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1 **TITLE: Herbicide mixtures at high doses slow the evolution of resistance in**
2 **experimentally evolving populations of *Chlamydomonas reinhardtii*.**

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20 Summary

21

- 22 • The widespread evolution of resistance to herbicides is a pressing issue in global
23 agriculture. Evolutionary principles and practices are key to the management of this
24 threat to global food security. The application of mixtures of xenobiotics has been
25 advocated as an anti-resistance strategy, without substantial empirical support
26 validating it.
- 27 • We experimentally evolved populations of single cell green chlorophyte,
28 *Chlamydomonas reinhardtii*, to the minimum inhibitory concentrations (MIC) of
29 single herbicide modes of action and to pair-wise and three-way mixtures between
30 different herbicides at various total combined doses.
- 31 • We found mixtures were most effective when each component was applied at or close
32 to their MIC. When doses were high, increasing the number of mixture components
33 was also effective in reducing evolution of resistance. Employing mixtures at low
34 combined doses did not retard resistance evolution, even accelerating evolution of
35 resistance to some components. When used at low combined doses, increasing the
36 number of herbicides in the mixture tended to select for more generalist resistance
37 (cross-resistance).
- 38 • Our results reinforce findings from antibiotic resistance literature and confirm that
39 herbicide mixtures can be very effective for resistance management but that should
40 only be employed where the economic and environmental context permits
41 applications of high combined doses.

42 Keywords: *Chlamydomonas reinhardtii*, environmental complexity, herbicide mixtures,
43 herbicide resistance, experimental evolution.

44

45 **Introduction**

46

47 The establishment of herbicides as the major method of weed control in agriculture (Powles
48 & Shaner, 2001) has resulted in widespread evolution of herbicide resistance (Powles & Yu,
49 2010). Mixture strategies that expose weeds to two or more herbicides with different modes
50 of action have been widely advocated for resistance management (Gressel & Segel, 1990;
51 Friesen *et al.*, 2000; Powles & Shaner, 2001). Similar strategies have been proposed for the
52 prevention of antibiotic resistance (Brown & Nathwani, 2005; Powles & Yu, 2010) and
53 management of resistance to antiretroviral and anti-cancer drugs (Pastan & Gottesman,
54 1987). Mixture strategies rely on the assumption that mutations conferring resistance to one
55 component of the mixture do not increase fitness in the presence of the second component.
56 Indeed, the most desirable situation arises when there is antagonistic pleiotropy between
57 resistance mechanisms (sometimes referred to as negative cross-resistance (Gressel, 2002)).
58 Where the assumptions of independent resistance are met, resistance to the mixture can only
59 arise via spontaneous evolution of resistance mechanisms to both (or all) mixture components
60 (Diggle *et al.*, 2003). The likelihood of this occurring decreases with each additional
61 herbicide in the mixture (Wrubel & Gressel, 1994).

62

63 Two broad categories of herbicide resistance have been documented: target-site and non
64 target-site (Powles & Yu, 2010). Target site resistance arises from modification or over-
65 expression of the herbicide target enzyme and results in resistance that is specific to a single
66 mode of action (specialist resistance) (Busi & Powles, 2009; Powles & Yu, 2010). Several

67 target-site resistance mutations can accumulate in the same individual, leading to multiple-
68 resistance (Powles & Yu, 2010). Non target-site resistance, based on enhanced metabolism of
69 the herbicide or sequestration away from the active site of the herbicide, often results in
70 resistance to multiple modes of action (generalist resistance) and may require multiple
71 mutations (Powles & Yu, 2010). Generalist resistance may be favoured in more complex,
72 multi-herbicide environments and this may compromise the potential efficacy of mixture
73 strategies.

