB+herbicide, LB+Cip, or LB+herbicide+Cip.
240   The chosen herbicide concentration was below NOEL (no observable effect level), so had no
241   impact on survival or selection of the bacteria on its own (Table 2). The ciprofloxacin
242   concentration was the same in all cultures to which it was added and was above MIC in cultures
243   not exposed to herbicide, but below MIC for bacteria in cultures simultaneously exposed to
244   herbicide. At the end of the experiment, bacteria were transferred to solid medium supplemented
245   with high levels of ciprofloxacin (above MIC) and no herbicide, which was permissive only to
246   the growth of variants with acquired resistance.

247   Expectedly, cultures supplemented only with ciprofloxacin did not grow, and genotypic resistant
248   variants were not detected. Cultures that grew for 25 generations without ciprofloxacin
249   supplementation produced resistant variants at similar low rates regardless of exposure to the
250   herbicide formulations. This indicated that the herbicides were not mutagens at these
251   concentrations. In a separate standard test of mutagenicity (Funchain et al. 2001), bacteria were
252   exposed to herbicides and plated on the antibiotic rifampicin. No difference in resistance rates
253   was observed (p =0.3873).

254   Populations of bacteria with continuous exposure to herbicide and antibiotic had significantly
255   higher numbers of ciprofloxacin resistant variants. The rate ranged from 10^2 times higher for the
256   combination of Cip+Kamba+*S. typhimurium* to 10^5 times higher for Cip+Roundup+*E. coli*
257   (Table 2). This is consistent with our prediction that herbicide-induced adaptive resistance that
258   increases MIC allows rare spontaneously arising ciprofloxacin resistant mutants to increase a
259   culture's MIC. Because ciprofloxacin is mutagenic (Cirz et al. 2005), it contributes quantitatively
260   to the genotypic heterogeneity, including acquired antibiotic resistance, among the bacteria that
261   survive because of the effects of the herbicide.

262   Ciprofloxacin resistant colonies were isolated at the end of each experiment from all treatments.
263   We determined MICs for 56 *S. typhimurium* isolates (isolated on 0.05 μg/mL Cip), 27 isolates
264   from Kamba+Cip and 29 isolates from Roundup+Cip treatments. The parental strain and 2

265 isolates from each LB, Kamba, and Roundup treatments, also isolated on 0.05 µg/mL Cip, were
266 included as controls.

267 The parental strain and 6 evolved isolates did not grow at 0.07 µg/mL, a concentration just above
268 the selection concentration. We observed MICs of 0.1 µg/mL ciprofloxacin for 21 isolates and
269 0.2 µg/mL ciprofloxacin for 28 isolates. Only 7 isolates displayed higher MICs (2 from
270 Kamba+Cip, 4 from Roundup+Cip, and 1 from LB), the highest being 1.25 µg/mL ciprofloxacin
271 reached by one isolate recovered from a Roundup+Cip culture. We observed no correlation
272 between level of resistance and original treatment, which indicates that there are no qualitative
273 differences between the ciprofloxacin resistant variants arising in the different treatments.

274

275 **Evolution of acquired antibiotic resistance in cultures with herbicide-induced decreases in**
276 **MIC**

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

282 Mixed cultures of *E. coli* were created to represent pre-existing antibiotic resistance
283 heterogeneity within natural environments. The phenotypic differences were created using
284 bacteria carrying isogenic plasmids with different alleles of *tetA* (Tet^{high}, Tet^{low}, Table 1). The
285 MIC for tetracycline of Tet^{low} was 1.5 µg/mL and for Tet^{high} it was 125 µg/mL.

286 Tet^{high} and Tet^{low} were competed in liquid LB, LB+Tet, and LB+Tet+Roundup media for about
287 42 generations. This herbicide lowers the antibiotic MIC (Fig. 1B). The same concentration of
288 Roundup was used in combination with various concentrations of tetracycline, all of which were
289 below the MIC of Tet^{low}. Roundup reduced tetracycline MIC for Tet^{low} to 0.5 µg/mL, but growth
290 of Tet^{high} was not affected for tetracycline concentrations up to 50 µg/ml, far above levels used in
291 this experiment.

