Heterogeneity of selection and the evolution of resistance

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The evolution of resistance to pesticides and drugs in pests and pathogens is a textbook example of adaptation to environmental changes and a major issue in both public health and agronomy. Surprisingly, there is little consensus on how to combine selection pressures (i.e. molecules used in the treatment of pests or pathogens) over space and time to delay or prevent this evolutionary process. By reviewing theoretical models and experimental studies, we show that higher levels of heterogeneity of selection are associated with longer-term sustainability of pest or pathogen control. The combination of molecules usually outcompetes other resistance management strategies such as responsive alternation, periodic application or mosaic, because it ensures "multiple intra-generational killing". A strategic deployment over space and/or time of several combinations can ensure "multiple inter-generational killing", further delaying the evolution of resistance.

The worrying issue of the evolution of resistance

Throughout history, humans have used a variety of strategies to control diseases and their vectors as well as pests impacting crops and domestic animals. As far back as the eighth century BC Homer refers to the use of sulfur to fumigate homes. Arsenic, an insecticide recommended by the Roman naturalist Pliny the Elder in the first century, was used in the tenth century by the Chinese to control garden pests. From the 1940s, the discovery of modern pesticides – such as DDT – and of most of the major classes of antibiotics appeared to offer

the key to controlling pests and pathogens. Most of these measures were relatively cheap and ensured very high levels of control. During two following decades those molecules have been widely used in fields, farms, homes and hospitals to treat crops, animals and humans saving yields and lives. Unfortunately, one of the drawbacks of these treatments for killing pests and pathogens is that they exert selection pressures on target populations, leading to the evolution of resistance mechanisms reducing the efficacy of treatments (for insecticides [1], for herbicides [2], for antibiotics [3, 4], for HIV-1 protease inhibitors [5]).

The evolution of resistance to pesticides and drugs has offered several case studies of adaptive evolution and can be considered a valuable example for other evolutionary changes more difficult to perceive and analyze. Hence, studies of the evolution of resistance to various pesticides have improved our understanding of the molecular mechanisms involved in adaptation [6] and dominance [7], the epistatic relationships between loci [8] and the fitness costs of adaptive mutations[9].

The evolution of resistance to pesticides and drugs is not only a textbook example of adaptation. It is, above all, a major issue for both public health and agronomy, because the number of drugs and pesticides with different mechanisms of toxicity and acting on independent targets has proved to be limited (see e.g. for antibiotics [10] and for pesticides [11]) (Box 1). Only a few new active molecules have been discovered in the last 30 years. A new wave of R&D on drugs and pesticides, with the exception of that relating to insecticidal toxins, would be unlikely to yield substantial public health and crop protection options within the next 10 to 15 years [12]. In the meantime, we need to protect the existing molecules.

Fortunately, most classes of pesticides and antibiotics [10] include several molecules that are still active and for which, at least in some cases, there is still no sign of resistance. This raises questions about how we can combine these molecules over time and space to preserve their efficacy for as long as possible.

In the literature, four principal basic strategies combining two (or more) molecules over time and/or space have been considered, to delay the evolution of resistance to drugs and pesticides: *Responsive alternation*, *Periodic application*, *Mosaic* and *Combination* (Box 2). Is one particular strategy intrinsically better than the others? Conversely, does the ranking of strategies depend on the target organism or the pesticide or drug considered? Theoretical models predicting the outcome of selection pressures and experimental selection on pests and pathogens can be used to test such predictions. Here, we review the results obtained with theoretical models and in empirical studies for various pesticides and drugs (generally considered separately, by ecologists and agronomists on the one hand and medical scientists on the other [13, 14]). We show that some consensus can be reached on the deployment of selection pressures over time and space to delay or prevent the evolution of resistance in pest and pathogen populations

Theoretical comparisons between strategies

We searched for articles that explicitly compared, in the same study, at least two of the four strategies — *Responsive alternation*, *Periodic application*, *Mosaic* and *Combination* (whether *half-* or *full-dose*, see Box 2) — in terms of their efficacy for delaying the evolution of

resistance to more than one pesticide or drug. We therefore excluded all studies that considered several molecules but modeled the evolution of resistance to only one molecule. A search of the Resistance to Xenobiotic (REX) bibliographic database [13, 14] for articles relating to the modeling of resistance evolution identified 20 relevant articles. Further searches in the Web of Science and Google Scholar and screening of the articles cited in the initial 20 articles yielded an additional nine articles. Half of those articles were related to either insecticide or antibiotic resistance.

