

Gene transfers into the environment

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Abstract

Global trade and climate change contribute to erase the bio-geographical boundaries separating the ecological niches of numerous species, favouring the emergence or re-emergence of crop pest or disease vector species. This report, commissioned by the Swiss Federal Ethics Committee on Non-Human Biotechnology (ECNH) will describe technologies implying the transfer of genetic material in the environment such as biological control, CRISPR-Cas9 and *Wolbachia* based technologies. It purposely focuses on technologies that are currently not used in Switzerland but that could be used in the future. The focus is set on potential future technologies. Every technology described is used somewhere in the world, enabling us to discuss biosafety concerns and provide an analysis of the regulation procedures mobilized to evaluate potential environmental risks.

A classical biological control case study focusing on risks associated with hybridization between introduced biological control agents and native species showed that the laws regulating releases of environment in the environment is suitable to ensure biosafety but that the environmental risk assessment procedure is incomplete. This case study revealed the need for an harmonized European environmental risk assessment and the need to integrate socio-economic aspects in environmental risk assessment procedures.

An analysis of a *Wolbachia*-based control strategy to control disease vectors showed that the Swiss laws regulating releases of organisms in the environment seems perfectly suitable to ensure biosafety. The environmental risk assessment scheme developed by Australian researchers seems to be adequate to evaluate the risks even if the technology is probably to new for evaluating its long-term appropriateness.

The chapters on the CRISPR/Cas9 technology that could be used to control disease vectors such as mosquitoes, herbicide resistant agricultural weeds or invasive alien plants highlighted the disruptive character of the technology, the need for more stringent biosafety measures in gene drive experiments compared to other genetic manipulations and potential gaps between law texts in force in certain countries and this rapidly evolving new technology. To overcome this problem, we encourage regulators to consider a product based regulation rather than a technology-based regulation.

To summarize, this works highlights the need to consider every case as a unique case necessitating a precise and thorough evaluation or expertise. We strongly encourage regulators to advocate for case-by-case environmental risk assessment.

Acronym list

BCA :	Biological Control Agent
CAS:	CRISPR Associated Systems
CHIKV :	Chikungunya Virus
CI :	Cytoplasmic Incompatibility
CRISPR:	Clustered Regularly Interspaced Short Palindromic Repeats
CSIRO :	Commonwealth Scientific and Industrial Research Organisation
ECNH :	Federal Ethics Committee on Non-Human Biotechnology
EFSA :	European Food Safety Authority
EPPO :	European and Mediterranean Plant Protection Organisation
ERA :	Environmental Risk Assessment
ES :	Endosymbiont
ESA :	US Endangered Species Act
FOAG :	Swiss Federal Office for Agriculture
FOEN :	Swiss Federal Office for the Environment
FPPS :	Federal Plant Protection Service
GMO:	Genetically Modified Organism
GLZ :	Gruppo Lavoro Zanzare del Cantone Ticino
gRNA:	guide-RNA
HDR:	Homology Directed Repairs (DNA repair mechanism)
HEG:	Homing Endonuclease Gene
HR:	Herbicide Resistance
IAP:	Invasive Alien Plant
INRA :	Institut National de Recherche Agronomique (France)
IOBC :	International Organisation for Biological Control
IPPC :	International Plant Protection Convention
mt-DNA :	mitochondrial DNA
NHEJ :	Non Homologous End Joining (DNA repair mechanism)
ODE :	Ordonnance sur la Dissémination dans l'Environnement
OPPh :	Ordonnance sur les Produits Phytosanitaires
OPV :	Ordonnance sur la Protection des Végétaux
PAM:	Protospacer Adjacent Motif
PCR :	Polymerase Chain Reaction
PLH :	Panel on Plant Health (EFSA)
RIDL:	Release of Insects carrying a Dominant Lethal gene
SIT:	Sterile Insect Technique
TALEN:	Transcription Activator-Like Effector Nuclease
ZFN:	Zinc Finger Nuclease

Introduction

Human activities reshape the biotic interactions at the surface of the planet at an unprecedented rate. Global trade and climate change contribute to erase the bio-geographical boundaries separating the ecological niches of numerous species, permitting biological invasions or geographic range expansions of numerous organisms. Quarantine insect species, causing great damages to crops all over the world are being transported on very large distances to a very rapid pace. Even if prevention is considered to be the most economic and efficient way to mitigate the impact of invasive species, border controls are no longer efficient enough to avoid the entry of crop pest species (Bacon et al. 2012) and global warming will dramatically change the establishment potential of those species entering in large numbers in Europe or Switzerland (Bacon et al. 2014). Anthropogenic activities also shuffled disease vectors around the planet. Since the XVth century ship transport was responsible for numerous disease vector invasions worldwide. *Aedes aegypti*, the *Culex pipiens* complex, and, most recently, *Aedes albopictus* invasions have been facilitated by worldwide ship transport (Lounibos 2002) and the current distribution of *A. albopictus* can best be predicted by models accounting for the evolution of air transportation and global warming patterns (Tattem et al. 2006). Increasing numbers of crop pest species and disease vectors will call for methods to control their populations. The tiger mosquito *A. albopictus* was selected as a model organism for the discussions about the use of Wolbachia-based and gene drive control strategies.

Emergence or re-emergence of crop pest species or disease vectors are not new. Agriculture and human health were always challenged by detrimental species affecting their crops or transmitting sometimes deadly diseases. Many techniques were developed during several centuries to reduce their impacts. Agricultural practices such as crop rotation were used to break the developmental cycle of pest species. Technology was mobilized to reduce the impact of such species since the Green Revolution. Very soon the application of chemical pesticides to kill arthropods was used at very large scales with sometimes-severe non-target impact such as health problems for humans and animals and extreme pollutions (Carson 1963). Constantly sprayed by chemicals many pest species developed resistance against a wide variety of molecules and a real war-fair was engaged to cope with the rapid evolution of resistance in several detrimental insect species. The XXth century also witnessed the emergence of sophisticated (bio)-technologies. The first one was the use of natural enemies to reduce the population of pest species. Biological control became popular after the publication of the book “Silent Spring” by Rachel Carson, describing environment and human health dangers linked to pesticide use, in 1963 (Carson 1963). The use of natural enemies to control pest species was then considered as “the magic bullet” to reduce the impact of crop pest species until sometimes-negative effects on the environment became evident in the 1980s 1990s (see chapter 1). In particular, the risk that introduced species hybridize with native species is discussed in this report.

Plant and animal breeding lead to the domestication of several species since 12'000 years. Artificial selection achieved through selective breeding enabled researchers to actively select organisms with desired beneficial traits. Genes coding for such traits were then passed on to the next generations and individuals not displaying the appropriate

traits were put aside. Being able to modify the nucleic acid sequence in a laboratory opened the door to the creation of recombinant DNA that can come from the same species (cisgenic) or that can come from a totally different organism (transgenic). Paul Berg produced the first recombinant DNA molecules in 1972 and the direct manipulation of genes using biotechnology was first accomplished by Herbert Boyer and Stanley Cohen in 1973 (https://en.wikipedia.org/wiki/Genetically_modified_organism, consulted on the 10.01.2016). Genetic engineering permitted the introduction of selected genes from a given species, into another distantly related organism. Recent methodological progresses allowed researchers to manipulate, add or remove selected genes of an organism. Being able to create new biological entities lead to the commercialization of GMOs since 1976, for agricultural or health purposes. The development of gene-editing technology such as CRISPR-Cas9 is currently revolutionizing the field of genetic engineering since 2013. This technique involves an endonuclease that will cut the DNA strand in a selected location, and force the cell to repair the DNA strand by adding a modified version of the initial gene. This process simplified greatly the deletion, edition or modification of selected genes. Gene drives can be used to ensure the dissemination of the selected gene in the target species population. Since its discovery in 2013, this technique was successfully used to modify yeast, fruit flies or mosquitoes (See chapter 4). This report develops the use of CRISPR-Cas9 technology and gene drives to fight against disease vector mosquitoes (see chapter 5) and as a way to restore herbicide susceptibility in agricultural weeds which became resistant against herbicides or to suppress invasive alien plants (see chapter 6).

Bacterial endosymbionts living in association with most insect species can be considered as natural gene drives (Curtis and Sinkins 1998). Endosymbionts such as *Wolbachia pipientis* are vertically transmitted from the mother to the next generation. To ensure their propagation in their host population, these bacteria manipulate the reproductive strategy of their host, in a process called cytoplasmic incompatibility, by increasing the fitness of infected females compared to the fitness of uninfected ones. *Wolbachia* is also known to reduce the vectorial capacity of disease vectors or can simply be pathogenic for their host. Mosquitoes harbouring *Wolbachia* have a shorter lifespan than those not harbouring the endosymbiont. Moreover, *Wolbachia* can also directly block the virus transmission. Researchers stated to use these characteristics in *Wolbachia*-based control strategies. An interesting aspect of this technology is that it is self-perpetuating thanks to cytoplasmic incompatibility. This mechanism evolved to give a fitness advantage to females carrying *Wolbachia*, to increase its vertical transmission to following generations and shows striking common features with gene drives (see chapters 2 and 3) (Curtis and Sinkins 1998). Recent work showed that it is possible to select naturally occurring *Wolbachia* strains, grow it on cell lines and injected it to a new species. The vectorial capacity of *A. aegypti* was successfully diminished after introducing a self-perpetuating strain of *Wolbachia* through laboratory manipulations, in Australia (Ritchie 2014).

This report, commissioned by the Swiss Federal Ethics Committee on Non-Human Biotechnology (ECNH) will describe technologies implying the transfer of genetic material in the environment such as biological control, CRISPR-Cas9 and *Wolbachia* based technologies. It purposely focuses on technologies that are currently not used in Switzerland (the biological control agent to control chestnut gall wasp populations in the Canton Ticino and Vaud arrived *accidentally* in Switzerland) but that could be used

in the future. The focus is set on potential future technologies. Every technology described is used somewhere in the world, enabling us to discuss biosafety concerns and provide an analysis of the regulation procedures mobilized to evaluate potential environmental risks.

An interdisciplinary team of a biologist and a biologist-anthropologist wrote this report. Biotechnologies, gene drives, *Wolbachia* based biological control strategies and biological control can all have detrimental effects on the environment. Biologist can predict these risks and develop environmental risk assessments as well as plans to mitigate them. This report shows that social aspects seem to occupy a prominent place in the implementation or the social acceptance of biotechnology tools such as biological control or *Wolbachia* based control strategies. Attachment to cultural traditions triggered the clandestine introduction of a biological control agent in Switzerland and elsewhere and social perception was identified as the most risky aspects of the *Wolbachia*-based control strategy of *Aedes aegypti* in Australia.

The questions posed by the authors of this report will be analysed by the Swiss Federal Ethics Committee on Non-Human Biotechnology and hopefully lead to fruitful discussions to further improve the procedure or reglementary gaps highlighted in this work.

Chapter 1

Biological control and the risks of gene transfers in the environment: biological and anthropological perspectives

Alexandre Aebi & Nicola Schoenenberger

Keywords: biological control, environmental risk assessment, hybridisation, non-target effects, gene transfer, anthropology

1.1. Biological control

1.1.1. Technology

Biological control is the use of one or several natural enemies of a crop pest species to reduce the impact of the latter by controlling its population. A biological control agent can be a predator, a parasite capable to control a pest feeding on plants or an herbivore that can feed on an invasive plant. Insects, nematodes, bacteria, virus or fungi are commonly used as biological control agents. Stating that biological control is the *use* of natural enemies to resolve an agronomic problem implies that humans take an active role. However this role can differ greatly from one biological control program to the other.

There are four kinds of biological control programs. First, inundative biological control is the repetitive introduction of biological control agents in a greenhouse, year after year to achieve control of a given crop pest. In this case, the long-term establishment of the biological control agent inside or outside the greenhouse is not desired. Of course, natural enemies used in inundative biological control programs may escape the glasshouses and cause damages in the neighbouring environment. An example of inundative biological control program is the use of aphid parasitoids or coccinelids to control aphids in glasshouses (Van Lenteren and Woets 1988). In Switzerland 26 species of insects and 8 species of mites are homologated for an indoor use (Aebi and Zindel 2013).

Conservation biological control is the capacity to benefit from natural enemies, already present in the environment surrounding the crop. By promoting diversified field margins such as trees, edges, wild-flower strips, natural enemies will have a chance to survive in the agro-ecosystem and provide an ecosystem service in the form of crop-pest control (Fabian et al. 2013). Actively improving the habitat and food sources of native predators, parasites or herbivores, or improving the connectivity among ecological compensations areas (Fabian et al. 2013) will ensure impact of their natural fauna on the pests attacking neighbouring fields. In this case, there are no biological control agent introductions in the environment. Swiss farmers commonly plant wild flower strips as ecological compensation areas. Their implementation as unique structure or as a network of ecological compensation areas, gives right to direct payments by the Swiss federal Office for Agriculture (FOAG).

If a suitable native biological control agent is identified in the field, entomologists may mass-rear it in laboratory facilities and then released it into the environment in an augmentative biological control program. Thus, no new biological control agent is introduced into the environment. An example is the use of native parasitoids to control the Mexican bean beetles in storage conditions against which efficient natural enemies were identified in the field and may be selected for mass rearing (Aebi et al. 2008). The best example, in Switzerland, is the use if native ladybird species such as *Adalia bipunctata* Linnaeus (Coleoptera: Coccinelidae) that are mass reared in laboratories and sold to private gardeners or professionals for an outdoor use on aphid infested plants or crops. These biological control strategies can be seen as alternatives to the classical biological control strategy described hereafter.

Classical biological control is the use of an exotic natural enemy to combat an exotic pest. Suitable natural enemies are identified in the native geographical range of the pest and introduced into the environment. Contrary to all other types of biological control, long term establishment of the natural enemy in nature is intended in the case of classical biological control. An example of a successful classical biological control program is the use of the neotropical parasitoid *Apoanagyrus (Epidinocarsis) lopezi* De Santis (Hymenoptera: Encyrtidae) against the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Homoptera: Pseudococcidae) and the cassava green mite *Mononychellus tanajoa* Bondar (Acari: Tetranychidae) (Herren 1987). The introduction of *A. lopezi*, maintaining the pests under a bearable threshold for cassava growers, probably saved millions of lives in Africa. It is interesting to note that to date, no classical biological control agent was authorized in Switzerland.