292 The MSC, i.e. the concentration of tetracycline at which one strain started to dominate the
293 culture, was determined from the calculated selection coefficients. A positive coefficient

294   indicated selection for the more resistant strain. The selection coefficient was significantly

295   different for competitions in environments supplemented with ≥0.1 µg/mL tetracycline compared

296   to competitions in LB medium (Fig. 2). Mixed cultures in LB+Tet+Roundup had significantly

297   different selection coefficients compared to the LB competitions beginning at a tetracycline

298   concentration of 0.05 µg/mL.

299   A reverse experiment was performed using Kamba instead of Roundup. This herbicide increases

300   the MIC of Tet^{low} from 1.5 to 3 µg/mL. When Kamba was part of the environment, the selection

301   coefficient was significantly different from the competition in LB medium at a tetracycline

302   concentration ≥0.5 µg/mL, compared to ≥0.1 µg/mL without Kamba (Fig. 2). In both cases, MIC

303   for the Tet^{low} strain and MSC moved in the same direction.

304

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

306   In addition to the observed increase or decrease of antibiotic MIC caused by herbicide

307   formulations, we also observed combinations of antibiotic and herbicide that did not change the

308   MIC of the antibiotic but did alter growth rate (Fig. 1C). For example, *E. coli* cultures in medium

309   supplemented with Kamba grew faster at some sub-lethal concentrations of ciprofloxacin, but

310   Kamba did not change the MIC (Kurenbach et al. 2015). At the concentrations of the antibiotic at

311   which the herbicide altered growth rate, possibly also the MSC of the antibiotic was changed.

312   To test this, we measured the frequency at which acquired ciprofloxacin resistance arose during

313   culture in the combination of Cip+Kamba+*E. coli*. The mutation rate of an *E. coli* monoculture

314   was measured over the course of about 25 generations in a standard rich growth medium, liquid

315   LB, and in LB supplemented with Cip, Kamba, or Cip+Kamba. The experiment was similar to

316   those described in the section "Evolution of acquired antibiotic resistance in cultures with

317   herbicide-induced increases in MIC", but here the ciprofloxacin concentration was below MIC

318   and high enough to slow the growth of bacteria in cultures not supplemented with herbicide. At

319   the end of the experiment, bacteria were transferred to solid medium supplemented with

320   ciprofloxacin at >MIC and no herbicide, which was permissive only to the growth of variants

321   with acquired resistance.

322   Acquired resistance rates were the same for cultures grown in LB, LB+Kamba or

323   LB+Cip+Kamba (for both treatment combinations p > 0.2; Table 3). The acquired resistance rate

324   in the LB+Cip culture was about 10^5 times higher than the other three cultures (p < 10^{-4} for all

325   treatment combinations), indicating that the ciprofloxacin concentration met or exceeded the

326   MSC. The addition of Kamba neutralized the selective effect of ciprofloxacin by shifting the

327   MSC to a higher level without changing the MIC.

328

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

330   Pairs of isogenic strains of *E. coli* (Table 1) with either different streptomycin (Str^{high}, Str^{low}) or

331   tetracycline (Tet^{high}, Tet^{low}) MICs were competed for about 40 generations. The frequency of

332   each strain after competition was measured. The concentration of the appropriate antibiotic did

333   not vary between cultures, but the concentration of herbicide did. Combinations were chosen that

334   lead to a decrease in MIC with the chosen herbicide, namely Str+Kamba, and Tet+Roundup.