Based on the 29 articles retained (Table S1), we identified a clear ranking of the strategies in terms of their efficacy for delaying resistance: *Combination* > *Periodic application* = *Mosaic* > *Responsive alternation* (Table 1). *Combination* was at least as good as, or outperformed *Responsive alternation*, *Periodic application* and *Mosaic* in more than 80% of the comparisons. *Half-dose Combination* was found to have been little studied and comparisons of *Combination* with other strategies were somewhat biased because a *full-dose Combination*, by doubling the dose of pesticide or drug used, increases overall selection pressure. *Responsive alternation* was less effective than *Periodic application* and *Mosaic* in all comparisons. The ranking of *Mosaic* and *Periodic application* was, by contrast, not straightforward. These two strategies were compared mostly to determine whether *Periodic application* (referred to as *Cycling* in clinical studies, see Box 2) could delay resistance to antibiotics more effectively than *Mosaic* (referred to as *Mixing* in clinical studies, see Box 2) in hospitals or, more specifically, in intensive care units. All the epidemiological models gave the same answer: *Mosaic* > *Periodic application*. By contrast, Roush [15] and Lenormand &

Raymond [16], who modeled the evolution of insecticide resistance, found *Periodic application* > *Mosaic*.

Combination and "multiple intra-generational killing" at the individual level

Combination is very effective due to "multiple intra-generational killing" [17], a key feature that can be explained as follows: if resistance alleles at each of two independent loci are present at very low frequency in the pest or pathogen population, then any given individual is extremely unlikely to carry resistance alleles at both loci. If resistance is recessive, then diploid pests and pathogens are only resistant if they are homozygous for the resistance allele at both loci. When resistance alleles are at low frequencies, this probability is very low. Thus, drug or pesticide *A* can kill individuals that are susceptible to this drug or pesticide, whatever their genotype at the locus conferring resistance to drug or pesticide *B* and *vice versa*. This is described as "multiple intra-generational killing", because most pest or pathogen individuals are susceptible to both molecules and are therefore "killed twice" (Figure 1).

The superiority of *Combination* over the other strategies appears to be very robust: in most models, this approach was effective for longer even if input and output parameters were varied. Its comparative advantage is particularly high when: (1) resistance to each pesticide or drug is initially rare [17-21], (2) resistance to each pesticide or drug in the combination are controlled by independent loci (no cross-resistance) [17, 22-24], (3) there is a high rate of recombination between the loci [17, 20, 23, 24], (4) in diploids, homozygous susceptible individuals have a high mortality [15, 21], (5) in diploids, resistance to each pesticide is

functionally recessive [17, 23, 25-27], (6) the pesticides or drugs are of similar persistence [15, 27] and (7) some of the population remains untreated [20, 22, 24, 25]. Even if these conditions are not completely met, *Combination* appears at least as good as the three other strategies.

Degree of treatment heterogeneity **and "multiple killing"**

The most recent approaches in medicine focus on antibiotic heterogeneity [28, 29], the idea being that higher degree of treatment heterogeneity (*DTH*) are associated with a slower evolution of resistance. Mani [30] explored this idea for insecticide resistance, more than 20 years ago. He showed that, after *Combination*, the most promising strategy was not to vary applications of a given molecule over time (*Periodic application*) or space (*Mosaic*), but to alternate the insecticides used over *both* time and space, thereby maximizing the *DTH* .