74

75 Mathematical models have been used to demonstrate the potential effectiveness of mixtures
76 for herbicide resistance management (Powles *et al.*, 1997; Diggle *et al.*, 2003; Neve, 2008).
77 However, these models predominantly focus on the evolution of target-site resistance.
78 Empirical evidence for the efficacy of herbicide mixture strategies is limited and often
79 anecdotal (Beckie, 2006), although these studies do tend to confirm the benefits of mixtures
80 over other management strategies (Manley *et al.*, 2002; Beckie & Reboud, 2009). Models
81 exploring the effectiveness of mixtures of insecticides or fungicides for managing resistance
82 provide conflicting evidence for its benefits (Mani, 1985; Denholm & Rowland, 1992;
83 Russell, 2005), as do experimental studies - some supporting mixtures as an effective method
84 of resistance management (McKenzie & Byford, 1993; Prabhaker *et al.*, 1998), others
85 cautioning against their widespread use (Immaraju *et al.*, 1990; Blumel & Gross, 2001; Castle
86 *et al.*, 2007). It is interesting to compare this to the situation in studies of antibiotic resistance,
87 where clinical trials predominantly report mixtures as effective strategies in slowing
88 resistance evolution (Bergstrom *et al.*, 2004; Brown & Nathwani, 2005; Beardmore & Peña-
89 Miller, 2010).

90

91 Increased economic and environmental costs are a major obstacle to the adoption of herbicide
92 mixtures in agricultural settings (Hart & Pimentel, 2002). Short term economic interests
93 favour the use of single herbicides as the level of control achieved prior to the evolution of
94 resistance may often be equivalent, and does not require investment in multiple herbicides
95 (Buttel, 2002). From an environmental perspective, herbicide mixtures raise concerns as they
96 increase inputs of pesticides into the environment (Hart & Pimentel, 2002). In response to
97 these problems, there have been calls to use synergistic mixtures of herbicides whereby the
98 total combined dose of herbicides in the mixture is reduced (Gressel, 1990). The implications
99 of such strategies for resistance evolution are not well understood. In antibiotic resistance it
100 has been shown that synergistic mixtures can exacerbate resistance evolution as appearance
101 of resistance to one of the components leaves a population exposed to an ineffective dose of
102 the other (Hegreness *et al.*, 2008).

103

104 Microbial experimental evolution offers the potential to explore conditions under which
105 herbicide mixture strategies may be effective, overcoming time and space limitations
106 associated with empirical studies with higher plants (Elena & Lenski, 2003). Here, we use the
107 unicellular green chlorophyte, *Chlamydomonas reinhardtii*, as a model organism.
108 *C.reinhardtii* grows asexually under laboratory conditions(Harris, 2008) and is susceptible to
109 a range of commercial herbicides (Reboud *et al.*, 2007). The techniques of experimental
110 evolution (Buckling *et al.*, 2009) are easily applicable to *C.reinhardtii* and have been adopted
111 to explore a variety of questions relating to herbicide resistance evolution and management
112 (Lagator *et al.*, 2012). We experimentally evolved populations of *C.reinhardtii* with exposure
113 to mixtures of two or three herbicides with different modes of action (atrazine, glyphosate
114 and carbetamide) at a variety of total combined doses, as well as in single exposure to each of
115 those herbicides. The objectives of this study were to investigate if (i) mixtures are effective

116 in delaying and/or preventing the evolution of herbicide resistance; (ii) the effectiveness of
117 mixtures is dependent on the total combined dose and the number of herbicides; (iii) increase
118 in the number of herbicides and a reduction in their combined dose increases the likelihood of
119 adaptation towards a generalist optimum.

120

121 **Materials and Methods**

122

123 *Founding population*

124

125 The *Chlamydomonas reinhardtii* strain used in the experiment is Seger's CC-1690 wild type
126 mt+ 21gr, obtained from the *Chlamydomonas* Resource Center's core collection. Prior to
127 selection experiments, the strain had been adapted to liquid Bold's medium through
128 continuous exposure for over 700 generations. Two weeks before the start of selection, 20 μ l
129 of the founding population (approximately 15,000 cells) was spread on an agar plate. After 7
130 days of growth, a single colony was picked and used to inoculate a Bold's medium liquid
131 culture. This colony was multiplied for 7 days and was used to found all experimentally
132 evolving populations.

133

134 *Culture conditions*

135

136 The culture media used in all experimental conditions is modified Bold's Medium
137 (subsequently BM)(Harris, 2008). Populations were cultured in disposable borosilicate glass

138 tubes, in 20ml of BM and maintained in an orbital shaker incubator, at 28°C and 180rpm,
139 under continuous light exposure, provided by six fluorescent tubes mounted in the incubator
140 lid (Osram L30 W/21-840, cool white; light intensity measured at the location of the tubes
141 was 161 $\mu\text{molm}^{-2}\text{s}^{-1}$). Cultures were propagated every seven days (see below), during which
142 time the ancestral population growing in the absence of herbicides would have reached
143 stationary phase (3.1×10^7 cells).