1.1.2. The emergence of biological control as a field of research

The history of biological control, as a research field was a rhythm of hopes and fears. Introducing non-native organisms in the environment to achieve the control of a pest may indeed cause environmental problems. Even if biological control is seen as an alternative to pesticides or genetically modified organism by many researchers in agronomy and by practitioners, this technique was widely criticized as negative impacts of classical biological control were regularly reported in the last decades (Howarth 1983). The cane toad *Rhinella marina* Linnaeus (Anura: Bufonidae), originating from Central and South America that was introduced in Australia to control the cane beetle *Dermolepida albobirtum* Waterhouse (Coleoptera: Scarabaeidae) is a good example. Initially considered as a success as the cane beetle population declined, *R. marina* rapidly threatened populations of about 30% of terrestrial Australian snake species because of toxins this toad is accumulating to avoid predation. The snake species that did not adapt their behaviour to avoid the new toxic prey will attack the cane toad and die by poisoning (Phillips et al. 2003).

The use of classical biological control as a way to suppress pest species was hotly debated during the last decades. We will present here the milestones of this debate as presented by Barratt and colleagues in 2010 (Barratt et al. 2010). One hundred and eighteen years ago, Perkins alerted the scientific community on potential effects of biological control by publishing an article describing the negative impacts caused by introduced ladybirds in Hawaii, in 1890 (Perkins 1897). Biological control became popular after the publication of the book "Silent Spring" describing environment and human health dangers linked to pesticide use, by Rachel Carson, in 1963 (Carson 1963). This technique was then considered as "the magic bullet" to reduce the impact of crop pest species. Economically, it also became evident that biological control was more cost-effective than pesticides as biological control agents are self-perpetuating in the field and don't require repetitive costly introductions (Barratt et al. 2010).

Several authors advocated against the application of biological control if it is done without prior sound environmental risk assessment. In their eyes biological control agent must be considered guilty of adverse environmental effects until proven innocent, in what can be called a precaution principle. Obtaining data on the biosafety of a candidate biological control agent is however long and sometimes very difficult. As a

consequence, only a few studies address environmental risks associated with biological control. It was then argued that the lack of proven negative environment effects was due to a lack of study addressing the issue (Howarth 1983).

Several researchers opposed their views on biological control between 1988 and 2000. Lockwood expressed his doubts on the introduction of an exotic biological control agent to control a native pest without carrying host-range testing (Lockwood 1993) and was severely criticised on the base of an absence of evidence of non-target negative effects (Carruthers and Onsager 1993). From a conservation biology point of view, Samways questioned the compatibility between biological control and nature conservation policies, in 1988 (Samways 1988). On the same line of argument, Hopper (1995) discriminated environmental risk assessment for endangered and for non-endangered species, arguing that if biological control had to consider every species, its cost would very quickly become unaffordable. Finally, Thomas and Willis (1998) stressed the importance of considering the alternatives to biological control to suppress a pest species before and not to forget to include the consequences of not doing anything in the balance. These two points are still not routinely considered in environmental risk assessment, sometimes for logistic or economic reasons.

Today biological control is widely used around the world. "Classical biological control of insects, where exotic natural enemies are introduced to control exotic pests, has been applied for more than 120 years, and release of more than 2000 species of natural enemies has resulted in the permanent reduction of at least 165 pest species worldwide. Augmentative biological control, where exotic or native natural enemies are periodically released, has been used for 90 years, and more than 150 species of natural enemy are available on demand for the control of about 100 pest species" (Bigler et al. 2006).

1.1.3. Current environmental risk assessment procedures

As described by Gibbs and colleagues (2011), a full environmental risk assessment (ERA) relies on the identification and evaluation of potential risks associated with the natural enemy to be released and the development of a plan to minimize them. The final step before an hypothetical release is to identify, evaluate and weigh up all adverse and beneficial effects in a risk-cost benefit assessment (Bigler and Kölliker-Ott 2006). The factors used to perform an ERA are listed in Table 1.1. Although all factors listed are of crucial importance for a comprehensive risk evaluation, there is growing awareness of the difficulties in obtaining relevant information for every aspect. Exhaustive study of the biology of a potentially beneficial arthropod can be very long and costly. The aim of the stepwise risk assessment procedure (Figure 1.1, Table 1.2) is to advise the release (or not) of a candidate biological control agent at an early step of the procedure, thus preventing unnecessary further studies and costs (Biggs et al. 2011).

1.2. Hybridization threats to the environment

Increasing and widespread movement of organisms across biogeographic barriers shuffles the geographic range of numerous species, favouring the occurrence of hybrids between species. Species movements can be anthropogenic (biological control, global-

Dispersal of the BCA
Host-range of the BCA
Direct effects of the BCA on other organisms in the ecosystem
Attack of non-target herbivores
Intraguild predation
Omnivory
Enrichment
Vectoring of pathogens
Indirect effects of the BCA on other organisms in the ecosystem
Competition
Intraguild predation
Apparent competition
Hybridization

Refer to van Lenteren et al. (2003) for definitions or details

Table 1.1. Factors investigated in current environmental risk assessment schemes for biological control agents (BCA) (Gibbs et al. 2011).

trade, voluntary introduction of species in the environment) or natural (geographic range expansion due to global warming).

As a consequence, rates of hybridization and introgression are increasing dramatically worldwide (Allendorf et al. 2001). From an environmental point of view, it is believed that hybridisation led to the extinction of numerous species. From an evolutionary point of view, hybridization is considered as a driver of the evolution of several animal and plant taxa. As stated by Allendorf (2001), determining whether hybridisation is natural or anthropogenic is crucial for setting conservation guidelines. Hybridisation is controversial as it is hard to define, and hybrids are difficult to detect. Hybridisation is defined as the interbreeding of individuals from genetically distinct populations, regardless of the taxonomic status of the population (Allendorf et al. 2001). Hybridisation can occur between species (Yara et al. 2000), strains (Benvenuto et al. 2012) or populations and each case can affect the outcome of a biological control program.

Being able to detect and to characterize hybrids is of outmost importance for conservation biologists. The first challenge is to be able to recognize and detect hybrids. Before the development of molecular tools, observation of morphological traits enabled researchers to detect hybrids until the mid-1960s. Morphological analyses have limitations as it implies that hybrid individuals will have an intermediate phenotype, expressing characters from both parents. The fact that hybrids containing a majority of genes inherited from one parent renders them undistinguishable from their parental taxon. Also, morphology cannot distinguish between first generation hybrid (F1), backcrosses or a later generation hybrid. Protein electrophoresis enabling the detection and visualisation of

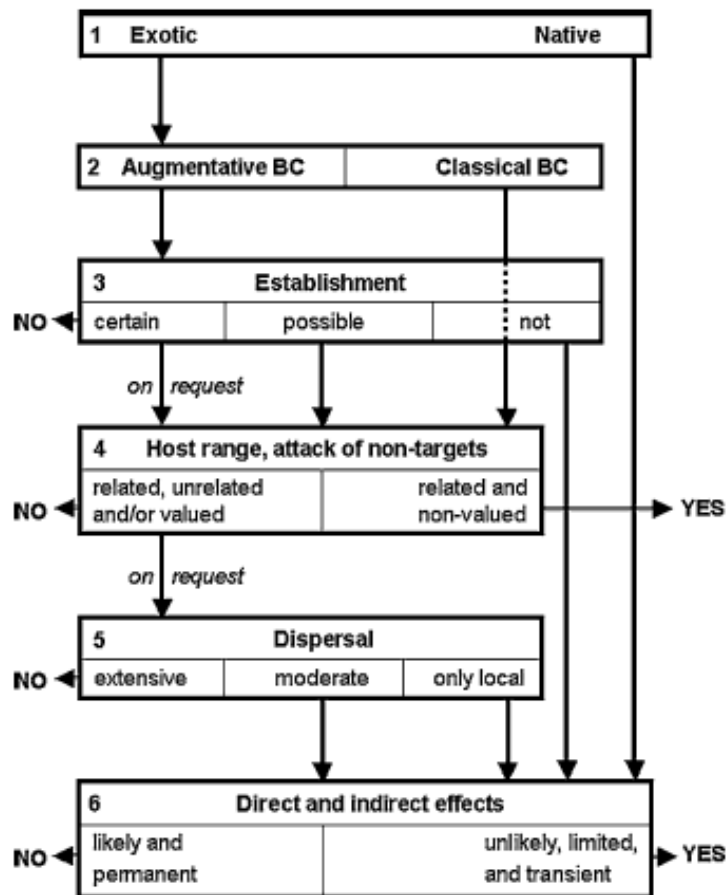


Figure 1.1. Simplified scheme of an environmental risk assessment of an invertebrate biological control agent (after van Lenteren and Loomans 2006).

Step	Question - answer	Action
1	Origin - native	GO TO 6
	Origin - exotic, either absent OR present in target area	GO TO 2
2	Augmentative biological control programme - establishment not intended	GO TO 3
	Classical biological control programme - establishment intended	GO TO 4
3	Establishment unlikely	GO TO 6
	Establishment possible to very likely, risk threshold not crossed	GO TO 4
	Establishment possible to very likely, risk threshold crossed	No release
4	If monophagous OR if oligophagous/polyphagous AND only related AND only related AND no-valued non targets attacked	Release
	If oligophagous/polyphagous AND related and unrelated non-target attacked AND/OR valued non targets attacked	No release
5	Dispersal local (L = 1-2)	GO TO 6
	Dispersal outside target area (L = 3 or more) AND extensive (M = 2 or more) Apply magnitude as weight factor	
	If risk threshold is not crossed (ERI = 5 or less)	GO TO 6
	If risk threshold is crossed (ERI = 6 or more)	No release
6	Direct and indirect effects inside dispersal area unlikely (L = 1-2) AND at most Transient and limited (M = 1-2)	Release
	Direct and indirect effects inside dispersal area likely (L = 3-5) OR permanent (M = 3-5)	No release

Table 1.2. Schedule for an environmental risk assessment for biological control agents (BCA).

allozymes in the mid 1960's (Toda et al. 2000 ; Yara et al. 2000) and the development of Polymerase Chain Reaction (PCR) enabled researchers to use several loci to screen small populations, reducing their impact on potentially endangered populations (Allendorf et al. 2001).

Allendorf and colleagues (2001) in their opinion paper based on fish conservation and fisheries described six types of hybrids. A precise description of each hybrid type could lead this team to discuss conservation policies in every situation. Their hybrid definition was adapted to the case of biological control. Depending on the outcome of undesired or desired crosses between two species or strains, several scenarios can be foreseen (see figure 1.2). After the introduction of a biological control agent in the field two scenarios can occur depending if only F1s are produced or if a hybrid population is produced. First, mating between two different species may result in the development of a sterile offspring and thus result in F1s only (Type 1). Here we have hybridization but without introgression (i.e. gene flow between populations whose individuals hybridize) and no widespread gene flow into the environment even if some amount of genetic mixing occurred and the production of very few hybrids after the first generation. The major detrimental effect of hybridisation, in this case is what can be seen as wasted reproductive effort to the extent that several authors consider type 1 hybridization as a threat to biological control program (Havill et al. 2012; Fisher et al. 2015; Goldson et al. 2003; Davies et al. 2009) as the introduced natural enemies will see its long term impact drastically diminish if mating with another species is leading to a sterile offspring (Allendorf et al. 2001).

In the second scenario, backcross (i.e. the cross of a F1 hybrid with individuals genetically identical to one of its parents) will lead to the creation of a hybrid population. In this case, the genetic integrity of native species is threatened. We can have limited introgression (Type 2), widespread introgression (Type 3) and complete admixture (Type 4) between populations, strains or species involved in a biological control program. Type 4 hybridization describes a situation where only a few pure populations remain, if any. In such situation, the hybrid has a fitness advantage over the parental species and no selection acts against the hybrids. Such a situation is of course detrimental for the environment. The loss of the genetic integrity of parental species is considered as a non-target effect (Allendorf et al. 2001). On the other hand, if hybrid BCA were selected and bred for the needs of a special biological control program (Benvenuto et al. 2012, see section 1.5.5. below), this situation would be regarded as favourable.

1.3. Biological control and the risk of transferring genes into the environment

The risk of interbreeding can be applied to voluntarily or involuntarily introduced species and depends on a variety of factors for which a likelihood of occurrence can be estimated. Hopper and colleagues proposed, in 2006, a methodology to evaluate these risks before the introduction of a biological control agent (pre-introduction) or in the stage when the population is augmented prior to release in the field (pre-augmentation).

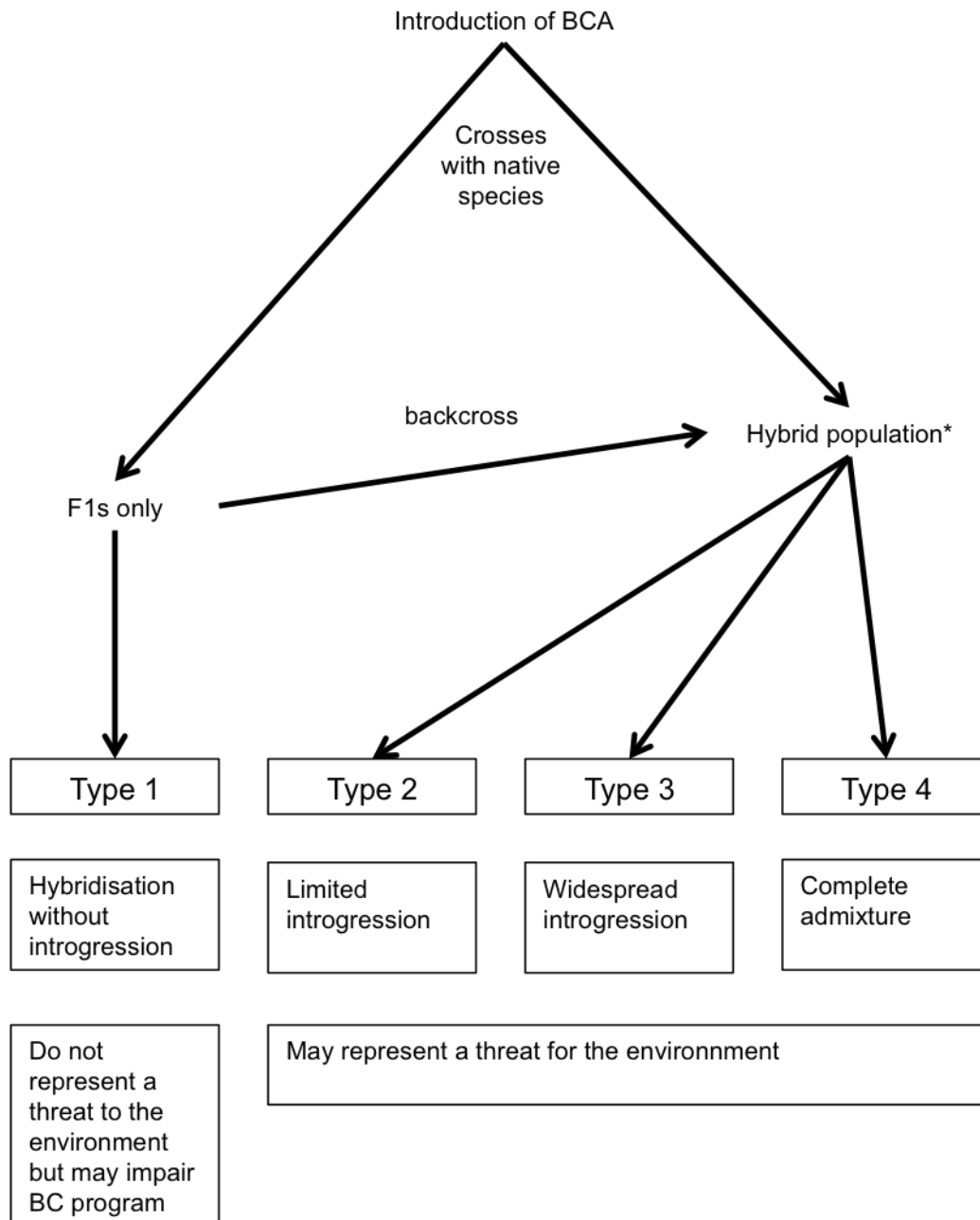


Figure 1.2. Foreseen hybridization types and consequences in biological control programs. BCA = Biological Control Agent, BC = biological control.