To our knowledge, the relationship between *DTH*, temporal or spatial selection heterogeneity, and the sustainability of efficacy for a given molecule has never been clearly formalized. We suggest that *DTH* should be defined as the probability that a set of resistance genes is confronted to more than one pesticide or drug within or between generations. In case of periodic application, offsprings from individuals resistant to one molecule will be treated with another molecule depending on the generation time of the pathogen or pest and on the period of application of the drug or pesticide. These offspring would be expected to be susceptible to the second molecule, particularly in the absence of cross-resistance and if resistance genes are independent of each other. In this case, *DTH* therefore ensures "multiple

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inter-generational killing" at the colony or family level (Figure 1), because the first molecule kills most individuals in the parental generation and the second molecule then kills the offspring of the few survivors. As explained before, *Combination* ensures "multiple intra-generational killing" and a maximal *DTH* because every individual suffers simultaneously both molecules. In a *Mosaic* set up the survivors to the first molecule can disperse and then be killed by the second molecule. If dispersal distances are larger than the scale of mosaic unit then *Mosaic* can also lead to "multiple intra-generational killing".

All things being equal, higher *DTH* should be associated with longer-term sustainability of pesticides or drugs. *Responsive alternation* systematically results in the lowest *DTH*, because the offspring are treated with the same molecules as their parents until the population size reaches unacceptable levels. Depending on the pattern of pest or pathogen dispersal, its generation time and the temporal and spatial scales of treatment, higher *DTH* can be achieved with either *Periodic application* or *Mosaic* strategies, or through the use of a combination of these two extreme strategies.

Empirical comparisons between strategies

In 1983, Georghiou [31] stated that: "*Perusal of pertinent literature reveals that there are more papers discussing the value of mixture* [i.e. combination] *(as well as rotation* [i.e. periodic application]*) than those that report actual research on the subject*". Unfortunately, this remains true in 2012. Using the Web of Science, Google Scholar and the references cited in recent articles on this topic, we found only 17 empirical studies – half of them being on

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insecticide resistance – comparing at least two strategies in laboratory, greenhouse or field conditions (Table S2).

In the 17 empirical studies identified, the ranking of efficacy was *Combination* = *Periodic application* > *Responsive alternation* (Table 1). Indeed, in five of eight comparisons, *Combination* was found to be as good as *Periodic application,* and *Responsive alternation* never outperformed either of these two strategies. It was not possible to rank *Mosaic* reliably, because too few comparisons included this strategy. *Mosaic* outperformed *Periodic application* and *Responsive alternation* in two independent studies but was found to be less effective than *Combination*, *Periodic application* and *Responsive alternation* in the other four comparisons (Table 1).

Although *Combination* appeared to be the best strategy in theoretical models, it did not clearly outperform *Periodic application* in empirical studies. This discrepancy between theoretical and empirical results can simply reflect time constraints. Indeed, in most experimental studies, treatments were applied during a fixed number of generations. In most cases, resistance emerged when molecules were used singly, but not when they were combined over space and/or time (*Periodic application*, *Mosaic* or *Combination*). Thus, several studies reported an absence of resistance development for at least one molecule for both *Combination* and *Periodic application* strategies [e.g. [32-36]], making it impossible to draw any firm conclusions concerning possible differences in efficacy between these two strategies. The conclusion that these two strategies are similar in efficacy is thus valid for the

number of generations over which selection took place, but might not hold absolutely true *per se*.

The discrepancy between theoretical and empirical results can also result from the use of experimental settings that decrease the advantage of *Combination* over other strategies. As mentioned above, empirical studies are limited by the number of generations that can be run. They are also limited by the number of individuals per generation that can be manipulated. These experimental constraints have two important consequences. First, empirical studies focus on the evolution of resistance alleles already present in populations rather than on resistance alleles acquired *de novo* by mutation or horizontal transfer. Hence, in all but one of the experimental studies (see Table S2), a deliberate decision was taken to have a high frequency (i.e. $> 10^{-3}$) of resistance to at least one pesticide or drug at the start of selection, thereby decreasing the efficacy of *Combination* by violating one of the favoring conditions [17-21]. Second, a sufficiently large number of individuals must survive pesticide or drug treatments to establish the next generation. Consequently, the selection pressure applied in such experiments generally varies between 0.5 and 0.8, corresponding to low doses. In such cases, resistance can be functionally dominant, further decreasing the comparative advantage of *Combination* over the other strategies [17, 23, 25-27]. Experimental settings with high initial frequencies of resistance alleles and low selection pressures can, in some cases, approach real conditions. Molecules newly released onto the market are sometimes used in combination with other molecules for which resistance has already been selected in the targeted pest or pathogen populations, for economic purposes. Selection at low doses can also

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occur in field conditions because of the dilution of the molecules and their degradation over time.