335   There was no differential in the fitness of paired strains when they were co-cultured in standard

336   rich medium (Fig. 3). In cultures containing antibiotic but not supplemented with herbicide

337   neither strain had a fitness advantage, indicating that the antibiotic concentration was below

338   MSC. Likewise, the herbicide alone did not affect fitness except for a statistically non-significant

339   effect at the highest tested concentration of Roundup.

340   In each test, the combination of antibiotic and herbicide reduced MSC. The more resistant strain

341   invariably increased relative to the isogenic competitor in a herbicide dose-dependent manner

342   when both antibiotic and herbicide were in the environment. Selection coefficients were

343   statistically significantly different from LB medium at 183 parts per million acid equivalent

344   (ppm ae) Kamba and 311 ppm ae Roundup.

345   The observed fitness differential was due to the faster reproductive rate of the Str^{high} and Tet^{high}

346   strains in environments with sub-lethal concentrations of antibiotic for both strains in each pair.

347   This was shown by measuring the growth rate of the strains in monoculture rather than in

348   competition (Supplementary Fig. S1). No differences were observed for the monoculture growth

349   rate (r) of matched isogenic pairs in LB medium, LB+antibiotic, or LB+herbicide (p>0.1 for all

350   combinations; Supplementary Fig. S1A and C). Only in the combined LB+herbicide+antibiotic

351   treatment did strains with higher MICs have shorter generation times in monoculture than their

352   lower MIC counterparts (p=1.6x10^{-5} (Kamba+Str) and p=4.7x10^{-10} (Roundup+Tet)).

353   Significant differences in the carrying capacity of the environments for all treatment

354   combinations were observed for the matched strains exposed to Kamba+Str measured in

355   monoculture. The high MIC strain achieved higher final optical densities (p<2x10^{-16};

356   Supplementary Fig. S1B). In contrast, the Str^{low} strain population grew to higher final optical

357   densities in LB, LB+Str, and LB+Kamba treatments. In cultures with Roundup+Tet, Tet^{high} and

358   Tet^{low} had similar densities (p=0.24, Supplementary Fig. S1D).

359

360   **Discussion**

361   In this study we report that when bacteria are simultaneously exposed to herbicides and

362   antibiotics, mutants with higher levels of resistance can evolve. In some cases, resistance evolved

363   100,000 times faster.

364   Herbicides can increase the MIC of some antibiotics. At what otherwise would be a lethal

365   concentration of the antibiotic, the bacteria can continue to reproduce. Each reproductive event

366   has a low but steady potential of producing a variant daughter with a higher MIC. We found that

367   these strains have a fitness advantage and accumulate differentially to their low MIC cousins.

368   Herbicides can also decrease the MIC of some antibiotics. At what otherwise would be a

369   concentration of antibiotic below the MSC, too low to have an effect on the fitness of two

370   bacteria differing in their MICs, we found that the bacteria with the higher MICs replaced the

371   bacteria with lower MICs. The shift in MSC seemed to be of a similar magnitude as the shift in

372   MIC observed for the lower MIC strain.

373   Finally, herbicides can also alter survival potential at some antibiotic concentrations but not

374   change the concentration to which the entire population is innately susceptible. In the case

375   presented here, MSC was shifted by the herbicides, but MIC was not. The herbicide mitigated

376   the selective pressure caused by the antibiotic, and no genotypes with higher resistance levels

377   established in the population. Although not tested, it is likely that in the reverse case, when MSC

378   is lowered by a substance, antibiotic resistance may arise at higher frequencies.

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

385   Antibiotics can influence acquired resistance evolution in a number of ways. First, some
386   antibiotics may also be mutagens (Cirz et al. 2005) and at sub-lethal concentrations could
387   increase the probability of a mutation conferring resistance arising in the population. Second,
388   they discriminate between the reproductive rate of those with higher and lower tolerances to the
389   toxic effects of the antibiotic, leading to different frequencies of genotypes that are less
390   susceptible. At the environmentally relevant concentrations of herbicide formulations that we
391   used, no mutagenic activity was detected. However, the herbicides did contribute to the ability of
392   antibiotics to change the frequency of resistant variants in populations.