* Hybrid swarms may be wanted in special cases, when an artificial hybrid is released in the field (Benvenuto et al. 2012).

Several aspects must be considered. The first point highlighted by Hopper and his team is the taxonomic issue of species complex. Closely related, sometimes cryptic species can be present and cause identification mistakes of the introduced species or the native species already present in the environment. The most likely scenario is when native species are found to be at risk from interbreeding with sibling species or closely related species augmented for biological control. The release of *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) was indeed authorized in Japan because the native lacewing *Chrysoperla nipponiensis* Okamoto was initially wrongly identified as *C. carnea* (Henry et al. 1993, 2001). Thankfully, different courtship songs, displayed by the two species maintained their genetic integrity (Naka et al. 2005) (see the section 1.5.3. below for

more details). The case of *Diadegma* Föster parasitoids released against the diamond back moth in Japan is another example highlighting the need for a sound taxonomy of potential introduced and native species complexes before the initiation of a biological control program (Davies 2009)(see the section 1.5.1., below for more details).

The second point raised by Hopper and colleagues is an analysis of the geographical distribution of hybrids and its parental species. The hybrid zone model is particularly relevant for introduced biological control agents as it is bound to change and evolve during the introduction and establishment phases of the biological control program (Barton and Hewitt 1981). The best-known example, for biological control is that of *Laricobius* Rosenhauer (Coleoptera: Derodontidae) species controlling Adelgids on hemlock in the United States, where the introduced *Laricobius nigrinus* Fender and the native *Laricobius rubidus* Leconte have distinct geographical ranges overlapping in a hybrid zone (Havill et al. 2012) (see the section 1.5.4., below for more details).

1.4. Factors favouring hybridization

Factors such as phylogenetic relatedness, geographical and habitat overlap or mate recognition will define the probability of hybridization between an introduced biological control agent and native species. Phylogenetic relatedness will affect the probability of interbreeding between two species. Phylogenetically close species will be more likely to interbreed than phylogenetically distant species. Molecular tools can be used to reconstruct the phylogeny of introduced and native species. In practice biological practitioners only have taxonomic keys based on morphological traits to identify the species that may interact in the field. As a rule of thumb, hybridization is most likely to occur within a genus or a species complex. As a matter of fact all the known cases documented in this chapter document hybridization between congeneric species. The choice of species to be tested when evaluating hybridization risks should follow a centrifugal approach, identifying species at risk in the same genus, and then in the same family in a risk assessment procedure (Hopper et al. 2006).

Bio-geographical barriers such as oceans, deserts or mountain ranges can define the geographical distribution of introduced and native species potentially at risk of interbreeding. Climatic conditions will also determine the geographical range of any introduced species. As stated by Hopper, “the risk of interbreeding depends on its distribution, the climatic tolerance of the introduced species and its ability to disperse across habitats and geographic barriers” (Hopper et al. 2006).

For hybridization to occur, spatial and temporal barriers to mating must be overcome. The phenology of both species but also their host-plant preference may interplay with the probability of interbreeding between them. For example hybridization occurred between the introduced *Torymus sinensis* Kamijo and the early-spring strain of *Torymus beneficus* Yasumatsu (Hymenoptera: Torymidae), while the late spring strain seem to be untouched (Yara 2014). Moreover, hybridization between these species was probably possible because *T. beneficus*, originally developing on oak gall wasps (Hymenoptera: Cynipidae) in Japan, was recruited by the chestnut gall wasp once it invaded Japan (Aebi et al. 2006) and started to exploit the same host plant as *T. sinensis* (see the section 1.6., below for more details).

Mate recognition is of course crucial for a successful mating between species whose ecological niche and phenology overlap. Depending on the taxonomic group, mate recognition involves secondary metabolites, courtship songs, dances but also body shapes and colours. Investigating mate recognition can be arduous as the outcome of pairs being brought together may vary from laboratory to field conditions (Hopper et al. 2006; Naka et al. 2005).

Copulation and sperm use vary a lot from one species to the other. Even if two species recognize themselves as potential mates, copulation may not be successful because of morphological differences. The outcome of interspecific mating may be death of one or both partners as a consequence of genitalia remaining blocked together. Finally, if sperm is successfully transferred, it may not necessarily fertilize heterospecific eggs. Sperm competitiveness or female reproductive behaviour (multiple mating for example) may explain reduced fertilization (Hopper et al. 2006).

1.5. Hybridization and biological control

The environmental outcome of hybrid progeny, occurring accidentally in biological control programs is poorly documented (see below for a precise description of the four known examples to date). One of the impacts could be a host-range shift. If the hybrid host-range differed from that of the introduced species unexpected non-target species might be affected by the offspring of the hybrids (even if they are sterile) (Hopper et al. 2006). Hybrid speciation and the creation of a new species might be the outcome of interspecific hybridization. In the case of biological control, if such speciation event occurred, it would be like if two species were introduced in the environment. The introduced species would display known traits while the hybrid species would display a combination of traits inherited from both parental species (Hopper et al. 2006).

Introgression (i.e. the transmission of DNA sequence from fertile hybrids back to parental species) may occur if hybrids are produced in the framework of a biological control program. Introgression might be rare. For example, if a hybrid's fitness is low and if backcrosses are rare, then the fate of introgression will depend on the genetic distance between the hybrid and the concerned parental species. In a closely related species, introgressed sequences will probably not dramatically change the sequence of a gene or its function. From a biological control point of view, beneficial arthropods are selected for traits that are absent in the target environment (otherwise, they would not have been selected). Therefore, according to Hopper, introgression might have dramatic effects (Hopper et al. 2006). If hybrids and backcrosses are common, neutral and even mildly deleterious genes could become common in the area of contact between the introduced and the native species and alter the adaptation of both species to their environment. Introgressed sequences that are strongly deleterious, either through direct effects on traits fitness components or through break up of co-adapted genes complexes, would be strongly selected against and thus unlikely to persist or spread. Impact of introgression is tightly linked with demographic processes. As stated by Hopper "a rare native, swamped by backcrosses with hybrids from a species introduced in large numbers, could have a high level of introgression" (Hopper et al. 2006).

Introgressed sequences appeared from rare hybrids and backcrosses will only persist and spread in the population if they confer their carrier with a selective advantage. Outcome of introgression could be tremendous if it affects traits such as climatic tolerances as they could allow the range expansion of introduced species. Hopper argued that such catastrophic outcome seems unlikely by chance given the similarity between genomes of species involved in biological control programs (Hopper et al. 2006).

More worrying for biological control, introgression of sequences affecting host-range may be problematic as introgression might alter the initial host-range of a biological control agent. As a result non-target species may be attacked and further host-range assessment would then be required to guarantee a safe biological control program for the environment. To date, none of these catastrophic scenarios were documented in actual biological control programs where hybrid specimens were actually found. Even if the information is sometimes scarce, we reviewed every known example, where a hybrid population arose after crosses between an introduced biological control agent and native species.

1.5.1. Hymenopteran parasitoids against the diamond back moth

Parasitoids of the genus *Diadegma* Förster (Hymenoptera : Ichneumonidae) are often used to control pest lepidopteran species, worldwide. This very large genus comprises more than two hundred species. *Diadegma* is used to control the very noxious diamond back moth, *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) causing billion dollar worth crop losses in the cabbage industry. *Diadegma semiclausus* Hellen (Hymenoptera: Ichneumonidae), a Palearctic larval parasitoid was successfully used to suppress diamond back moth populations in Southeast Asian agro-ecosystems. This successful record and the fact that the native parasitoid *Diadegma fenestrale* Holmgren was considered as an inefficient natural enemy of the diamondback moth motivated researcher to release this species in Japan. A study published by Davies and colleagues was motivated by the complicated taxonomy of the genus *Diadegma* with the presence of several morphospecies with varying efficacy as biological control agents (Davies et al. 2009). Hybridization and introgression to the F1 generation occurs with one way crosses between female *D. semiclausum* and *D. fenestrale* in the laboratory, proving that hybridization is indeed possible between these two species, even if evidence for hybridization in the field is lacking between these species. From a biological control point of view, hybridization between *D. semiclausum* and *D. fenestrale* may alter the efficiency of the latter to control the diamondback moth. It is interesting to note that Japanese researchers were able to improve the biological ability of *D. fenestrale* by crosses with commercial strains. In this case hybrid vigour led to an improved sex-ratio (Takashino et al. 2005). On the other hand, Davies and colleagues highlight hybrid depression as a potential outcome of interspecific hybridization (Davies et al. 2005).

1.5.2. Parasitoids against the clover root weevils

The clover root weevil is endemic to Europe and widely distributed on this continent. The genus *Sitona* Germar (Coleoptera: Curculionidae) comprises 100 species worldwide.

Sitona lepidus Gyllenhal is a species preferring white clover, distributed throughout Europe. Larvae feed on clover root nodules and roots, causing devastating damages to pastures. Goldson and colleague fear severe pasture nitrogen stress and a general loss of vigour (Goldson et al. 2003) in New Zealand. In New Zealand, another species of root weevil, *Sitona discoideus* Gyllenhal was successfully controlled by a Moroccan parasitoid *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae). Hopes that this species could control the new pest species were soon deceived. However, a screen of potential parasitoids to control *S. lepidus* revealed that a European strain of the same parasitoid was probably the most effective biological control agent. But introducing a European strain of *M. aethiopoides* was then perceived as a threat to the on-going biological control program. « Should a European strain of *M. arthiopoides* be introduced into New Zealand in an attempt to control *S. lepidus*, there could be the danger that it would cross with the Moroccan strain already present and lose efficacy through strain hybridization » (Goldson et al. 2003). This experience highlighted the importance of thorough analysis, to the strain level, of candidate biological control agent, and analysis of the context in which the selected natural enemy will be released before selecting a natural enemy for a biological control program (Goldson et al. 2003). Such analyses involve costs. The aim here is to ensure public money is well invested to reach efficacy of the biological control program. Lab crosses showed a significant reduction of impact on parasitoid efficacy to control clover root weevil.

1.5.3. Lacewings against aphids, mites and caterpillars.

Naka and colleagues documented a potential case of hybridization between an introduced BCA and a native species between 2005 and 2006. The green lacewing *Chrysoperla carnea* Stephens (Neuropteran : Chrysopidae) is a commonly used BCA against aphids, mites, and early stages of caterpillars. Until recently this species with a broad Holarctic distribution was considered as a single morphological species until the discovery that many species, characterized by different courtship songs formed what can be named a species complex (Henry et al. 1993, 2001). In Japan, the native *chrysoperla* species was considered to belong to the species *C. carnea* until its revision and its categorization as *Chrysoperla nipponiensis* Okamoto on the base of differing morphological traits. The green lacewing was registered as a phytosanitary product in Japan in 1996. Since then researchers feared genetic crosses between the native *C. nipponiensis* and the introduced *C. carnea*, leading to the loss of the genetic integrity of the former. Laboratory tests were performed to evaluate the risk of hybridization between both species. Fertility, pre-oviposition period, viability, development time and sex ratio of the offspring were measured. These tests showed that if *C. nipponiensis* and *C. carnea* are brought together in a petri dish, they will recognize themselves as mates, mate and produce a viable and fertile offspring. However, the fertility of the produced offspring was lower than that of parental crosses. The hybridization rate of parental cross in the introduced green lacewing was 90 per cent. No hybrid breakdown was reported. Even if the prezygotic barrier between species seems to be broken in this case, it is interesting to note that the courtship songs displayed by these lacewings is an important barrier to interspecific hybridization. The courtship songs of *C. nipponiensis* and *C. carnea* are indeed quite different, reducing the chance of mating between these species in natural conditions (Naka et al. 2005)

Cryptic species in arthropod taxonomy is a recurrent problem in applied entomology. The *Chrysoperla* example in Japan highlights the importance of a sound taxonomy and an accurate monitoring of native species distribution and on the identity and origin of every beneficial arthropod shipment in a new geographic region.

The examples concerning *D. semiclausum*, *M. aethiopoides* and *Chrysoperla carnea* highlight the difficulties to obtain sufficient information to predict the outcome of hybridization events on the environment and on the outcome of a biological control program, as information such as the extent of interbreeding and the relative fitness of hybrid versus parental lineages, in the ecological context are needed (Havill et al. 2012). Probably the best documented case of hybridization between a native and an introduced biological control agent is that of *Laricobius* spp., against Adelgids in eastern United States. (Havill et al. 2012). This example is the fourth example of hybridization involving biological control agents.