Nevertheless, one specific feature of empirical studies clearly differs from practice. As pointed out above, the presence of untreated individuals from refuges increases the success of *Combination*. However, nine of the 17 empirical studies were conducted without such refuges (Table S2). This is unfortunate, because such refuges could easily be included in studies of the selection of pesticide resistance. Leaving a fraction of the population free of pesticide exposure would have better mimicked the conditions in fields, hospitals and care units. Indeed, a significant proportion of the pests or pathogens often remain untreated unintentionally. Dormant weeds, resting spores of fungi, hidden mosquito breeding sites, soil seed banks or field borders, alternative hosts, or people outside the medical system are very common and constitute unplanned refuges of pest and pathogens.

Can *Combination* be outcompeted?

One particular condition can render *Combination* inferior to other strategies. This condition is the occurrence of fitness costs, resulting in resistant individuals being less fit than susceptible individuals in the absence of the pesticide or drug. Such costs might lead to the counterselection of resistance alleles and would therefore delay, if not prevent, the development of resistance. The expression of this cost would require spatial or temporal variation in pesticide or drug selection, with locations or periods of time in which one of the pesticide or drug is absent. *Combination* is the only strategy combining two molecules that

does not generate such variation and therefore it is the only strategy that does not allow the expression of a resistance cost. Consequently, fitness costs can facilitate the mitigation of resistance in all strategies except *Combination*. This can explain why Dobson *et al.* [37] (theoretically) and Immajaru *et al.* [38] (experimentally) found *Combination* to be less effective than *Periodic application* and *Mosaic* (see Tables S1 and S2). Indeed, their theoretical and biological models were characterized by very high fitness costs and an absence of refuges (see below).

In practice, fitness costs might make *Combination* worse than other strategies only rarely. First, the "multiple intra-generational killing" provided by combination approaches might be sufficient to ensure the superiority of this strategy in many cases, even in the presence of fitness costs. Second, although mutations conferring resistance are often costly (e.g. for herbicides [39], for insecticidal proteins [40], for antibiotics [41], for anti-virus [42]), decreases in fitness can be attenuated or even completely abolished by compensatory mutations (for herbicides [43], for antimicrobial drugs [44], for antibiotics [45], for anti-virus [46]) or through interactions with other resistance mutations [47]. Over time, costly resistance mutations can also be replaced by resistance mutations associated with lower fitness costs [48]. Finally, when part of the population remains untreated, fitness costs counteract the selection of resistance alleles, even for *Combination*. Untreated individuals can be actively preserved by the use or maintenance of refuges for pests and pathogens. The use of refuges is not possible in hospitals, because it would be unethical not to treat infected humans with antibiotics or other drugs. However, the community outside hospitals constitutes a refuge for

253 most pathogen populations and individuals carrying pathogenic bacteria or viruses but
254 displaying no symptoms, or only minor symptoms, are left untreated. Finally, pathogens or
255 pests generally escape treatments even within the host or the field, because treatment coverage
256 is rarely complete.

257

258 **Increasing *Combination's degree of treatment heterogeneity***

259 The number of molecules that can actually be used in a *Combination* is limited by resistances
260 that have already developed (Box 1) and several challenges underlined in Box 3. Generally,
261 the concomitant use of a large number of molecules entails higher costs, which can outweigh
262 the benefits of delaying or preventing resistance in the eyes of the stakeholders. Thus,
263 combinations containing all the available molecules are unlikely to be used. However, it might
264 be possible to use several different *Combinations* to treat a given pest or pathogen. These
265 combinations would ideally be used so as to ensure the highest *DTH*, yielding "multiple intra-
266 generational killing" (at the individual level) and "multiple inter-generational killing" (at the
267 colony or family level). Depending on the distances over which dispersal occurs, the highest
268 *DTH* can be provided by *Mosaic, Periodic application* or a complex temporal and spatial
269 combination of the various combinations.