144

145 *Herbicides*

146

147 We selected for resistance to three herbicides – atrazine, glyphosate, and carbetamide. The
148 herbicides have different modes of action (atrazine – photosystem II inhibitor; glyphosate –
149 inhibitor of aromatic amino acid synthesis; carbetamide – mitosis inhibitor). Prior to
150 selection, we determined the minimum inhibitory concentration (MIC) of each herbicide, this
151 being the minimum concentration that prevented detectable population growth over seven
152 days. We also determined the ‘MIC equivalent’ value when herbicides were used in
153 combination (subsequently MICeq), this being the equal proportion of each herbicide in the
154 mixture that completely inhibited growth of the founding population over seven days. In all
155 pairwise and three-way herbicide mixtures, the growth inhibitory effects of herbicides were
156 synergistic, such that complete growth inhibition was achieved with each herbicide at 45% of
157 its MIC in a two-way, and at 30% of its MIC in the three-way mixture.

158

159 *Selection regimes*

160

161 Three experimental conditions involved continuous exposure to a single herbicide (A0
162 denoting continuous exposure to atrazine, G0 to glyphosate and C0 to carbetamide).
163 Conditions containing pairwise mixtures of herbicides at MIC_{eq}, 50% (MIC), 75% (1.5MIC)
164 and 100% (2MIC) of each herbicide MIC were created (AG_{eq}, AG, AG1.5 and AG2
165 denoting a mixture between atrazine and glyphosate at MIC_{eq}, 50%, 75% and 100% of each
166 herbicide MIC, respectively). For a three-herbicide mixture, MIC_{eq}, 33%, 50% and 66%
167 doses of each herbicide's MIC were used to create selection conditions (AGC_{eq}, AGC,
168 AGC1.5 and AGC2, respectively). Each experimental condition (19 in total) was replicated 6
169 times, for a total of 114 evolving populations. Six populations were propagated in the absence
170 of herbicides and were used as controls and as source populations to sustain the evolving
171 populations (see below).

172

173 Approximately 125,000 cells (estimated by absorbance at 750nm) from the founding
174 population provided the initial population for all selection regimes. Transfers into fresh media
175 containing appropriate herbicides were carried out at seven-day intervals. Absorbance at
176 750nm (OD₇₅₀) of all evolving populations was measured prior to transfers. At each transfer,
177 200µl of the evolving culture was transferred into fresh media. If the number of cells in 200µl
178 of culture medium was estimated as less than 125,000, then the appropriate number of cells
179 from one of the source populations was added to make the total cell number at the transfer
180 approximately 125,000. Therefore, the minimum number of cells at the beginning of each
181 cycle was 125,000. For each of the six replicates, the same source population was used for
182 immigration throughout the experiment. The experiment was carried out for 15 transfer
183 cycles (15 weeks), at which time populations were transferred into BM and allowed to grow
184 for 7 days to multiply evolved populations.

185

186 *Cross-resistance assays*

187

188 To test for selection of generalist cross-resistance, we assayed the growth of evolved
189 populations at the MIC (determined in the manner described above) of four herbicides to
190 which they had no previous exposure (tembotrione, iodosulfuron-methyl-sodium,
191 fluorochloridone and S-metolachlor). 125,000 cells of the evolved populations were
192 inoculated into tubes containing one of these herbicides and population growth (OD₇₅₀) was
193 measured after seven days. Each condition was replicated twice.

194

195 *Statistical analyses*

196

197 We are addressing three questions – how do (i) the number of herbicides and (ii) the
198 combined dose affect rates of resistance evolution, and (iii) how do rates of resistance
199 evolution compare between dose treatments within herbicide mixture combinations? None of
200 these questions requires a comparison of all treatment groups. Rather than analysing subsets
201 of the data set to address the different questions, we analysed the entire data set, using
202 appropriate nesting (see below for details of the nesting structure used in each case) to
203 separate treatments of interest from other treatments. This approach ensures that all
204 hypotheses are being tested using the same measure of between-observation variability, and
205 maximises the degrees of freedom (and hence statistical power) associated with this source of
206 variation.