1.5.4. Predatory beetles against hemlock bug

The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) is a major pest of eastern hemlock, *Tsuga canadensis* L. (Carrière) and Carolina hemlock, *T. caroliniana* Engelman, in eastern United States. Originating from Japan, it was introduced in this region of the United States in the early 1950s. Western North American populations of *A. tsugae* are controlled by a guild of predators that controls the infestation level below a bearable threshold. Western North American populations of *A. tsugae* were studied by researchers in their search for a potential biological control agent (Havill et al. 2012). The predatory beetle *Laricobius nigrinus* Fender (Coleoptera : Derodontidae) was selected as an appropriate biological control agent and released in the United States, where *A. tsugae* is causing environmental problems. After being established, it successfully reduced the population of the adelgid pest, even if hemlock forests health could not yet be documented. *Laricobius* are considered as specialists feeding only on adelgids. Oviposition tests and life table analysis showed that *L. nigrinus* is specialized on *A. tsugae*. *Laricobius rubidus* is an endemic species in eastern North America, associated to *Pineus strobe* on *Pinus strobus* even if it was already recorded on other non-native adelgids: *A. tsugae* on *T. canadensis*, *Adelges picea* on *Abies* spp. and *Pineus pini* on *Pinus sylvestris* (Havill et al. 2012). Attack of several adelgid species, sympatrically living on a set of host-trees is the first condition for interspecific hybridisation (Hopper et al. 2006). Another criteria proposed by Hopper and colleagues are the low genetic distance between species that might hybridize. The genetic diversity within and between *L. nigrinus* and *L. rubidus* was evaluated with a mitochondrial marker. The results showed that these species were in fact recently diverged sister species (Havill et al. 2012). Specimens were collected in the native and introduced geographical ranges of *L. nigrinus* and *L. rubidus* on their entire host-plant range. The results of the genetic analysis showed that hybridization did occur between the two species, even if the hybridization seems to be at an early stage. As stated by Halvill “there is strong evidence for hybrid fertility and advanced generation hybrids, which show that hybridization is broadly occurring. However, the persistence of individuals identified as pure species and asymmetrical Introgression suggest that complete admixture may not occur” (Havill et al. 2012). From a biological point of view, the relative fitness of parental species and the hybrid contributed to predict the impact of

this hybridisation event on the ability of the biological control agent to control adelgid populations. Halvill and colleagues proposed two scenarios. In the first one, a negative effect could occur if the hybrids started to feed less on the target pests. The second scenario refers to wasted reproductive efforts if interbreeding led to unfertile offspring. The outcome of hybridization between *L. nigrinus* and *L. rubidus* is strongly influenced by the ecology of the two parasitoid species and that of their host. Host-plant preference is well documented for herbivore species and parasitoids are known to rely on chemical cues emitted by a plant under herbivory by their herbivore hosts (Turlings et al. 1990). The ecological link among plants, herbivores and parasitoids may enable or disable hybrid formation by defining clear ecological niches of tri-trophic partners. On the other hand, hybridisation might alter the parasitoid host-range (Havill et al. 2012). From a community ecology point of view, potential outcomes of hybridization which might all negatively affect the outcome of a biological control program between these species are: genetic assimilation or displacement of *L. rubidus* or *L. nigrinus*, reinforcement of premating isolation resulting in decreased hybridization and permanent separation of both species, creation of a third species or maintenance of a stable hybrid zone (Fischer et al. 2015). After conducting field and laboratory analyses, Fischer and colleagues concluded that the risk of genetic assimilation was low because of variation in populations of both species across sites, local selection pressure (occurrence and abundance of the host plants). Moreover, both species appear to have different habitat preferences and pure populations of one of the species, *L. nigrinus* occurs outside of the hybrid zone. As mentioned above, introgression could alter the host-preference of hybridizing species.

1.5.5. Using strain hybridization to improve biological control agents?

Other authors suggested a potential use of strain hybridization for biological control. Benvenuto and colleagues state that « the underlying rationale for the positive effect of hybridization relies on the fact that hybrids may exhibit, at the individual level, heterosis and/or, at the population level, high genetic variance for relevant phenotypic traits ». Heterosis and such genetic variance may enhance an hybrid's capacity to adapt to varying environmental conditions, leading to improved adaptation in comparison to parental lineages (Benvenuto et al. 2012). Of course, genetic improvement of commercially available BCA cannot be generalized, even if genetic improvement of BCAs has been tried for several groups of arthropods such as insects, mites or nematodes (Benvenuto et al. 2012). Benvenuto and colleagues propose that genetic improvement approaches could be used on broadly used biological control agents in inoculative or inundative biological control (Benvenuto et al. 2012). Furthermore, their experience highlighted the fact that hybridizations can be obtained by mere chance, or by labour intensive crossing experiments. Is such effort worth it? Benvenuto and colleagues propose that a more ambitious goal of such experiments would be to define predictors of hybrids' quality, such as phenotypic distance between parents or the level of heterosis. They argue that the outcome of hybridization cannot be evaluated by a simple measurement of the fitness of a viable offspring because allopatric populations might become reproductively incompatible through premating isolation, post-mating prezygotic mechanisms or post-mating postzygotic mechanisms. These authors stress that the task of genetic improvement of BCA can be challenging. Nevertheless, if researcher desire to pursue in this direction, Benvenuto and colleagues encourage

researchers to try to test relevant hypotheses to better understand the outcome of reproductive isolation and hybrid's performance (Benvenuto et al. 2012).

1.6. A Swiss example.

1.6.1. The chestnut gall wasp

The chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) is a Chinese gall-inducer inflicting severe losses to chestnut growers all around the world to the point that it is considered as the most severe pest species of chestnut trees (Aebi et al. 2006). Parthenogenetic females induce the formation of the gall by ovipositing an egg in a bud. Galls, forming on leaves or twigs will protect and feed the developing next generation. The hard wall protects the larvae against parasitism by hymenopteran parasitoids and specialized vegetal tissues on the wall of the gall chamber will provide food to the developing larvae.

Dryocosmus kuriphilus is an invasive species present on three continents. Originating from China, it arrived in Japan in 1940 (Oho and Umeya 1975) and in Korea in 1958 (reviewed in Aebi et al. 2006). It inflicted considerable losses in American chestnut orchards after arriving in Georgia in 1974 (Cooper and Rieske 2007). In 1999 it was found in Nepal (Abe et al. 2007). The chestnut gall wasp arrived in Europe in 2002. It was discovered in Italy, in chestnut orchards located in Piedmont (Brussino et al. 2002). Europe's colonisation accelerated as it was detected, in France and Corsica in 2005 (Aebi et al. 2006), in Switzerland in 2009 (Föster et al. 2009), In Hungary in 2009 (Csoka et al. 2009), in Slovenia in 2005, in Croatia in 2010 (Matosevic et al. 2010) (reviewed in Aebi et al. 2006). The invasive success of *D. kuriphilus* can be explained by its parthenogenetic reproduction where one single un-mated female can found a new population. The relative minute size of this wasp also explains its propensity to be carried by wind or accidentally carried by human transportation (Aebi et al. 2011). Global trade can explain intercontinental movements. Indeed, the arrival of the chestnut gall wasp in Piedmont could be traced back to a shipment of chestnut material for grafting, from China. A single minute egg, concealed in a bud is then sufficient for the wasp to colonize a new biogeographic region of the world.

Dryocosmus kuriphilus is considered as a quarantine species, a status implying an obligation to take measures to eradicate the invasive species in any new territory (EFSA 2010). Measures such as the imposition of a closed quarantine zone, forbidding any movement of chestnut material out of the perimeter, or a surveillance scheme to follow its progressions were enforced. Of course, the task is tremendous and almost impossible. An example from Slovenia illustrates the difficulty of the task. In 2005, Slovenian authorities discovered galls of the chestnut gall wasp on their territory. Custom data enabled authorities to trace the origin of the contaminated shipment and allowed phytosanitary offices to launch an eradication program. After identifying almost all orchards that acquired plant material from this shipment, every potentially infected tree was destroyed. Despite these efforts, the chestnut gall wasp established itself in Slovenia in 2005 (Seljac G. personal communication).

Once established, the chestnut gall wasp is very difficult to eradicate. Insecticides are made obsolete by the fact that the vegetal walls of the galls protect the developing stages and by the fact that their use is forbidden in forests to protect the surrounding environment (Swiss Federal Council 2010a,b). Pruning and burning infested branches may slow down the propagation of the gall wasp in an orchard but this technique is not adapted to forest that can literally be covered by galls. The breeding of chestnut varieties resistant to the chestnut gall wasp was tried for twenty years until the emergence of a *D. kuriphilus* variety able to attack these resistant varieties. Native parasitoids were rapidly recruited by the chestnut gall wasp. This jargon means that native species, normally attacking oak gall wasps very soon recognized the chestnut gall wasp as a potential host and started exploiting this resource. A total of 15 species belonging to six families were consistently recorded on chestnut gall wasp all along its invasion route (Aebi et al. 2006). Very soon, researchers thought that these parasitoids could control the chestnut gall wasp, but this was without considering major difficulties or knowledge gaps about that biological system. First, it is impossible to rear these univoltine parasitoid wasps in a laboratory as their hosts (oak gall wasps) follow a complicated life cycle, alternating between a sexual generation on Turkish oaks and an asexual generation on English oaks. A program relying on native parasitoids would then have to rely on wild-caught specimens. It is worth noting that this is exactly what is done for *T. sinensis*, but the difference relies in the abundance of chestnut and oak galls. While chestnut galls literally cover chestnut trees, oak galls are very rare. It would then be impossible to find enough oak gall parasitoids to launch such program. Taxonomical knowledge on this group of parasitoid represents another challenge. The biology of these minute wasps is poorly known. Parasitoid can be primary parasitoid or facultative hyperparasitoid depending if they are specialized in attacking the gall inducer or other parasitoid that already parasitized a gall. Moreover, from a genetic point of view, current molecular techniques (sequencing) highlighted the presence of cryptic species in families commonly found in galls such as Eurytomidae, Torymidae or Eulophidae (Nicholls et al. 2010).

Classical biological control is then probably the best way to keep the population of *D. kuriphilus* under a bearable threshold for chestnut producers (Aebi et al. 2007). Japanese researchers were the first to study the community of parasitoid naturally attacking the chestnut in its native geographical range. A delegation of the Ministry of Agriculture and Forestry was sent to China in 1975. A total of 69 galls were collected in His-an, Shensi were brought back to Japan. The rearing of these galls revealed a complex community of parasitoids. Eight species belonging to six families emerged from these samples. All the species discovered by the biologists were polyphagous and known to attack oak gall wasps, except one: *Torymus sinensis*. This parasitoid was then selected as the only specialist parasitoid of the list. Between 1979 and 1981, 260 mated female *T. sinensis* were released at the Fruit Tree Research station in Ibaraki Prefecture (Otake et al. 1984). In 1990, Moriya published a paper showing that a stable population of *T. sinensis* established itself in Japan despite mortality inflicted by facultative hyperparasitoids. To date, this study is the only documented evidence that *T. sinensis* is effectively controlling the population of *D. kuriphilus* under its damage threshold (30% of buds infected). In the United States and in Europe, the efficiency of *T. sinensis* was only documented on the base of field observations.

1.6.2. Biological control of the chestnut gall wasp in Japan.

Torymus sinensis was introduced in Japan for the control of the chestnut gall wasp *D. kuriphilus* from 1982 to 1999 (Yara 2014). Interspecific competition with a native parasitoid *Torymus beneficus* was feared by researchers working on the biological control of *D. kuriphilus* (Moriya et al. 1992), as their morphological and ecological characteristics were very similar (Yara 2014). Moriya showed that F1 between *T. sinensis* and *T. beneficus* could be produced in the laboratory and that the female offspring was fertile and proved that mating between *T. sinensis* and *T. beneficus* was possible. Moriya and his team discovered individuals considered to be hybrids, on the base of the length of the ovipositor sheath in the field in 1992 (Moriya et al. 1992). Molecular analysis using malic enzyme electrophoresis failed to demonstrate that morphologically intermediate individuals are hybrid between *T. sinensis* and *T. beneficus* (Yara et al. 2000). This result was confirmed by mt-DNA analysis (Yara 2004). Another factor is complicating the task of researchers trying to document the outcome of this biological control program in Japan. Indeed, the native *Torymus beneficus* species is actually consisting of two strains differing in their phenology, the early spring strain and the late spring strain. The presence of another parasitoid, *Torymus koreanus* Kamijo, endemic to Korea further complicates the story, as this species is considered as the other parasitoid effectively controlling *D. kuriphilus* in Japan. Field investigations based on morphological identification of Japanese parasitoids suggested the displacement of *T. beneficus* by *T. sinensis* and hybridization between these species (Moriya et al. 1992). To date, these concerns are only partially documented as discrimination among *T. sinensis* and *T. beneficus* (early strain, late strain) are still posing problems (Yara and Kunimi 2009). In a paper published in 2012, Yara and colleagues concluded that “early-spring and late-spring strains of *T. beneficus* had been rapidly displaced by the introduced *T. sinensis* and that hybridization between the two species played a role in the displacement if the late-spring strain but not in the displacement of the early-spring strain” (Yara et al. 2000, 2007). The population of *T. sinensis*, *T. beneficus* early spring and late spring strains and *T. koreanus* were investigated between 1992 and 1996. In the absence of *T. sinensis*, in 1992, the early strain of *T. beneficus* was the dominant species even if the late strain of *T. beneficus* and *T. koreanus* were present in low numbers. In 1994, *T. sinensis*, *T. beneficus* (both strains) and *T. koreanus* were present. F1 hybrids between *T. beneficus* (both strains) and *T. sinensis* were detected. The early strain of *T. beneficus* still predominated. Contrastingly, all the *Torymus* individuals collected in 1996 were identified as *T. sinensis* or as F1 hybrids between *T. sinensis* and the late strain of *T. beneficus*. In contrast, *T. koreanus* population did not vary after the arrival of *T. sinensis*. (Yara et al. 2012).

The precise mechanisms that lead to the displacement of *T. beneficus* by *T. sinensis* are still unknown (Yara et al. 2012). Hybridization between *T. beneficus* and *T. sinensis* leading to hybrid vigour is an explanation. The high reproductive potential of *T. sinensis* (*T. sinensis* female can oviposit more eggs than *T. beneficus*, Piao and Moriya 1992) can be another explanation. Finally the longer ovipositor of *T. sinensis* may confer its female a better ability to exploit large galls, compared to *T. beneficus* (Yara et al. 2012). It is interesting to note that 448 *Torymus* larvae were analysed and that hybrid between *T. sinensis* and early-spring *T. beneficus* were discovered in a large proportion of the samples, more than in the adult stages that were analysed. This result suggests that

hybridization between both species occurred but that offspring survival is somehow impaired through possible unknown post-zygotic mechanisms.