270 This might have practical consequences. For example, in antibiotic resistance management,
271 treatment heterogeneity is currently defined at the level of the hospital rather than the
272 pathogen. The theoretical and empirical studies reviewed here show that diversity in antibiotic
273 use between care units or beds at a given time (i.e. a *Mosaic* strategy) is more sustainable than

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cycling different antibiotic regimens over time (i.e. a *Periodic application* strategy). This is because, at the scale relevant to bacterial populations, *Mosaic* imposes greater *DTH* than *Periodic application* [29, 30, 51, 52]. This is particularly true when the cycle of each antibiotic regimen is very long, extending over several months. Indeed, due to its short generation time, a bacterial colony is more likely to encounter the second antibiotic in a *Mosaic* implemented at the scale of the bed or at the scale of the care unit than in a *Periodic application* based on the cycling of antibiotics over several months. Another hypothesis has been put forward by Boni et al [49] to explain the higher performance of *Mosaic* over *Periodic application*: *Periodic application* degrades the mean fitness of the parasite population more quickly than *Mosaic*, making it easier for new resistant types to invade and spread in the population.

Although difficult to implement, we suggest that *Periodic application* at the level of the patient, rather than the hospital (or care unit), might result in greater *DTH* than *Mosaic*. Alternating antibiotics to treat patients would increase the likelihood of "multiple inter-generational killing" — i.e. the probability of colonies resistant to a given antibiotic being treated, and therefore killed, by another antibiotic in the next generation.

Beyond *Combination* and *degree of treatment heterogeneity*: protecting populations against the emergence of resistance alleles

The question of how to combine pesticides and drugs over time and space is only one part of the overall debate on resistance management. The dose of the molecules used must also be

considered. Resistance management strategies sometimes include the use of high doses of pesticides and drugs. For bacterial and HIV infections, this has been referred to as the "Hit hard and early" approach [50]. Interestingly, different rationales are applied to pesticides and drugs. For drugs, the reason for treating "hard" is to decrease the size of the pathogen population as much as possible, to prevent the appearance of resistance alleles. For pesticides, high-dose strategies are designed not only to avoid the emergence of new resistance alleles [51, 52], but also to avoid the building of polygenic resistance [53] and to make resistance of diploid pests functionally recessive [54]. The use of a high dose can also enlarge the spectrum of pests targeted. This is particularly true for herbicides, as fields generally contain more than one weed species that must be controlled.

The drawback of hitting hard is that it increases the costs associated with resistance management (Box 3). This strategy can actually be counterproductive if resistant pathogens are already established [52, 55]. However, in the case of new molecules for which no resistance has been detected, this approach can well be the most appropriate, provided that the costs are sustainable. Unfortunately, most mathematical models of the evolution of pesticide resistance assume that population size is infinite – but see e.g. [53, 56]. Consequently, resistance alleles are generally assumed to be initially present at all resistance loci in the population. In fact, natural populations are limited in size and might contain no resistance alleles. In such cases, the appearance and early increase in frequency of resistance alleles is a stochastic process dependent on the balance between mutation rates and population size. This stochasticity is also largely ignored in empirical studies. As indicated above, empirical studies

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316 are always performed at locations or using strains in which resistance to at least one molecule
317 occurs at a relatively high frequency which constitute an unfavorable situation for high-dose
318 strategies.

319 There is therefore a need for both theoretical and empirical studies to further investigate the
320 evolution of resistance in conditions allowing stochastic events [57]. In such situations,
321 'hitting hard' likely results in a greater efficacy of *Combination* than of other strategies and
322 *full-dose Combination* certainly provides populations with the highest level of protection
323 against the emergence of resistance alleles. The use of high-dose *Bt* crops could be seen as a
324 life-size experiment testing this hypothesis. Interestingly, in the US, populations of the pink
325 bollworm, *Pectinophora gossypiella*, targeted by Bt cotton, and of the European corn borer,
326 *Ostrinia nubilalis*, targeted by Bt maize, have been declining from year to year [58, 59]. For
327 the pink bollworm, sterile moths releases have been successful in suppressing the emergence
328 of resistance alleles to *Bt* cotton [60], a cornerstone for the multi-tactic eradication program of
329 this pest [61]. This provides some hope that pest populations could be eliminated over a wide
330 area before resistance alleles emerge and spread.