207

208 **Effect of the number of herbicides.** To analyze for the effects of herbicide number, we
209 modelled the temporal dynamics of population size using a linear mixed model within
210 ANOVA (aov function in R 2.15.0). To do so, we compared the regimes that evolved in
211 single herbicide environments to those in mixtures at MIC_{eq} doses, as these regimes offered
212 the same initial level of population control and therefore rates of adaptation could
213 meaningfully be compared. Regimes selected in mixtures at MIC, MIC1.5 and MIC2 were
214 not relevant to this question. As discussed above we used a nested model to allow our
215 hypothesis of interest to be tested based on an analysis of the entire data set. The response
216 variable was population size (measured as OD₇₅₀ at the end of each transfer period). Nested
217 within the entire dataset, we fitted an initial fixed term with two levels; the first level
218 included all treatments relevant to this question (A0, G0, C0, AGeq, ACeq, GGeq, AGGeq),
219 whilst the second level included all other treatments. Within the first level we nested a factor
220 with three levels to allow comparison of the treatments with different numbers of herbicides
221 (one, two or three). We then nested further terms to account for variation amongst the three
222 single herbicide treatments, and three different herbicide pair treatments. Within the second
223 level of the initial fixed term we included a nested factor with 12 levels to account for
224 variation amongst the 12 treatments that are not directly relevant to this question. The random
225 (error) term consisted of time (weeks, 15 levels) nested within each regime (19 levels), nested
226 within replicate (population, 6 levels). Significance of fixed effects was tested with F-tests.

227

228

229 **Effects of combined dose.** When investigating the effects of combined dose on the dynamics
230 of resistance, we were only interested in regimes with more than one herbicide as the single

231 herbicide environments had only one dose. We adopted similar approaches to above to
232 partition the data within the entire dataset. An initial fixed term separated the treatments into
233 two groups: the 16 treatments of interest (all of the regimes involving more than one
234 herbicide) and the remaining three treatments (A0, G0 and C0). Within the first level, two
235 nested factors accounted for the variation due to differences between herbicide mixtures (AG,
236 AC, GC, AGC), and due to differences between doses (4 different levels), and the fixed
237 model also included the interaction between these two factors to account for all variation
238 among the 16 treatments of interest. Within the second level, a nested factor accounted for
239 the variation between the three single herbicide treatments (though not of direct interest for
240 this question). The error term was same as above, and the significance of fixed effects was
241 tested with F-tests.

242

243 **Comparing the time of resistance evolution in selection regimes.** To analyse the dynamics
244 of resistance evolution in herbicide mixtures and single herbicide exposure regimes, we
245 modelled OD₇₅₀ as the response in a further set of linear mixed models using ANOVA in
246 GenStat (13th edition). We separately modelled resistance for regimes associated with each
247 herbicide mixture (AG, AC, GC, AGC), enabling comparison between all four dose regimes
248 for each mixture as well as the two or three relevant single herbicide regimes (i.e. A0 and G0
249 for the AG mixture, and all three single herbicide conditions for the AGC mixture), following
250 the nesting approach outlined above. An initial term in each model compared the mean for
251 the six or seven regimes of interest with the mean of the remaining treatments, with nested
252 terms accounting for the variation among the treatments not of direct interest. Each model
253 also included the time term, using a series of linear contrasts to identify the time periods over
254 which there were changes in the level of resistance across the six or seven treatments of
255 interest, and the interaction of these contrasts with the treatment terms identified above, to

256 detect where there were differences in the patterns of resistance evolution between
257 conditions. Each linear contrast assessed the slope of the linear regression over four
258 consecutive time points (the first for weeks 1-4, the second for weeks 2-5, and so on),
259 allowing identification of both the first point and last point at which a significant change in
260 resistance was seen for each condition. To illustrate, as all regimes started with a slope of
261 linear regression that was not significantly different from 0 (no resistance), the point when a
262 slope of one regime started becoming significantly different from the slopes of other regimes
263 indicated when resistance in that regime started evolving. It was in this way that we analysed
264 the rates of resistance evolution as a comparison between the linear regression slopes at each
265 of 12 contrasts to assess the time when each population started exhibiting measurable growth.
266 These 12 linear contrasts are not independent, so that they do not provide a complete
267 partitioning of the between-time variation, and some care is needed in the interpretation of
268 significant effects for overlapping periods.