Torymus sinensis was never legally authorized as a biological control agent. In other terms, it was never released in a country conducting environmental risk assessment prior to beginning a biological control program. The releases done in Japan and in the United States were done prior the environmental concerns described above. In Italy, there is no procedure in place to regulate new biological control agents and Italian researchers and chestnut producers could mobilize economic arguments to justify the release of *T. sinensis* against the chestnut gall wasp, inflicting severe damages to the chestnut industry in Piedmont as Italy is among the biggest worldwide exporter of chestnut. In France, *T. sinensis* was released just before the enforcement of a regulation to protect the environment. *Torymus sinensis* was put on a list of organism used for biological control not necessitating an environmental risk assessment.

1.6.3. Socio-anthropology of environmental problems: how the fear of losing traditions favoured the geographical expansion of *T. sinensis*?

As suggested by Dewey (2010) problematizing an issue (such as an environmental problem) cannot be done without considering its publics. The definition of a problematic situation and the description of the communities involved are obligate aspects to consider when one want to understand a problematic situation with unanswered questions (Cefaï and Terzi 2012, Cefaï 1996, Lascoumes 2012). Anthropological enquiry involving semi-directed interviews and participatory observations (Beaud and Weber 2010) were applied since 2012, by an interdisciplinary research team, at the University of Neuchâtel to investigate the environmental concerns raised by the chestnut gall wasp and its natural enemy, *T. sinensis*. A team bringing together biologists and anthropologists investigated what seemed (at the beginning) a very straight forward and simple biological problem: « The chestnut gall wasp is causing damages to chestnut orchards in Ticino and in Chablais, Italian researcher found a solution by introducing the biological control agent *T. sinensis*. Swiss chestnut growers, aware of the Italian biological control program want to introduce *T. sinensis* in Switzerland ». Semi-directed interviews with chestnut growers in Chablais, with regulators from the Swiss Federal Office for the Environment (FOEN), with cantonal forestry and phytosanitary services officers in Ticino and in Chablais revealed the complexity of this environmental problem.

According to Lascoumes and Le Galès (2012): “The construction of a public problem can be defined as a process in which a group of private and public actors interact in order to impose their representations and interpretations of an issue, and to guide its management and the actions to be taken.” In other words, building a public problem means naming and framing it, assigning blame or responsibility and finally claiming its place on the political agenda (Felstiner et al. 1980). For chestnut growers, the problem as initially described was a reduced chestnut yield. For the phytosanitary service in Ticino, the problem was the threat of landslide posed by declining chestnut tree populations. For the phytosanitary service in Ticino, the problem was to find a way to reassure the population, concerned by the vision of declining chestnut trees. For biologists, *T. sinensis* might hybridize with native *Torymus* species and may attack non-target oak galls (Aebi et al. 2011). Interestingly the same environmental issues as the

biologists animated regulators, but their position was complicated by their official position. Indeed, our data revealed that they were concerned by the fact that the *T. sinensis* was never homologated elsewhere in the world and that its potential first ever homologation in Switzerland may pose responsibility questions. Moreover, our data showed that the FOEN officials standing between chestnut growers vindictively pushing for a solution and cantonal services confronted to the population, without a clear communication strategy is another aspect of the problem that need to be taken into consideration.

Ticino was the first Swiss canton to be affected by the chestnut gall wasp. After the discovery of *D. kuriphilus* galls in 2009 by Foster (Foster et al. 2009), the phytosanitary service contacted Italian researchers from the Regione Piemonte and the University of Turin, to seek advices in the best way to handle the situation. Italian researchers provided guidance to the Ticino cantonal administrations (Phytosanitary and forest services) to submit an application to the federal authorities to obtain a release permit for *T. sinensis*. The application went through a complicated administrative process to clarify under which Swiss law the case could be regulated. The Federal office for agriculture being in charge of the “Ordonnance sur la protection des végétaux » (Swiss Federal Council 2010), and the « Ordonnance sur les produits phytosanitaires » (Swiss Federal Council 2010) and the Federal office for the environment respectively being responsible for the « Ordonnance sur la dissémination dans l’environnement » (Swiss Federal Council 2008) that just went through a revision process at that time (in 2012). Chestnut trees proved to be juridical complicated objects as they can be considered as an orchard tree and then as a part of agriculture, but also as a forest species and then as part of the environment. After deliberations, it was decided that the introduction of *T. sinensis* was going to be regulated under the “Ordonnance sur la dissémination dans l’environnement” as *T. sinensis* is an exotic species. It is important to understand that both federal offices collaborate in the application of phytosanitary measures to prevent the introduction and spread of particularly harmful pests and diseases that affect plants and plant products. The Federal Plant Protection Service (FPPS) run jointly by the Federal Office for Agriculture (FOAG) and the Federal Office for the Environment (FOEN) is responsible for the implementation of this task. In practice, the environmental risk assessment scheme used by the FOAG was applied to *T. sinensis*. Based on environmental risk criteria, the application was rejected at the check of completeness stage, as hybridisation and host-range risks were not treated in the application despite being documented in the scientific literature on biological control (reviewed in Yara 2014).

The rejection of two homologation permits exacerbated the feeling that federal authorities consider the environmental problem of the canton Ticino, and the cantonal-federal opposition was brought in the public space by the media. Following the thoughts of Felstiner et al. (1980) chestnut producers and cantonal services might have failed to blame the state for their environmental problem and our data showed that frustration and fear to lose their tradition became the dominant sentiment of chestnut producers. Mobilizing the press who wrote numerous articles on the lengthy administrative procedures of Federal offices had two consequences. First, in the eyes of Becker, the problem becomes public (Becker 1985) and second, the emotions of the public, concerned by the sick sight of chestnut trees, could be used by chestnut producers and cantonal services fighting for an introduction of *T. sinensis*. Emotions play an enormous

role in the emergence of environmental problems as shown by the example of Quéré in his paper on the influence of emotions on the management of green tides in Britany, France (Quéré 2012).

It is in this context that we followed a chestnut producer in his combat to obtain releases of *T. sinensis* in the Chablais. The chestnut producer we followed is a moral entrepreneur in Becker's view. Retired, passionate about chestnuts, he made the cause his and decided to mobilize the actors around him to obtain releases of *T. sinensis* in his region (Becker 1963). By following him, we identified a new category of actors involved in such environmental cases: retired amateur naturalists. This sociological group is constituted of autodidact entomologists, people passionate about nature or simply chestnut or chestnut tree lovers. Conducting semi-directed interviews with them and following them in their daily activities (through participatory observation) highlighted their attachment to chestnut trees and more generally to the traditions around chestnuts (Dupré 2002). We showed that the emotions driven by their passion motivates their actions (Quéré 2012). By following them, we understood that this attachment to such values was the ecological bond bringing together chestnut trees, producers and the chestnut gall wasp. Only their attachment to the trees can motivate them to take care of their trees in adverse situations as the chestnut gall wasp, inflicting severe losses (up to 90%) during 5-8 years to chestnut trees may discourage chestnut tree owners to take care of their orchards. Sadly, we think that by not pruning dead branches, demotivated tree owners might favour outbreak of the chestnut canker, *Cryphonectria parasitica* Murill (Diaporthales: Cryphonectriaceae) (Rigling et al. 2014), an ascomycete fungus with the potential of inflicting severe losses to chestnut trees.

The European union has no harmonized environmental risk assessment procedure for the homologation of biological control agents (Bigler et al. 2005). Despite a very successful European project (<http://www.rebeca-net.de/?p=999>, consulted the 13.1.16), leading to straightforward guidelines, individual countries handle the homologation of biological control agents in very diverse ways. In some countries like Switzerland or France (see figure 1.3), a sound environmental risk assessment is regulating the introduction of exotic beneficial insects. In other countries, such as Germany, a similar procedure is being adopted thanks to the work of an expert panel from EPPO and IOBC. But in other countries, such as Italy, such regulation is inexistent.

The chestnut gall wasp and its parasitoid *T. sinensis* is a classroom example highlighting the difficulties inherent to this international complicated situation. Indeed, under the pressure of chestnut producers, Italian researchers could introduce *T. sinensis* without prior complete environmental risk assessment. In this situation, economic arguments were brought forward to justify these releases. On the contrary, in Switzerland, where chestnut does not represent a major source of income, biosafety arguments were mobilized to justify the non-homologation of the biological control agent. France recently decided to enforce a regulatory system. During the transition period, organisms already released, such as *T. sinensis* were authorized for releases without environmental risk assessment. Countries with very distinct legislative tools to handle classical biological control agents surround Switzerland. This situation is hardly bearable as the decision process of a country might be affected by the decisions taken in neighbouring countries and because arthropods' geographical range does not coincide with political frontiers.

Our interdisciplinary study enabled us to document how the attachment to these trees also motivated cantonal services in Ticino and our moral entrepreneur to obtain *T. sinensis* releases in Italy and in France, right next to the Swiss border, in total legality. In Ticino, our enquiry showed how scientists from the phytosanitary office were aware of *T. sinensis* releases at a few kilometres of the Swiss border by Italian researchers. More interestingly, data obtained from semi-directed interviews and participatory observations showed how a Swiss chestnut producer contacted the French city council of Lugrain and how the latter was convinced to ask a *T. sinensis* release in Franche-Comté, a region sharing bio-geographical conditions with the Swiss Chablais to the French “Institut National de Recherche Agronomique”, in charge of the biological control program using *T. sinensis*.

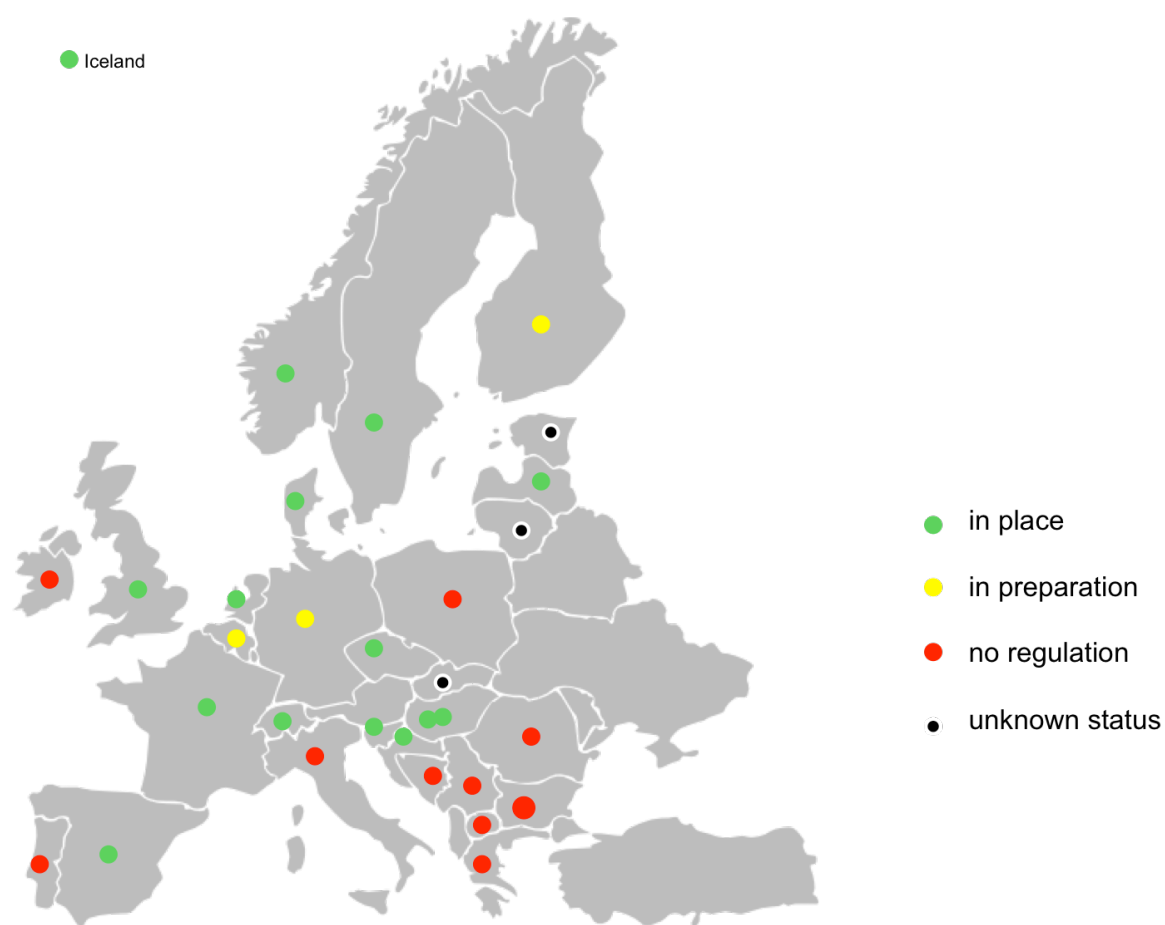


Figure 3. Regulation procedure for biological control agents in Europe (modified from Bigler et al. 2005)

The chestnut gall wasp arrived in France, in the Alpes-Maritimes department, where it caused tremendous damages to the chestnut industry, to the point that a trade association of chestnut producers (Syndicat National de la châtaigne) was constituted. Pressed by a vivid demand from the trade association, researchers decided to introduce *T. sinensis* in France. As *T. sinensis*, released in the whole Italian peninsula was going to arrive in France anyway, scientists from the INRA designed a clever experiment aiming to document the establishment and spread of the biological control agent in relation to the number of release points and the number of mated females released in each site.

Scientists from the INRA and chestnut producers from the trade association worked together to make sure the experimental design corresponded to the expectations of chestnut producers (who wanted a rapid diminution of the chestnut gall wasp population) and the researchers (desiring to learn something from this experience through a sound and scientific experimental design). The release of *T. sinensis* in France is then a scientific experiment aiming at understanding the factors favouring the establishment and spread of a biological control agent in the environment.