393   Combination treatments using a non-antibiotic to increase an antibiotic's effectiveness have been
394   suggested to help preserve the usefulness of antibiotics (Allen et al. 2014; Schneider et al. 2017;
395   Wright 2016). Indeed, various combinations are in clinical use (Worthington & Melander 2013).
396   While this might improve or enable treatment of an infection, our results indicate that decreasing
397   bacteria's survival by making an antibiotic more potent may promote resistance evolution by
398   creating more environments in which a resistance genotype is more fit.

399   Herbicides and different ingredients in product formulations can have antimicrobial activities,
400   with some being more potent antimicrobial agents than are others (Kurenbach et al. 2017). For
401   example, Roundup was more toxic than Kamba to the bacteria that we used. The low level
402   toxicity is likely how they induce adaptive resistance, a source of phenotypic heterogeneity
403   (Kurenbach et al. 2015). In addition, biocidal agents that are mutagens may introduce genotypic
404   heterogeneity.

405   Antibiotic and herbicide gradients may be viewed as two environmental dimensions
406   concentrating competition between bacteria of different genotypes and physiotypes. The
407   particular concentrations of antibiotic and herbicide at any point of intersection of the gradients
408   above a threshold level increases the frequency of the most fit strains and species, amplifying

409   any linked or associated traits in the more fit competitor. While antibiotics can do this without

410   herbicides (Andersson & Hughes 2014; Baquero et al. 1998a; Denamur & Matic 2006), the

411   herbicides used in this study increased the range of concentrations under which the antibiotic

412   affects the evolution of resistance.

413   The concentrations of herbicides we used were below recommended application levels and hence

414   within relevant environmental ranges, suggesting that what we observe in the laboratory has the

415   potential to occur in many places where both antibiotics and herbicides are found together.

416   Simultaneous herbicide and antibiotic exposures are common. Herbicides are used in agriculture,

417   where spray drift or walking through treated fields exposes farm livestock and pets, which may

418   be on therapeutic or prophylactic antibiotics. Most ingested antibiotic is not metabolized and thus

419   excreted (Chee-Sanford et al. 2009), becoming mixed with soil as crop fertilizer which *in situ*

420   may be subsequently sprayed with herbicide. Microbes from these mixes may be carried by

421   blow- and house-flies (Zurek & Ghosh 2014). Likewise honeybees may be exposed to herbicide

422   spray or residues as they forage and return to an antibiotic-treated hive. Additionally, herbicides

423   are used in urban environments for purposes like gardening and lawn care, including parks and

424   roadsides (Atwood & Paisley-Jones 2017). Worldwide, herbicide use was approximately 1×10^9

425   kilograms in 2012 with up to 2×10^8 kilograms of the active herbicidal ingredients glyphosate, 2,

426   4-D and dicamba used in the US in 2012 (Atwood & Paisley-Jones 2017).

427   Other chemicals also have been shown to cause adaptive resistance and to increase resistance

428   frequencies (Egeghy et al. 2012; Gustafson et al. 1999; Levy 2001). Non-antibiotic prescription

429   medicines and food emulsifiers select antibiotic resistant gut bacteria (Kurenbach et al. 2017;

430   Maier et al. 2018). Approximately 8 million manufactured chemical substances are currently in

431   commerce (Egeghy et al. 2012; Shen et al. 2011). According to the US Environmental Protection

432   Agency, annual production of each of the top 3,000 chemicals is greater than 6×10^{11}

433   kilograms/year (EPA 2008). They are not regulated for effects on antibiotic resistance and not

434   tested for such effects.

435   The susceptibility of bacteria to antibiotics must be seen as a non-renewable resource, one that

436   requires careful stewardship worldwide (Amabile-Cuevas 2016; Heinemann & Kurenbach 2017).

