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

Author list:

Mato Lagator[1], Tom Vogwill[2], Andrew Mead[1], Nick Colegrave[3], Paul Neve[1]

[1]School of Life Sciences, University of Warwick, Coventry, CV4 7AL

[2]Department of Zoology, University of Oxford, Oxford, OX1 3PS

[3]School of Biological Sciences, Institute of Evolutionary Biology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JT

Corresponding author:

Mato Lagator, e-mail: M.Lagator@warwick.ac.uk, telephone: (0)7500895706

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Summary

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Founding population

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

Culture conditions

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

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

Herbicides

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

Selection regimes

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

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

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186 *Cross-resistance assays*

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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 (OD_{750}) was
193 measured after seven days. Each condition was replicated twice.

194

195 *Statistical analyses*

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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.

Effect of the number of herbicides. To analyze for the effects of herbicide number, we modelled the temporal dynamics of population size using a linear mixed model within ANOVA (aov function in R 2.15.0). To do so, we compared the regimes that evolved in single herbicide environments to those in mixtures at MICeq doses, as these regimes offered the same initial level of population control and therefore rates of adaptation could meaningfully be compared. Regimes selected in mixtures at MIC, MIC1.5 and MIC2 were not relevant to this question. As discussed above we used a nested model to allow our hypothesis of interest to be tested based on an analysis of the entire data set. The response variable was population size (measured as OD_{750} at the end of each transfer period). Nested within the entire dataset, we fitted an initial fixed term with two levels; the first level included all treatments relevant to this question (A0, G0, C0, AGeq, ACeq, GCeq, AGCeq), whilst the second level included all other treatments. Within the first level we nested a factor with three levels to allow comparison of the treatments with different numbers of herbicides (one, two or three). We then nested further terms to account for variation amongst the three single herbicide treatments, and three different herbicide pair treatments. Within the second level of the initial fixed term we included a nested factor with 12 levels to account for variation amongst the 12 treatments that are not directly relevant to this question. The random (error) term consisted of time (weeks, 15 levels) nested within each regime (19 levels), nested within replicate (population, 6 levels). Significance of fixed effects was tested with F-tests.

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

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

Comparing the time of resistance evolution in selection regimes. To analyse the dynamics of resistance evolution in herbicide mixtures and single herbicide exposure regimes, we modelled OD_{750} as the response in a further set of linear mixed models using ANOVA in GenStat (13^{th} edition). We separately modelled resistance for regimes associated with each herbicide mixture (AG, AC, GC, AGC), enabling comparison between all four dose regimes for each mixture as well as the two or three relevant single herbicide regimes (i.e. A0 and G0 for the AG mixture, and all three single herbicide conditions for the AGC mixture), following the nesting approach outlined above. An initial term in each model compared the mean for the six or seven regimes of interest with the mean of the remaining treatments, with nested terms accounting for the variation among the treatments not of direct interest. Each model also included the time term, using a series of linear contrasts to identify the time periods over which there were changes in the level of resistance across the six or seven treatments of interest, and the interaction of these contrasts with the treatment terms identified above, to

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

Cross-resistance. We analysed differences in the cross-resistance profile of selected populations by ANOVA with population growth after seven days (measured as OD_{750}) as the response variable. Fixed factors were genotype (selection regime, 14 levels, as we excluded regimes that did not give rise to any resistant populations) and environment (novel herbicide environment, 4 levels), while the error term consisted of the source population. We were particularly interested in the genotype x environment interaction as this represents the differences in the range of novel herbicides that a population expressed cross-resistance to. A subsequent analysis was conducted using Tukey's honestly significant pairwise tests between the mean OD_{750} of the populations selected in each regime across all four novel herbicide environments. This test treated cross-resistance as a composite measure that included both the

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

Results

Dynamics of herbicide resistance

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

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

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

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

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

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

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

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

Patterns of cross-resistance

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

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

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359 We observe that regardless of herbicide identity, populations exposed to the two lowest
360 combined doses (MICeq 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 then to
380 single herbicides at high relative doses (Fig. 1).

381

382 *Mixtures increase the likelihood of cross-resistance*

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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

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

Mixtures in a wider applied setting

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

Antibiotics acting synergistically – offering the same control of susceptible populations at lower combined doses (Trindade *et al.*, 2009) – have been shown to elevate rates of resistance evolution (Michel *et al.*, 2008), as a lower effective dose is experienced once resistance evolves to one of the components in synergistic mixtures, as opposed to a mixture of non-interacting or antagonistic antibiotics (Hegreness *et al.*, 2008). Our results support these findings and extend the implications to alterations of the dose of components in a 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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Table 1. Patterns of cross-resistance measured as populations growth (mean OD_{750}) after 4 days of growth in a novel herbicide for each regime/standard error of the mean. F - 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+Ceq	0/0	0/0	0/0	0.232/0.087
A+Cx	0/0	0/0	0/0	0.065/0.041
G+Ceq	0/0	0.048/0.033	0/0	0/0
G+Cx	0/0	0.144/0.028	0/0	0/0
G+C1.5	0/0	0.0532/0.034	0/0	0/0
AGCeq	0.073/0.033	0.084/0.042	0/0	0.186/0.017
AGCx	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.

566

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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