269

270 **Cross-resistance.** We analysed differences in the cross-resistance profile of selected
271 populations by ANOVA with population growth after seven days (measured as OD₇₅₀) as the
272 response variable. Fixed factors were genotype (selection regime, 14 levels, as we excluded
273 regimes that did not give rise to any resistant populations) and environment (novel herbicide
274 environment, 4 levels), while the error term consisted of the source population. We were
275 particularly interested in the genotype x environment interaction as this represents the
276 differences in the range of novel herbicides that a population expressed cross-resistance to. A
277 subsequent analysis was conducted using Tukey's honestly significant pairwise tests between
278 the mean OD₇₅₀ of the populations selected in each regime across all four novel herbicide
279 environments. This test treated cross-resistance as a composite measure that included both the

280 number of herbicides a population was resistant to and the growth rates achieved in each of
281 those herbicides.

282

283 **Results**

284

285 *Dynamics of herbicide resistance*

286

287 **Evolution of resistance.** Adaptation to the selection regimes occurred in many experimental
288 populations, under various single- and multiple-herbicide conditions. Resistance (defined
289 here as elevated growth rates in herbicide regimes) evolved in all populations under exposure
290 to atrazine and glyphosate, and in two of six populations under carbetamide exposure (Fig.
291 1). Resistance was observed in all populations exposed to mixtures of atrazine and glyphosate
292 at MICeq, MIC and MIC1.5, as well as in four populations at AG2 (Fig. 1a). Populations
293 exposed to a mixture of atrazine and carbetamide evolved resistance in three populations at
294 ACEq and two populations at AC. Resistance did not evolve in AC regimes at AC1.5 or AC2
295 (Fig. 1b). Mixtures of glyphosate and carbetamide gave rise to resistance in all populations
296 evolving at GCEq and GC, two populations at GC1.5, and was never observed at GC2 (Fig.
297 1c). In the three-herbicide regimes, resistance evolved in all populations at AGCEq and AGC,
298 in two populations evolving at AGC1.5 and never at AGC2 (Fig. 1d).

299

300 **Effects of herbicide number and combined dose.** We identified a significant effect of the
301 number of herbicides in the mixtures on the dynamics of resistance evolution (measured as

302 the mean population size at transfer over the 15 week selection regime), with resistance
303 evolving more slowly with an increase in the herbicide number ($F_{2,90}=7.85$; $P<0.001$). We
304 also found that an increase in the total combined dose slowed resistance evolution, as the
305 interaction between herbicide mixture and overall herbicide dose was significant ($F_{9,90}=6.49$;
306 $P<0.001$).

307

308 **Rates of resistance between regimes.** We analysed the rates of resistance evolution as a
309 comparison between the linear regression slopes at each of 12 contrasts and we report the F
310 statistic indicating the differences between all 6 or 7 treatments at each time interval (Table
311 S1-S4). Considering comparisons between the AG mixtures and continuous exposure to
312 glyphosate or atrazine (Fig.1a; table S1), we first observed resistance to the continuous
313 glyphosate regime (between weeks 2-5, $F_{5,90}=16.50$; $P<0.001$). Resistance in populations
314 exposed to AG and AGeq followed (between weeks 6-9, $F_{5,90}=2.84$; $P=0.015$), with the
315 populations exposed to atrazine (A0) and AG1.5 evolving resistance subsequently (between
316 weeks 10-13, $F_{5,90}=2.43$; $P=0.004$). Resistance evolved most slowly in populations selected at
317 AG2, and since it occurred only in four populations near the end of the selection procedure.
318 Growth rates (slopes of regression lines) for AG2 populations never became significantly
319 different from 0.

320

321 In populations exposed to mixtures of atrazine and carbetamide and the individual component
322 herbicides (Fig.1b, Table S2), the populations exposed to atrazine evolved resistance first
323 (between weeks 10-13, $F_{5,90}=2.34$; $P=0.048$), closely followed by the populations growing at
324 ACeq (between weeks 11-14, $F_{5,90}=5.07$; $P<0.001$). The slopes of regression lines for

325 exposure to carbetamide (C0), AG, AG1.5 and AG2 never become significantly different
326 from 0.