Very soon, chestnut producers perceived the experimental design proposed by the researchers as not ambitious enough. Obtaining scientific data is surely important but rapidly reducing the chestnut gall wasp population is outmost important. After a couple of years, researchers found important populations of *T. sinensis* in locations where no official releases were ever made. Moreover, the population of parasitoids observed in these sites exceeded all the other populations, ruling out natural propagation of the insect (Borrowiec N. personal communication). These were the first European observations of clandestine releases of *T. sinensis*. Unexplained occurrences of *T. sinensis* are probably scattered all along the invasion route of *D. kuriphilus*. The first observation of *T. sinensis* in a place where it was never released is the Oki Islands, in the Japan Sea, at 40 kilometres from Honshu Island and 150 km from the Korean Peninsula. The dispersal capacity of 12 km in 6 years as measured by Moriya could not explain this discovery, apart from the fact that an extremely large body of water makes a natural dispersion impossible. Moreover, the observation that *T. sinensis* was dominating the chestnut gall wasp parasitoid community on the Oki Islands and the parasitism rate equalling that expected two years after a release further argues for human-mediated introduction of this parasitoid in this region. As stated by Toda and colleagues “ if only a few individuals of *T. sinensis* had invaded from elsewhere, it would have been difficult to increase as they have in Tsukuba. Therefore, *T. sinensis* must have been present in the Oki Islands for a long time before being released in Tottori Prefecture” (Toda et al. 2000). Furthermore, the fact that researchers could observe the growth of the *T. sinensis* is in our eyes another sign that *T. sinensis* was indeed released there and that its presence isn't due to a lack of precise knowledge about its geographical range but possibly the result of clandestine introduction.

On the Swiss border, INRA researchers, after accepting to consider the Chablais as a release site for *T. sinensis* (to complement their experimental design, suffering from clandestine introduction in France) found out that *T. sinensis* was already present and that its population was actually suggesting that clandestine releases were done a couple of years earlier (N. Borrowiec personal communication).

A citizen science project, led by the University of Neuchâtel, in collaboration with the chestnut growers showed, that *T. sinensis* had crossed the border and that it was well established in Switzerland, all along the Rhone Valley, in the cantons of Wallis and Vaud, in 2015. Similarly an on-going research project, financed by the Federal Office for the Environment showed that *T. sinensis* was also present in the whole Ticino canton. Very recently, *T. sinensis* was discovered North of the Alps in Switzerland, in the canton Zug (M. d'Alessandro, FOEN, personal communication).

This example shows that in certain circumstances, it is crucial to take into account social sciences to understand the motivations of practitioners to obtain a solution to their

problem. Here, it is the fear to lose the cultural traditions linked to chestnut production and to lose the special landscapes associated to this emblematic tree that triggered the mobilisation of chestnut producers. Anthropology doesn't aim to judge if the activities of actors are legal or illegal or good or bad. Anthropologists just wish to understand why and how actors make sense of their reality. In this case, regulators form the federal office for the environment applied the law (Swiss Federal Council 2008, 2010a, 2010b) and mobilised biosafety arguments to support their decision not to provide a release permit for *T. sinensis*. Scientists, have good reasons to believe that an hybridisation risk does occur among the introduced *T. sinensis* and native *Torymus* species (Yara 2014; Aebi et al. 2011). Cantonal services, in close contact with local populations played their role of intermediary between federal authorities and the populations.

Thus, the introduction of genes in the environment cannot be analysed with the unique glasses of biology. In the case of the chestnut gall wasp, the socio-economic value of chestnut and their importance in the eyes of chestnut producers triggered the introduction of *T. sinensis* in Switzerland. This result calls for interdisciplinary research even if we are not arguing that a complete biology anthropology analysis should be performed for every biological control program as such study would be too expensive but this example suggests, that, when a crop or a tree is heavily loaded in traditions, socio-economic factors cannot be ignored.

1.7. Regulatory gaps

The use of biological control agents is regulated by three following federal Swiss laws. There are, in our opinion, no regulatory gaps as the ordinance enable regulator to evaluate an homologation application in a way that protects the environment. However, the environmental risk assessment procedure might be further improved (see the section 1.8. below).

Swiss Federal Council (2008) Ordinance on the Handling of Organisms in the Environment (Release Ordinance, RO). Of 10 September 2008 (Status as of 1 June 2012) SR 814.911.

Swiss Federal Council (2010a) Ordonnance sur la mise en circulation des produits phytosanitaires (Ordonnance sur les produits phytosanitaires, OPPh) du 12 mai 2010 (Status as of 17 November 2015). SR 916.161

Swiss Federal Council (2010b) Ordonnance sur la protection des végétaux (OPV) du 27 octobre 2010 (Status as of 1 January 2015). SR 916.20

1.8. Questions raised

Costs versus biosecurity.

A baseline challenge concerning the environmental risk assessment of potential biological control agent is to find the balance between “answering every question biologist have on biosecurity” and “not imposing tremendous research and development costs to biological control agent retailers”. Biological control firms on the base of literature and experimental assays make environmental risk assessments. Some firms collaborate with research institutes such as Universities or research stations to obtain

biological data when missing in the literature. As stated by Hopper (1995) “if biological control had to consider every species, its cost would very quickly become unaffordable”. Another question raised by the scientific community working on the environmental risk assessment of biological control agent is the necessity to impose an homologation to the strain and not to the species level.

1. Can we impose unlimited research and development costs to biological control firms?
2. Could unaffordable research costs limit the development of biological control as a tool to reduce the impact of pest or invasive species?
3. Could unaffordable research costs favour the use of other techniques such as genetically modified organisms (see chapter 3) or pesticides to reduce the impact of pest or invasive species?

Consequences of not doing anything against a pest species

Another baseline challenge is to systematically consider the alternatives to biological control to suppress a pest species before and to include the consequences of not doing anything in the environmental risk assessment. These two points are still not routinely considered in environmental risk assessment, sometimes for logistic and financial reasons.

4. Can we impose unlimited research and development costs to biological control firms?
5. Can we impose the delays linked to such research to practitioners facing a problem due to a pest species?

Need for a sound taxonomy

A sound and precise taxonomy is the base of a successful biological control program. Beneficial arthropods can be closely related or be constituted of sometime cryptic species assemblages. Identification mistakes of the introduced species or the native species already present in the environment are therefore common.

6. What would be the outcomes of identification mistakes?
7. Can we create an identification system for every potential beneficial species (molecular barcode for example)?
8. Who would finance such tool?

Impact of hybridization

One of the impacts of hybridization could be a host-range shift. If the hybrid host-range differed from that of the introduced species un-expected non-target species might be affected by the offspring of the hybrids (even if they are sterile) (Hopper et al. 2006). Hybrid speciation and the creation of a new species might be the outcome of interspecific hybridization. In the case of biological control, if such speciation event occurred, it would be like if two species were introduced in the environment. The introduced species would display known traits while the hybrid species would display a combination of traits inherited from both parental species. (Hopper et al. 2006). More worrying for biological control, introgression of sequences affecting host-range may be problematic as introgression might alter the initial host-range of a biological control agent. As a result non-target species may be attacked and further host-range assessment would then be required to guarantee an environmental safe biological control program. Introgressed sequences appeared from rare hybrids and backcrosses will only persist

and spread in the population if they confer their carrier with a selective advantage. Outcome of introgression could be tremendous if it affects traits such as climatic tolerances as they could allow the range expansion of introduced species. Hopper argued that such catastrophic outcome seems unlikely by chance given the similarity between genomes of species involved in biological control programs (Hopper et al. 2006).

9. Currently full environmental risk assessment and hybridization risk assessment procedures (Hopper et al. 2006) do not consider the evaluation of an hybrid that might appear in the environment. How can we consider it?

10. The hybrid might hybridize with another native species. When do we stop?

11. Who is responsible to perform the environmental risk assessment of the hybrid?

12. Are the current laws addressing this point? The “Ordonnance sur la dissemination dans l’environnement” is dealing with organisms introduced in the environment, not with hybrid naturally forming after the introduction of a beneficial arthropod.

13. How can we be sure that catastrophic outcome are unlikely by chance given the similarity between genomes of species involved in biological control programs (Hopper et al. 2006)?

Need for an interdisciplinary approach?

The *T. sinensis* example illustrates the fact that the introduction of genes in the environment cannot be analysed with the unique glasses of biology. In the case of the chestnut gall wasp, the socio-economic value of chestnut and their importance in the eyes of chestnut producers triggered the introduction of *T. sinensis* in Switzerland. This result calls for interdisciplinary research. We are not arguing that a complete biology anthropology analysis should be performed for every biological control program, but this example suggests, that, when a crop or a tree is heavily loaded in traditions, socio-economic factors cannot be ignored.

14. When should we perform a social science study to evaluate the risk of human-mediated introduction of biological control agents?

Chapter 2

Could Wolbachia-based control strategies be used against the tiger mosquito *Aedes albopictus* in Switzerland?

Alexandre Aebi & Nicola Schoenenberger

Keywords: biological control, endosymbiont, environmental risk assessment, *Wolbachia*, cytoplasmic incompatibility, *Aedes albopictus*.

2.1. The tiger mosquito *Aedes albopictus*

The tiger mosquito *Aedes albopictus* Skuse (Diptera: Culicidae) is listed among the worst 100 invasive species in Europe (<http://www.europe-aliens.org/default.do>, consulted on the 14.1.16) because of its environmental impact (displacement of native mosquitoes) and its capacity to transmit viruses to humans. The tiger mosquito is a vector of 26 arboviruses including chikungunya and dengue (Neteler et al. 2013; Medlock et al. 2012). This species originating from Southeast Asia is considered to be the most invasive mosquito species in the world as it spread in the last decades in North, Central and South America, parts of Africa, Northern Australia and in several European countries. The tiger mosquito is now reported in 20 European countries including Albania, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, France (including Corsica), Germany, Greece, Italy (including Sardinia and Sicily), Malta, Monaco, Montenegro, the Netherlands, San Marino, Serbia, Spain Switzerland, Turkey and the Vatican City (Medlock et al. 2012) (Figure 2.1).

Current and future climate explain the actual and predicted geographical range of *A. albopictus* (Tatem et al. 2006). Permanent populations of the tiger mosquito are for the moment confined to the Canton Ticino in southern Switzerland where it was detected for the first time in 2003 (Wymann et al. 2008). Predictions based on actual and future climatic data show that this species will most likely invade the northern part of the country (Neteler et al. 2013, Petrini et al. 2012). Suitable areas for adult survival and overwintering of diapausing eggs will be available under future climatic regimes and Neteler and colleagues identified the following regions as suitable for *A. albopictus* in Northern Switzerland: Rhine Valley, Lake Constance and the lake of Neuchâtel (Neteler et al. 2013) (Figure 2.2-2.4). Trade of used tires and lucky bamboo are the most common invasion pathway for *A. albopictus* (Medlock et al. 2012, Neteler et al. 2013) but accidental transport by passenger cars cannot be ruled out. A monitoring of the tiger mosquito along Swiss highways is running since 2011, to be able to detect new populations and take appropriate measures such as pesticide application on the surrounding vegetation and water bodies if new population were to be discovered in Northern Switzerland (Elias et al. 2015, Office fédéral de l'environnement 2011). Sentinel traps were positioned along Swiss highways and revealed the presence of eggs from *A. albopictus* in the Northern part of Switzerland (Müller et al. 2013). The long-term establishment of reproducing *A. albopictus* populations in Northern Switzerland is in our opinion certain in the coming years.

Invasion of disease vectors can have rapid and important health impacts. Swiss health authorities are concerned and fear chikungunya and dengue outbreaks in Switzerland (Wymann et al. 2008; Elias et al. 2015). According to Elias "every expert consulted, agree that there is a high risk of dengue and chikungunya epidemics in Switzerland in coming years" (Elias et al. 2015). These authors call for the elaboration of a national plan of action with a clear strategy to reduce the probability of dengue and chikungunya epidemics in the country.

Dengue and chikungunya outbreaks occurred in the invasive range of *A. albopictus*. Cases of autochthonous dengue fever were recorded in France (Gould et al. 2010; La Ruche et al. 2010), in La Réunion (France) (Delatte et al. 2008) and in Croatia (Gjenero-Margan et al. 2010). Cases of autochthonous chikungunya fever were recorded in southern France (Delisle et al. 2014; Grandadam et al. 2011) in La Réunion (France) (Delatte et al. 2008) and in Italy

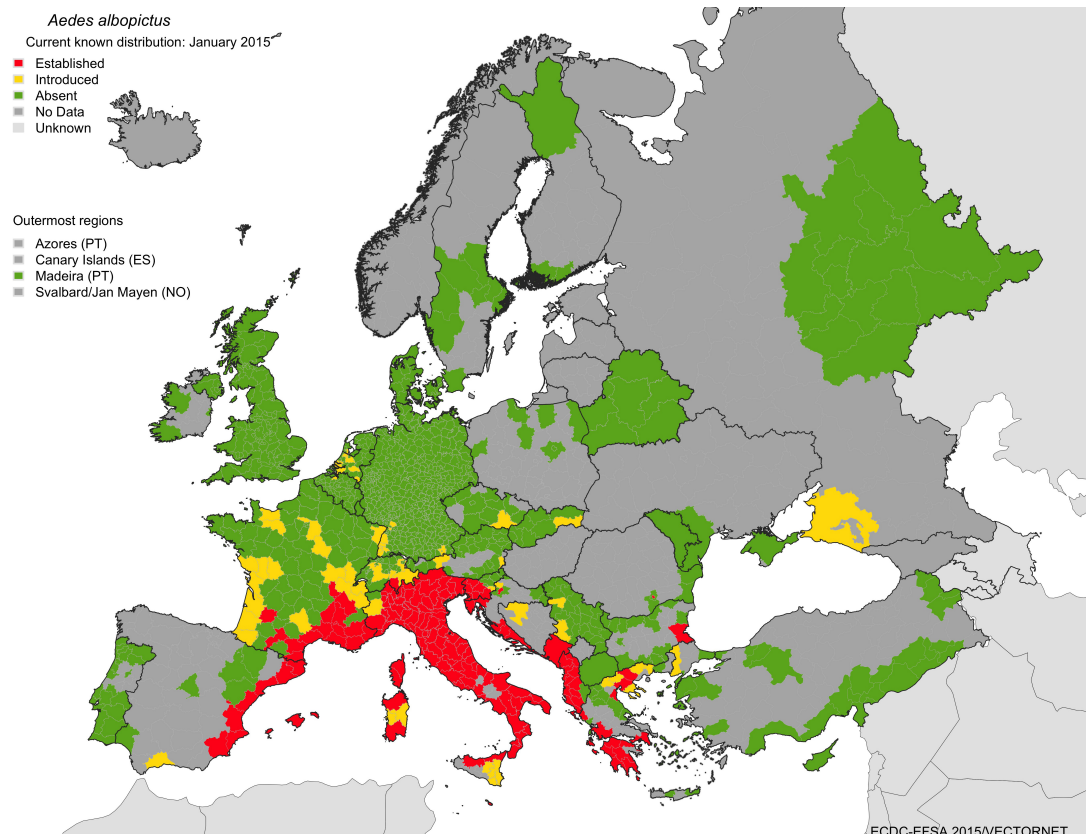


Figure 2.1 : Geographical distribution of the invasive mosquito *Aedes albopictus* (GLZ 2014)

(Angelini et al. 2007a,b). The Italian example proved a very high vectorial capacity of *A. albopictus* and highlighted the conditions in which an outbreak might occur “Overall, the epidemic in Italy can be said to be the result of the combined effect of the globalisation of vectors and humans, which occurred through a two-step process: i) the introduction and adaptation of the vector *A. albopictus* to a new environment (i.e., a temperate climate); and ii) the introduction of CHIKV in a previously infection-free country, with totally susceptible subjects, as the result of population movement (i.e., travelling human hosts, acting as a sort of Trojan Horse) » (Angelini et al. 2007b).