437   Evidence that antibiotic resistance evolution is influenced by exposure of bacteria to a wide

438   range of substances may require us to make changes in how we manage both antibiotics and

439 other manufactured and widely distributed chemical products. This is because many facets of the

440 extrinsic environment induce adaptive changes, a complexity frequently ignored in standard

441 studies of resistance. As our results show, complex effects of exposures to non-therapeutic

442 chemicals may undermine strategies to preserve the effectiveness of antibiotics through altering

443 just their use. To our knowledge, there has been no attempt to systematically test common

444 chemicals to which pathogenic bacteria are chronically exposed for effects on antibiotic

445 resistance.

446 **Conclusions**

447 Neither reducing the use of antibiotics nor discovery of new ones may prevent the post-antibiotic

448 era. This is because bacteria may be exposed to other non-antibiotic chemicals that predispose

449 them to evolve resistance to antibiotics more quickly. Herbicides are examples of some of the

450 most common non-antibiotic formulations in frequent global use. More research is necessary to

451 see to what extend other different manufactured chemicals may contribute to this effect.

452 Moreover, depending on how the manufactured chemicals are used, or how they move through

453 the waste stream, there may be combinatorial effects caused by mixtures of different products.

454 Future work should take into account likely combinations as well as different ways that microbes

455 could be exposed to chemical products.

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458

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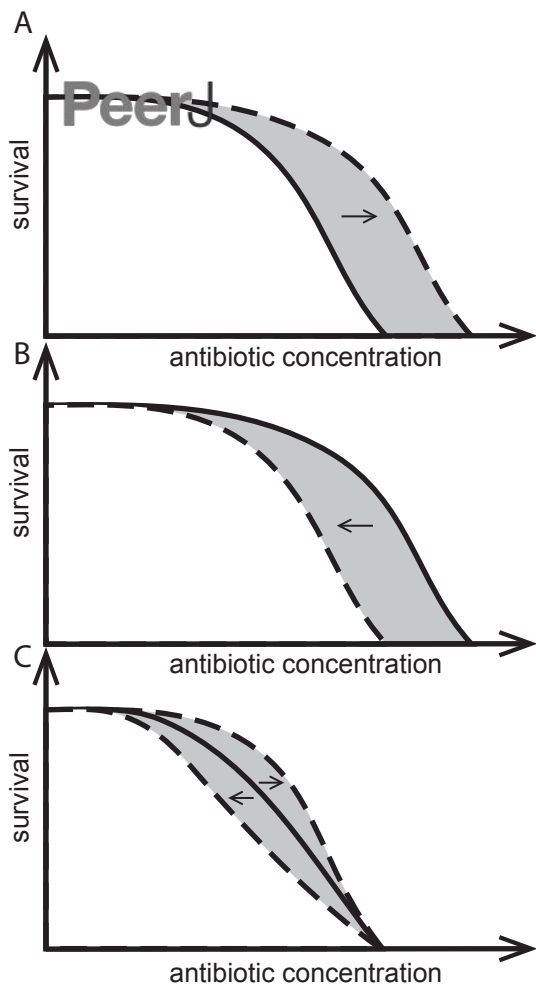
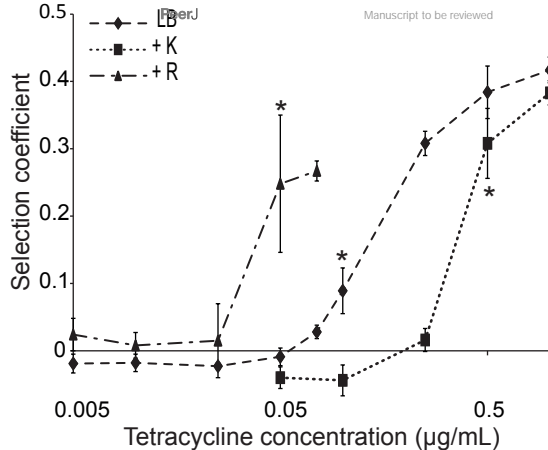
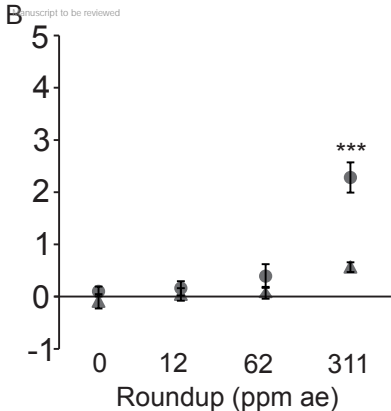
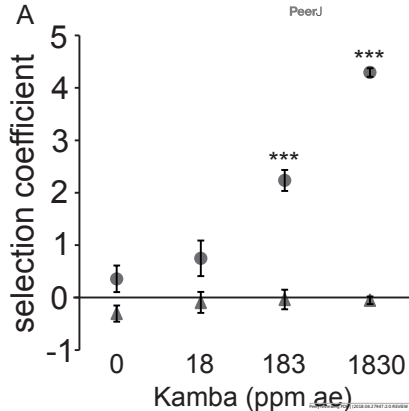
597