327

328 In the GC comparisons, resistance evolved most rapidly in the populations exposed to
329 glyphosate only (between weeks 2-5, $F_{5,90}=16.93$; $P<0.001$). Populations exposed to GCeq
330 were the second to evolve resistance (between weeks 9-12, $F_{5,90}=5.05$; $P=0.001$), with the
331 populations exposed to GC exhibiting resistance in the subsequent interval (between weeks
332 10-13, $F_{5,90}=10.12$; $P<0.001$).

333

334 In the AGC comparisons, resistance evolved most rapidly in the G0 regimes ($F_{6,90}=15.43$;
335 $P<0.001$), followed by the populations selected at AGCeq and in A0 (between weeks 10-13,
336 $F_{6,90}=6.32$; $P<0.001$). Exposure to AGC of the mixture gave rise to resistance in the
337 subsequent interval (between weeks 11-14, $F_{6,90}=6.21$; $P<0.001$).

338

339 *Patterns of cross-resistance*

340

341 We identified an overall effect of the regime-by-herbicide (genotype-by-environment)
342 interaction ($F_{42,295}=3.37$, $P<0.001$), indicating the emergence of phenotypes with different
343 cross-resistance profiles (Table 1). Populations evolving at MIC and MICeq of a three
344 herbicide mixture were significantly more cross-resistant than all other evolved populations,
345 with the exception of the populations evolved in a mixture of atrazine and glyphosate at MIC

346 (Table S5). There were no significant differences in cross-resistance between populations that
347 evolved in any other regimes.

348

349 **Discussion**

350

351 Results indicate that herbicide mixtures may be successful at preventing or slowing evolution
352 of resistance when all components are used at or close to the MIC. The benefits of increasing
353 the number of herbicides in the mixture depend on the combined dose in the mixture: lower
354 combined doses of a three-way mixture led to significant levels of cross-resistance, while
355 higher combined doses were successful at preventing adaptation in those regimes.

356

357 *Lower combined doses of mixtures do not effectively slow resistance evolution*

358

359 We observe that regardless of herbicide identity, populations exposed to the two lowest
360 combined doses (MIC_{eq} and MIC) evolved resistance more rapidly to the mixture than they
361 did when exposed to the least resistance-prone of the mixture components at MIC (Fig.1). At
362 lower combined doses, resistance is likely to evolve rapidly to the more resistance-prone
363 component of the mixture, leaving populations exposed to lower-than-MIC doses of the other
364 herbicide(s). Such dynamics allow populations to rapidly circumvent the effectiveness of
365 mixture strategies as these elevated growth rates enable rapid population growth and this in
366 turn may increase mutation supply rates for rarer mutations that increase population fitness in
367 the presence of the second (and further) herbicide(s) (Drlica, 2003; Busi & Powles, 2009). As

368 such, low dose mixture strategies may facilitate the accumulation of multiple resistance
369 mechanisms in the same individual (Wrubel & Gressel, 1994). Growth assays conducted at
370 the termination of selection procedures indicated that this was likely the case in this study as
371 all populations that had evolved resistance to mixture regimes were individually resistant to
372 all mixture components at MIC (data not shown). An alternative explanation is that exposure
373 to lower doses selected for generalist mutation(s) that provide resistance to all herbicides in
374 the mixture (Neve & Powles, 2005). If the number of mutations required for such a
375 mechanism is low, resistance could emerge as rapidly as we have observed. Appearance of
376 such a mechanism would have to be dose specific, as we do not observe it at higher combined
377 doses. Our findings are in line with some previous studies (Immaraju *et al.*, 1990; Birch &
378 Shaw, 1997), indicating that the use of equivalent or lowered MICs poses a significant risk
379 for resistance management as resistance to these mixtures may evolve more rapidly than to
380 single herbicides at high relative doses (Fig. 1).

381

382 *Mixtures increase the likelihood of cross-resistance*

383

384 The requirements for successful mixture strategies (Wrubel & Gressel, 1994) may be
385 overcome if evolution proceeds towards a single generalist phenotype instead of requiring
386 resistance to multiple herbicides through independent mutations (multiple resistance) (Rubin,
387 1991; Elad *et al.*, 1992). We observed a significant trend towards cross-resistant phenotypes
388 as the number of herbicides in the mixture was increased (Table 1). Increase in the number of
389 herbicides can lead to a generalist optimum either because the likelihood of acquiring non-
390 target site resistance is greater than the likelihood of acquiring multiple resistance mutations;
391 and/or because the accumulation of fitness costs associated with each independent resistance

392 becomes too large (Poisot *et al.*, 2011). From an applied perspective, use of more complex
393 mixtures elevates the risk for management, as wider cross-resistance patterns can reduce the
394 number of available herbicides that could be used for subsequent control.