331

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Glossary

Cross-resistance: a resistance to a pesticide or drug that also confers resistance to another pesticide or drug.

Degree of treatment heterogeneity (DTH): the probability that a set of resistance genes is confronted to more than one pesticide or drug during a certain amount of time, be it within or between generations.

Insecticidal toxins: toxins produced by bacteria, mostly *Bacillus thuringiensis* and *B. sphaericus*, and used in sprays or in genetically engineered plants to control insects.

Multiple intra-generational killing: a strategy that consists in using a variety of pesticides or drugs on each pest or pathogen individual, in order to maximize the probability that each individual is killed. An individual resistant to molecule A but susceptible to molecule B will be killed if treated simultaneously by molecules A and B.

Multiple trans-generational killing: a strategy that consists in using a variety of pesticides or drugs on successive generations of pests or pathogens, in order to maximize the probability that the offspring of resistant individuals is killed. The offspring of an individual resistant to molecule A but susceptible to molecule B will be killed by molecule B.

Recessive resistance allele: an allele that confers resistance to diploid pests and pathogens only if present in homozygous state. A dominant resistance allele confers resistance when both in heterozygous and homozygous states.

Refuge: areas, fields or group of pests or pathogens remaining untreated by pesticides or drugs.

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Resistance: a heritable change in a population that is reflected in the ability of individuals to survive and reproduce in the presence of environmental conditions that once killed most individuals of the same species.

Resistance cost: a negative pleiotropic effect of a resistant genotype that results in a lower fitness of resistant individuals compared to susceptible ones in absence of pesticide or drug.

Resistance gene: a gene at which one or more alleles confer resistance to pesticides or drugs.

Resistance management strategy: a strategy devoted to delay or prevent the evolution of resistance in a population of pests or pathogens. *Mosaic*, *Periodic application*, *Combination* and *Responsive alternation* (see box 2) are simple resistance management strategies using more than one pesticide or drug.

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Box 1. The evolution of resistance to pesticides and drugs

Almost 8,000 cases of resistance to 300 insecticide compounds have been reported in more than 500 species of arthropods [1] (Arthropod Pesticide Resistance Database (APRD, www.pesticideresistance.com)). Similarly, 300 cases of field resistance to 30 fungicides have been reported in 250 species of phytopathogenic fungi (The Fungicide Resistance Action Committee database (FRAC, http://www.frac.info)). The International Survey of Herbicide-Resistant Weeds (ISHRW, www.weedscience.com) has suggested that there are currently about 370 resistant biotypes in 200 weed species in 570,000 fields. The situation is most critical for antibiotic resistance. Genes conferring resistance to antibiotics are ubiquitous in bacteria and highly diverse. The Antibiotic Resistance Genes Database (ARGD, http://ardb.cbcb.umd.edu/), developed by Liu and Pop [62], lists more than 23,000 potential resistance genes of about 400 types, conferring resistance to 250 antibiotics in 1,700 species of bacteria from 270 genera. Strains from highly pathogenic bacteria, such as *Tuberculosis bacilli,* resistant to all known classes of antibiotics have recently been described [63].

In addition, most of the major classes of antibiotics were first isolated between 1940 and 1960 [64]. The more recently commercialized drugs and pesticides are often variants of previously isolated or synthesized compounds and are therefore not particularly effective against the prevailing resistance mechanisms (see for herbicides [65], for insecticides [66], for antiviral drugs [67] and for antibiotics [68]). The cost of developing new drugs and pesticides has been further increased by the tightening of requirements by regulatory authorities, necessitating a larger number of toxicological, clinical and environmental trials [69]. Hence,

according to Larson [70], it currently takes about 10 years and up to US$1 billion to develop a new antibiotic. Similarly 10 to 12 years are required to develop and launch a new pesticide on the market [71].