Record breaking warm winter like the one we are experiencing at the moment (2015-2016) and climate change will surely favour chikungunya and dengue outbreaks in Switzerland in a near future, enhancing the need for an action plan to fight against the invasive mosquito *A. albopictus*. Here we propose to evaluate the technology based on *Wolbachia* developed by Australian researchers to control the dengue vector *A. aegypti* and limit its dengue vector capacity to control *A. albopictus* and reduce its dengue and chikungunya vectoring capacity.

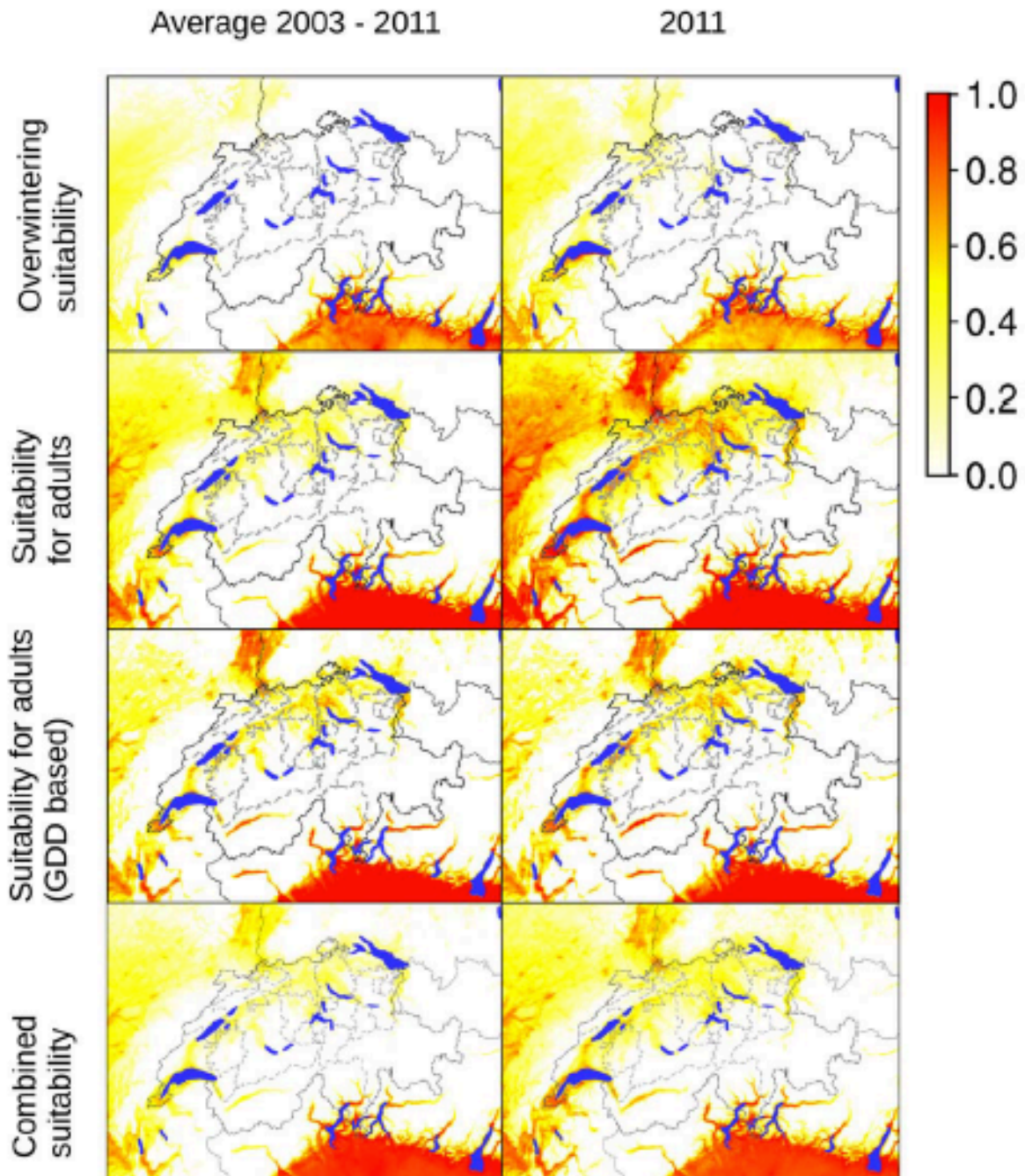


Figure 2.2. : Suitability indicators for *A. albopictus* in Switzerland (Neteler et al. 2013)

Combined suitability for *Ae. albopictus* in 2035,
climate change scenario:

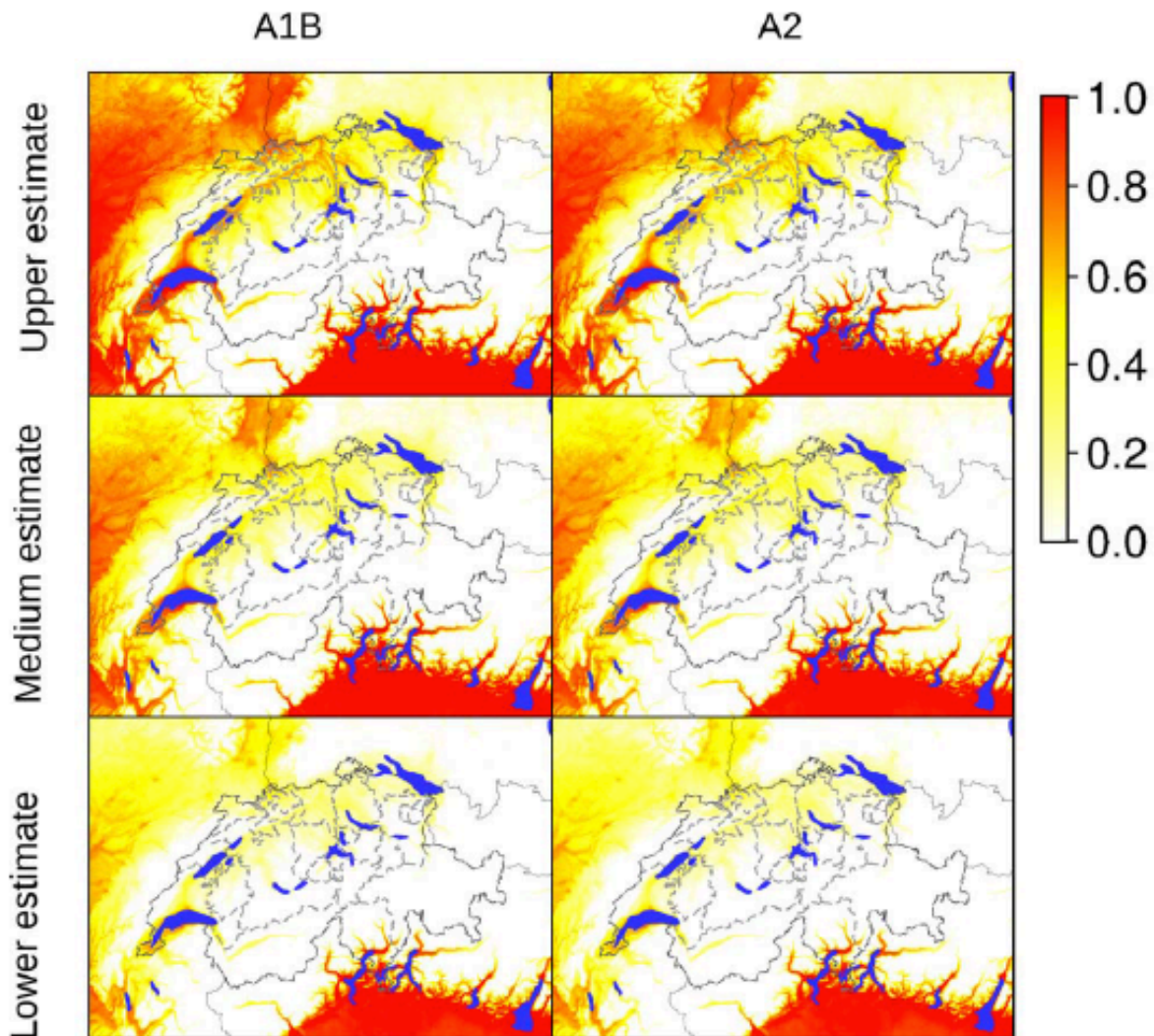


Figure 2.3. : Combined Suitability indicators for *A. albopictus* in Switzerland in 2035 (2020-2048) (Neteler et al. 2013)

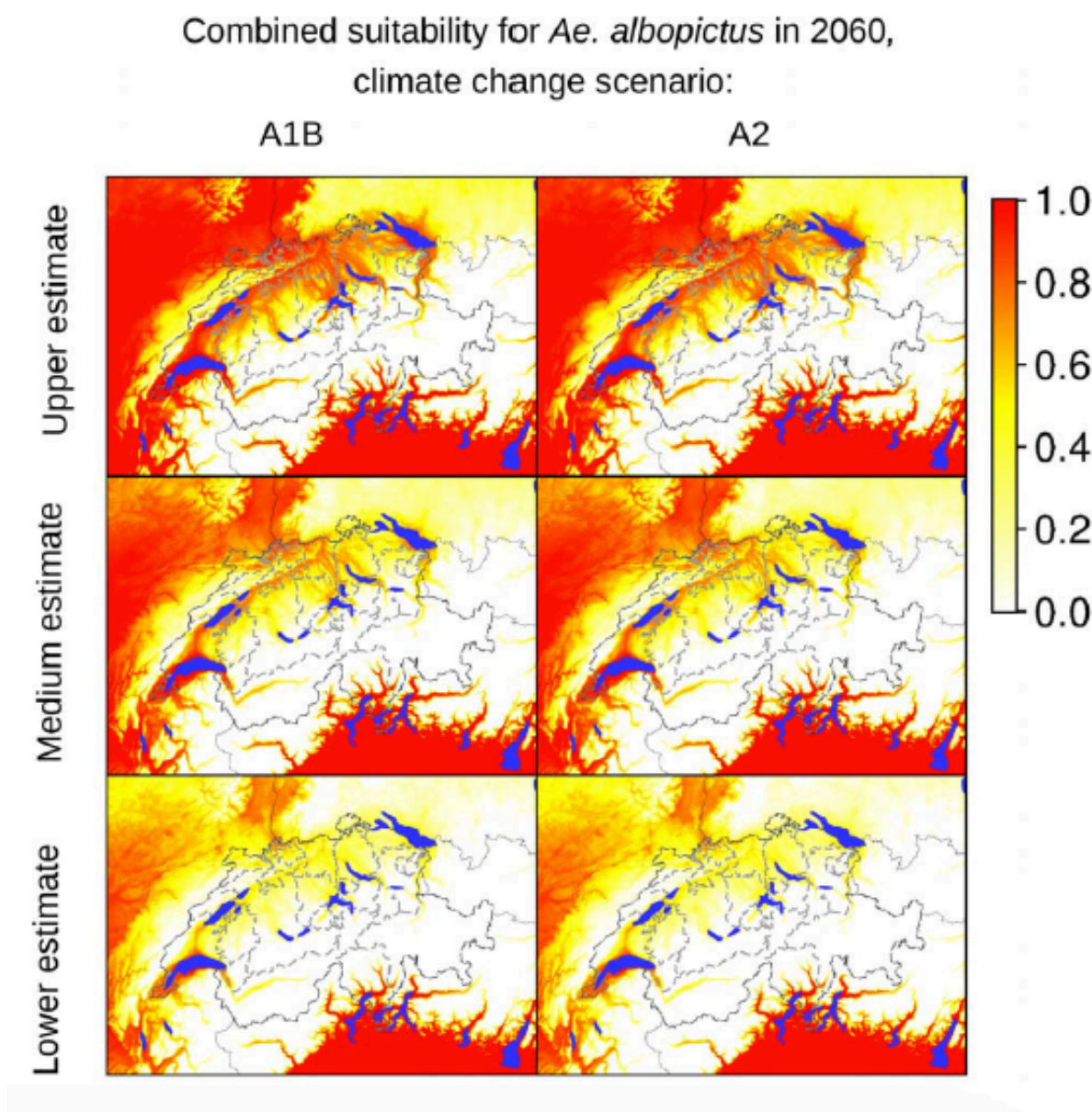


Figure 2.4. : Combined Suitability indicators for *A. albopictus* in Switzerland in 2060 (2045-2074) (Neteler et al. 2013)

2.2. Wolbachia

Arthropods are widely used in augmentative or classical biological control (Bigler et al. 2006), and most insect or mite species are hosts to one or several endosymbiotic bacteria (Zchori-Fein and Perlman 2004; Weinert et al. 2007; Duron et al. 2008; Hilgenboecker et al. 2008; Aebi and Zindel 2013). The two most studied maternally inherited bacterial endosymbionts (ESs) are *Wolbachia pipientis* Hertig (Rickettsiales: Rickettsiaceae) hereafter referred to as *Wolbachia* and *Candidatus Cardinium hertigii* (Bacteroidales; Bacteroidaceae); infecting 66% and 6%–7% of insect species, respectively (Hurst and Jiggins 2000; Kittayapong et al. 2003; Zchori-Fein and Perlman 2004). As stated by Zindel and colleagues « Given that arthropods

represent a large part of our planet's biomass, maternally inherited endosymbionts are probably the most common bacteria living in association with living organisms on Earth » (Zindell et al. 2011).