395

396 *Mixtures in a wider applied setting*

397

398 As in medical settings, where high doses of multiple antibiotics have to be balanced against
399 toxicity to patient cells (Gluckman *et al.*, 2011), the use of multiple pesticides in agricultural
400 settings has to be considered in the light of environmental concerns and economic constraints
401 (Carroll *et al.*, 2011). Our results, in line with previous studies (Gressel, 1997; Diggle *et al.*,
402 2003; Russell, 2005; Beckie, 2006), support the use of mixtures at full dose of each
403 component herbicide. We show that reductions in the combined dose lead to more rapid
404 resistance and potentially to cross-resistant phenotypes, questioning the suitability of
405 mixtures for sustainable management unless these can be applied at high doses.

406

407 Antibiotics acting synergistically – offering the same control of susceptible populations at
408 lower combined doses (Trindade *et al.*, 2009) – have been shown to elevate rates of
409 resistance evolution (Michel *et al.*, 2008), as a lower effective dose is experienced once
410 resistance evolves to one of the components in synergistic mixtures, as opposed to a mixture
411 of non-interacting or antagonistic antibiotics (Hegreiness *et al.*, 2008). Our results support
412 these findings and extend the implications to alterations of the dose of components in a
413 mixture. In line with previous studies (Manley *et al.*, 2002; Beckie, 2006; Neve *et al.*, 2011),

414 the importance of the composition of the xenobiotic mixture is also highlighted, as the rates
415 of evolution in a mixture depend on how resistance-prone individual components are.

416

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419 appreciated contributions. The project was funded by Leverhulme Trust.

420

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431

432

433 **Table 1.** Patterns of cross-resistance measured as populations growth (mean OD₇₅₀) after 4
 434 days of growth in a novel herbicide for each regime/standard error of the mean. F -
 435 fluorochloridone; T - tembotrione; I - iodosulfuron-methyl-sodium; and S - s-metolachlor.

Regime (genotype)	F	I	S	T
A	0/0	0.021/0.021	0/0	0/0
C	0/0	0/0	0/0	0.055/0.035
G	0/0	0/0	0/0	0/0
A+Geq	0/0	0.067/0.031	0/0	0.081/0.028
A+Gx	0/0	0.118/0.039	0/0	0.06/0.028
A+G1.5	0/0	0/0	0/0	0/0
A+G2	0/0	0/0	0/0	0/0
A+Ce _q	0/0	0/0	0/0	0.232/0.087
A+C _x	0/0	0/0	0/0	0.065/0.041
G+Ce _q	0/0	0.048/0.033	0/0	0/0
G+C _x	0/0	0.144/0.028	0/0	0/0
G+C1.5	0/0	0.0532/0.034	0/0	0/0
AGCe _q	0.073/0.033	0.084/0.042	0/0	0.186/0.017
AGC _x	0.139/0.030	0.127/0.03	0/0	0.096/0.044
AGC1.5	0/0	0/0	0/0	0/0

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557 **Supporting Information**

558 Table S1: Comparisons of dynamics of resistance evolution for different dose regimes in atrazine and
559 glyphosate (AG) mixtures.

560 Table S2: Comparisons of dynamics of resistance evolution for different dose regimes in atrazine and
561 carbetamide (AC) mixtures.

562 Table S3: Comparisons of dynamics of resistance evolution for different dose regimes in glyphosate
563 and carbetamide (GC) mixtures.

564 Table S4: Comparisons of dynamics of resistance evolution for different dose regimes in atrazine,
565 glyphosate and carbetamide (AGC) mixtures.

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567 **Figure legends**

568 Figure 1. Mean population size at transfer (measured as OD_{750}) during 15 weeks of
569 adaptation to herbicide selection regimes. a) Dynamics of resistance in regimes containing
570 mixtures of atrazine and glyphosate; b) atrazine and carbetamide; c) glyphosate and
571 carbetamide; d) atrazine, glyphosate and carbetamide. Individual selection regimes are
572 indicated in the legend with the number of replicates (of 6) in which resistance evolved
573 shown in parentheses. Bars are standard errors of the mean.

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