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.

Competition between strains of different potential to resist tetracycline.

Competition between tetracycline resistant (AH201, Tet^high) and susceptible (AH214, Tet^low) strains of *E. coli* 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 means of three independent experiments. To represent sampling uncertainty in the mean value for treatment groups we present error bars depicting standard errors (standard deviation/√n). * indicate the lowest tetracycline concentrations for each treatment where selection coefficients were significantly different from the -Tet/-herbicide treatment, based on a Bonferroni corrected contrast described in the Materials and Methods section.

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

Dose-response of *E. coli* 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 three independent experiments. To represent sampling uncertainty in the mean value for treatment groups we present error bars depicting standard errors (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, based on a Bonferroni corrected contrast described in the Materials and Methods section, with *: p<0.05; **: p<0.01; ***: p<0.001.

Bacteria and plasmids

*Kamba: added at 1830 ppm ae, Roundup added at 1240 ppm ae.

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*	Cip/Cip+Kamba/ Cip+Roundup*: Solid medium: 0.01/0.07/0.07 µg/mL Liquid medium: 0.04/0.05/0.09 µg/mL	(Baba et al. 2006)
SB21	*hsdS, leuB6, thr*		(Heinemann & Sprague 1989)
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	(Heinemann et al. 1996)
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*	Cip/Cip+Kamba/ Cip+Roundup*: Solid medium: 0.035/0.1/0.1 µg/mL Liquid medium: 0.05/0.1/0.2 µg/mL	(Roantree et al. 1977)
Plasmids			
pBR322	Amp[R], Tet[R]	Amp: 100 µg/mL, Tet: 125 µg/mL	(Sutcliffe 1979)
pAH14	pBR322 derivative, Cam[R], Amp[S], Tet[S]	Cam:20 µg/mL	This study
RSF1010	Str[R]	Str: 250 µg/mL	(Scholz et al. 1989)

| pAH11 | RSF1010 derivative, Cam^R, Str^S | Cam: 20 µg/mL, Str: 1 µg/mL | This study |

Mutation rate (mutant frequency per generation)

Rates of acquired resistance were determined with or without herbicide and Cip exposure. Values are means of at least four independent experiments. To represent sampling uncertainty in the mean value for treatment groups we present standard errors (standard deviation/√n) in brackets. Cip concentrations used were 0.07 µg/mL for *S. enterica* and 0.05 µg/mL for *S. enterica* in liquid culture and : 0.07 µg/mL for *S. enterica* and 0.06 µg/mL for *E. coli* for final plating. 1,250 ppm ae Roundup or 1830 ppm ae Kamba were used. [a]Herbicide+antibiotic mutation rates 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

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

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

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

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