At the turn of the 21st century, the combination of approaches such as genomics [72], proteomics [73] and metabolomics [74] with target-based high-throughput screening strategies [71, 75] appeared very promising for the discovery of new drugs and pesticides with little or no impact on environment and health. However, these new methods and strategies have proved relatively unsuccessful, both for antibiotics [76] and for pesticides [77]. The situation is very different for insecticidal toxins, mostly proteins from *Bacillus thuringiensis,* whether formulated for application in sprays or produced by transgenic plants. The number of toxins identified is increasing [78] and the populations of most of the pests targeted remain resistance-free ([66], but see [79]).

Box 2. Strategies for combining molecules over time and space

Four principal basic strategies combining two (or more) molecules over time and/or space have been considered for drugs and pesticides. These strategies differ in the way the pesticides or drugs are combined. In the *Periodic application* and *Responsive alternation* strategies, molecule use is uniform over space but heterogeneous over time. *Periodic application* involves temporal cycles of pesticide or drug application, a strategy first suggested by Coyne [80]. By contrast, *Responsive alternation* corresponds to successive applications of molecules, but without a cycle. In this approach, a molecule is used repeatedly until the emergence of resistance, after which the second molecule is introduced, and so on. *Mosaic* — a strategy first suggested by Muir [81] — concerns a spatial pattern of application for at least two molecules. Molecule application remains uniform over time and the spatial distributions of the molecules used do not overlap. Finally, *Combination* is the concomitant use of two or more molecules over time and space. *Responsive alternation*, *Periodic application*, *Mosaic* and *Combination* have been referred to by various names within and between the different classes of pesticides and drugs, as summarized in the following table.

	Names used to define strategies			
Strategy	**Antibiotics or antiviral drugs**	**Insecticides or Bt toxins**	**Fungicides**	**Herbicides**
Responsive alternation	sequential use	sequence, sequential use, serial use	_	sequence, threshold strategy
Periodic applicatio	cycling, antibiotic rotation, periodic application,	rotation, alternation,	rotation, alternation,	rotation

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n	sequential use	sequential use	sequence	
Mosaic	mixing, 50-50 treatment, antibiotic diversity, multiple first-line therapy	mosaic	_	mosaic
Combination	combination, antibiotic diversity, simultaneous strategy	mixture, pyramiding	mixture, combination	mixture, combination, double knockdown

In practice, molecules in *Combinations* are combined in variable ratios and at different doses. Strategies based on both *full-dose* and *half-dose Combinations* have been proposed. In the *full-dose Combination* strategy, each pesticide or drug is applied at the dose at which it would be used if applied alone, whereas, in the *half-dose* strategy, the dose of each pesticide or drug is half that used when the compound is applied alone. Consequently, the final overall dose of the *full-dose* strategy is equivalent to twice that applied if each molecule were to be used alone, whereas the final overall dose of the *half-dose Combination* strategy corresponds to the dose at which each molecule would be applied if used alone.

Practical recommendations on the strategy to be used depend on the target organism. For instance, *Combinations* are currently recommended in the treatment of HIV [82], tuberculosis [83] and malaria [84]. Pesticides are also increasingly used as combination rather than as individual compounds, as exemplified by the new generation of *Bt* crops, which produce several independent toxins against the target pests [85]. However, *Combination* is not the current *default* strategy in antibiotic treatment, particularly in the community, and is not

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430 recommended for several pesticides (e.g. for the control of *Anopheles,* the vector of malaria,

431 [86]).

Box 3. Challenges with *Combinations*

Imagine that two molecules are available and that all conditions are satisfied for their combination to outperform all other strategies for delaying the evolution of resistance. Would *Combination* become THE optimal strategy for use with any given set of pesticides and drugs? Probably not! There are several obstacles to the universal recommendation and implementation of this strategy.

The possibility of antagonistic effects between molecules – which may seriously reduce pest or pathogen control – constitutes a first obstacle to the use of the *Combination* strategy [87]. Synergistic molecule combinations can be advantageous in controlling pests and pathogens. However, resistance to such combinations can evolve faster than resistance to antagonistic molecule combinations and, in some cases, to individual molecules themselves [88].

A second obstacle for using *Combination* is that the molecules prescribed by physicians and used by farmers not only control pests and pathogens, they may also injury crops and have undesirable effects on non-target organisms and human health. The World Health Organization (WHO) has reported that there are about three million human cases of pesticide poisoning annually, resulting in 220,000 deaths worldwide [89], hepatoxicity, neurotoxicity and lipodystrophy [90-92]. Chemical pesticides have a significant impact on non target plants, fungi and arthropods [93]. Pesticide use can disrupt biological control through direct toxicity [94], indirectly changing the community structure [95] and their predators or parasitoids [96].

Trends in ecology & evolution (2013), Vol. 28, N°.2, p. 110-118, DOI: 10.1016/j.tree.2012.09.001,
Journal homepage: http://www.cell.com/trends/ecology-evolution

A trade-off thus exists between controlling the pest with the right dose and limiting the side effects of treatment. Undesirable effects occur when single molecules are used, but they are probably worsened by the use of combinations, because synergy between molecules [97] can increase the threat to the environment [98] and to human health [99].

Stakeholders – i.e. companies, users, prescribers and public authorities - diverge on their respective interests, goals and their sensitivity to strategies' costs, depending on the policy implemented. For example, refuges increase the risk of pest/pathogen damage, and in the short term this cost is met directly by users. Similarly, *Combination,* by "multiple intra-generational killing", can be more efficient for controlling pests/pathogens but, because of the higher dose applied, implies financial costs to farmers or patients (or public authorities if there is social health coverage) as well as increased magnitude of undesirable effects on health and the environment and, thus, the costs to be covered by public authorities.

The willingness of the various stakeholders to share the costs depends directly on the extent to which they are likely to be affected by or considered responsible for the emergence of resistance. Hence users are confronted with the so-called "*Tragedy of the Commons"* when exploiting a common property resource [100], even if they are likely to be strongly affected by the evolution of resistance. In most cases, by not playing their part in the management of resistance, each user maximizes their own short-term benefit but favors the selection of resistant pests/pathogens, thus having potentially long-term negative effects for the community.

**Trends in ecology & evolution (2013), Vol. 28, N°.2, p. 110-118, DOI: 10.1016/j.tree.2012.09.001,
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Figure Legends

Figure 1. Schematic representation of the effect of the different strategies (*Responsive alternation*, *Mosaic*, *Periodic application* and *Combination*) on the targeted pests or pathogens – here, a mosquito. These strategies can lead to multiple intra-generational killing at the individual level (for *Combination*) or multiple inter-generational killing at the family or colony level (for *Periodic application* and *Mosaic*). This depends on the balance between the spatial and temporal scales of the treatments and the dispersal capacities and generation time of the targeted pests or pathogens. At each generation (G), pests or pathogens are selected by molecule 1, molecule 2 or a combination of these two molecules. Individuals S, R_1 and R_2 are susceptible, resistant to molecule 1 and resistance to molecule 2 respectively. Individuals R_{12}, harboring genes conferring resistance to molecule 1 as well as genes conferring resistance to molecule 2, can survive in a patch treated with a combination of these two molecules. The *Degree of Treatment Heterogeneity* (*DTH*) defined here as the probability that a set of resistance genes is confronted to more than one pesticide or drug varies among the strategies. *Combination* displays the largest *DTH*, followed by *Periodic applications* and *Mosaic* depending on the generation time, dispersal distance, period and spatial scales of application, and by *Responsive alternation*.

703 **Table 1.** Side-by-side comparisons of the four strategies in terms of their relative efficacies for delaying or preventing resistance.

Strategies	**Theoretical studies**	**Empirical studies**								
1	**2**	**N** [a]	**1 > 2**	**1 = 2**	**1 < 2**	**Conditional** [b]	**N**	**1 > 2**	**1 = 2**	**1 < 2**
Combination	*Responsive alternation*	14	11	0	0	3	10	8	2	0
Combination	*Periodic application*	16	14	0	1	1	8	2	5	1
Combination	*Mosaic*	7	5	0	1	1	1	1	0	0
Periodic application	*Responsive alternation*	7	3	4	0	0	9	7	2	0
Periodic application	*Mosaic*	11	2	3	5	1	3	2	0	1

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Mosaic	*Responsive alternation*	3	2	1	0	0	2	1	0	1

[a] N = number of comparisons in all theoretical and empirical studies

[b] The ranking of the strategies depends on the setting for one or several input or output parameters.