Endosymbiotic bacteria can be divided into obligatory (primary) and facultative (secondary) symbionts (Zindell et al. 2011). Obligatory symbionts are crucial for the functioning of vital functions of their host (Baumann 2005) and their hosts would not survive without them. One of the most studied examples is *Buchnera aphidicola* Munson, Baumann & Kinsey (Enterobacteriales: Enterobacteriaceae), the primary symbiont of the pea aphid *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae), providing its host with essential amino acids (Douglas 1998). Facultative endosymbionts, on the other hand, are not essential for the survival of their host. Moreover, their presence can be neutral, beneficial or detrimental to their host (Oliver et al. 2003; Perotti et al. 2006). Facultative endosymbionts can be involved in their hosts' feeding (Gunduz and Douglas 2009; Hosokawa et al. 2010), reproductive (O'Neill et al. 1997; Werren et al. 2008) or defence strategies (Haine 2007; Brownlie and Johnson 2009).

Endosymbiotic bacteria have to guarantee their maintenance in a host population to improve their own fitness. Many facultative endosymbionts are maternally inherited. As vertical transmission rates (from mother to offspring) are usually less than 100%, they would eventually be lost from the population in the absence of some measure of horizontal transmission (Lipsitch et al. 1995; Lively et al. 2005). To ensure its persistence in a host population, an endosymbiont can reduce the fitness of non-infected female hosts by manipulating their reproductive strategies. Such manipulations include cytoplasmic incompatibility (CI) between infected males and uninfected (or between individuals infected by different strains) females, selective male killing in broods, feminization of genetic males or parthenogenesis induction (O'Neill et al. 1997).

2.2.1. Cytoplasmic incompatibility and its use in biological control

Cytoplasmic incompatibility is probably the reproductive manipulation by *Wolbachia* that has the most potential to control disease vectors or pest species. It relies on mechanisms that can be best understood by the citation of Stephen Dobson «the simple rule of cytoplasmic incompatibility (CI) is... if the male has an infection that is not present in his mate, it's an incompatible cross » (<http://dobsonserv.ca.uky.edu> consulted on the 8.1.16). The figure 2.5 explains in a visual way the outcome of crosses between *Wolbachia* infected and non-infected female and male mosquitoes. In uni-directional cytoplasmic incompatibility, uninfected parents will produce a normal offspring. A female infected with a CI-inducing cytoplasmic incompatibility mated with an uninfected male will produce an infected offspring. Two infected parents will produce an infected offspring but an uninfected female mated with an infected male will produce no offspring because of developmental arrest. The precise mechanism of cytoplasmic incompatibility is poorly understood and a key research question of the scientific community working on *Wolbachia*.

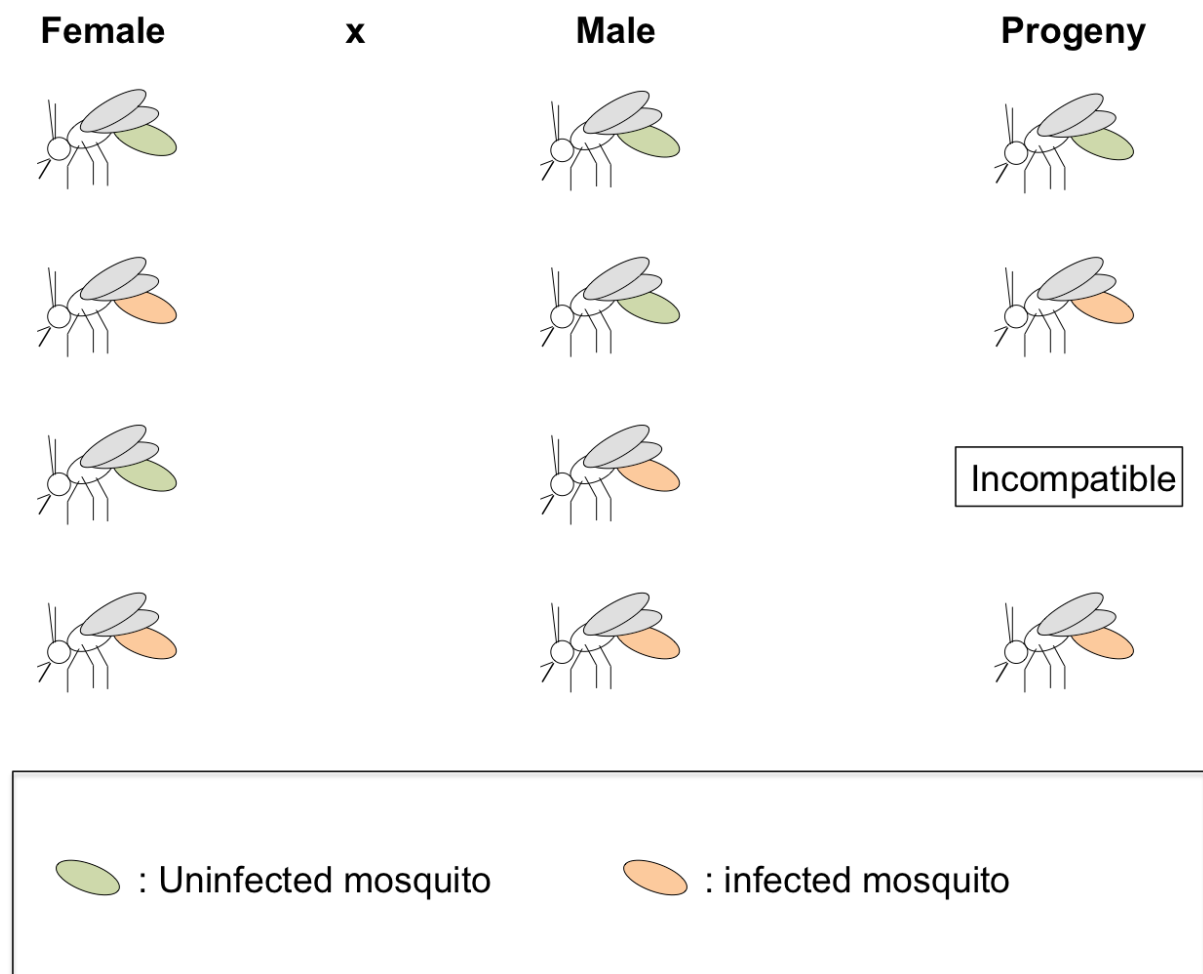


Figure 2.5. : Unidirectional cytoplasmic incompatibility induced by *Wolbachia* endosymbionts

Bidirectional incompatibility (between individual mosquitoes infected with different strains) is explained in figure 2.6, in a visual way. Here as well, the Dobson rule can be applied « if the male has an infection that is not present in his mate, it's an incompatible cross ». If a male and a female harbour a different strain of CI-inducing *Wolbachia*, no offspring will be produced. If both parents carry the same *Wolbachia* strain a normal progeny will be produced and if a females carries both strains of *Wolbachia* and the male carries only one strain, both *Wolbachia* strains will be passed on to the next generation. However, if the male carries both strain of *Wolbachia* and the female just one, the cross will be incompatible. Finally, if both parents carry both *Wolbachia* strains both will be passed on to the next generation.

Cytoplasmic incompatibility suppresses the development of offspring from crosses between infected males and uninfected females. There have been attempts to use CI-inducing ESs directly in BC, to deplete uninfected pest populations by releasing an excess of males carrying

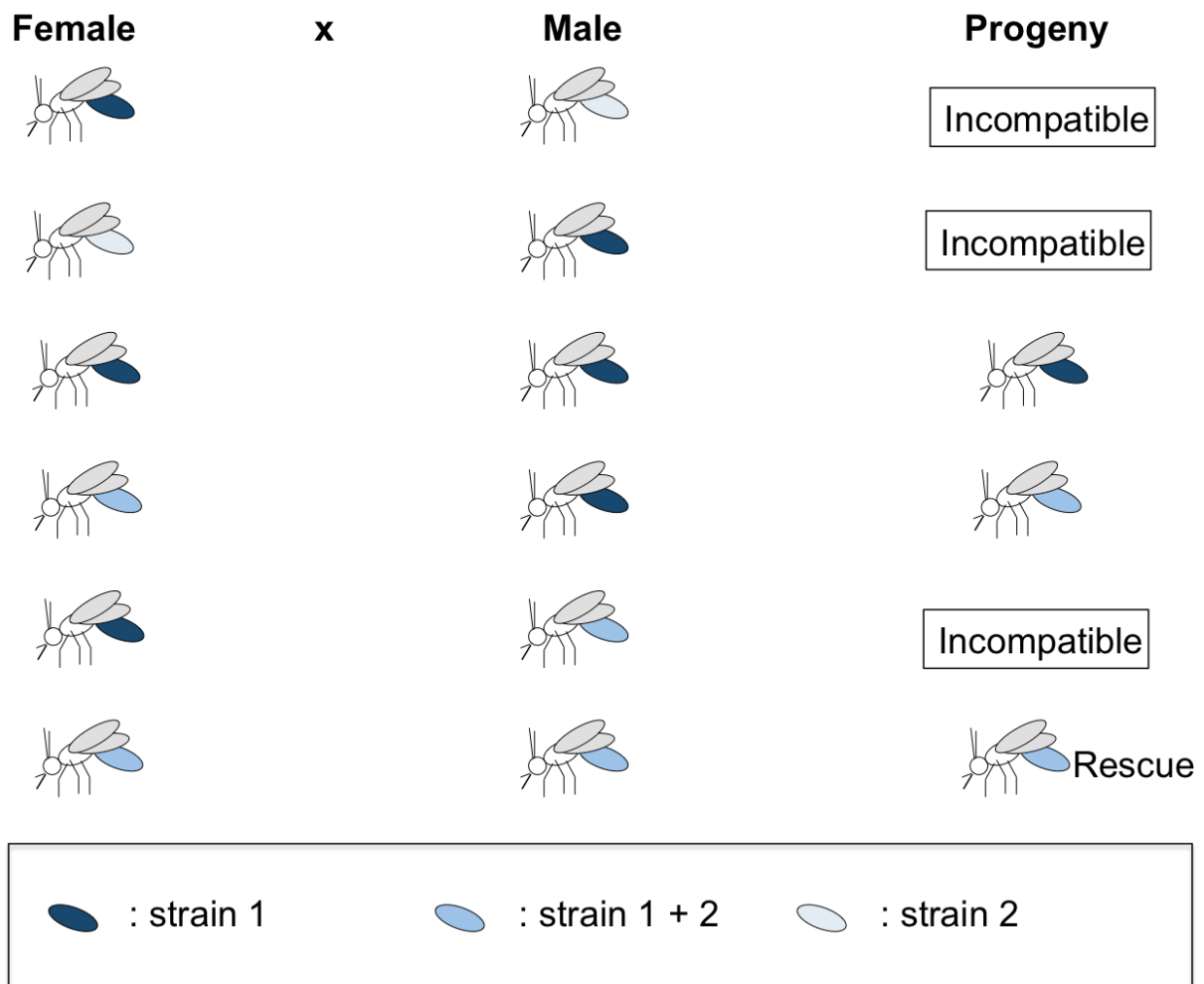


Figure 2.6: Bidirectional cytoplasmic incompatibility induced by *Wolbachia* endosymbiont

CI-inducing ESs. This technique is analogous to the sterile insect technique (SIT), considered to be one of the only strategies that can successfully eradicate a detrimental insect population (Krafsur 1998). Zabalou et al. (2004, 2009) demonstrated a rapid decrease in laboratory Mediterranean fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) populations inundated with males artificially infected with a CI strain isolated from a closely related species, *Rhagoletis cerasi* Linnaeus (Diptera: Tephritidae). This approach could be taken much further. Given that the host's genes, located on mitochondrial DNA, will spread into a population in the same way (because mitochondria and ESs are both vertically transmitted), the use of CI-inducing ESs has been suggested for the introduction of a gene impeding malaria transmission into an *Anopheles* Meigen (Diptera: Culicidae) population (Curtis and Sinkins 1998). Brelsfoard and colleagues in (2009) discuss the use of CI-inducing ESs in combination with a low dose of radiation for the control of lymphatic filariasis-transmitting mosquitoes. The repeated release of incompatible males only could deplete the mosquito population. It is however crucial not to release any females infected with the CI ES into the population to avoid a spread of infection that would lead to population replacement (uninfected to infected). To prevent accidental release of females, low-dose irradiation of BCAs was suggested to sterilize any females present in the material to be freed.

2.2.2. *Wolbachia* based control strategy against the dengue virus vector *Aedes aegypti*.

Endosymbionts may influence the vectorial capacity of its host by i) interactions between the vector and its symbionts, and ii) interactions between the symbionts (microbes always present) and the pathogens (transient microbes), within the vector. The vector competence or the vectorial capacity are two ways to describe the capacity of an arthropod to serve as a carrier and then transmitter of pathogens along the food chain in an ecosystem (Reisen 2002). To mobilize the definitions proposed by Benedict (2008), The vector competence is the genetic capability of a mosquito to serve as a host for the complete development and/or replication of a specific pathogen (Hardy et al. 1983). The Vectorial capacity is a numerical index of the potential for a mosquito population to transmit a pathogen, and includes vector competence, feeding preferences, longevity, etc. (Garrett-Jones 1969). Zindel and colleagues (2011) described the numerous ways microbes may influence the survival of the pathogen within its vector host.

Endosymbionts can increase the vectorial capacity of their hosts by contributing to the transmission of the pathogen. For example, in the B biotype of the whitefly *Bemisia tabaci*, plant viruses have been shown to positively (Jiu et al. 2007) or negatively (Rubinstein & Czosnek 1997) affect vector fitness. Recently, Gottlieb et al. (2010) showed that the efficacy of Tomato Yellow Leaf Curl Virus (TYLCV) transmission by *B. tabaci* depends on the presence of the whitefly symbiont *Hamiltonella*. The interaction between a specific *Hamiltonella* GroEL and the virus coat protein protects the virus from proteolysis in the haemolymph, enhancing its chances of infecting the whitefly salivary gland and increasing its probability of being transmitted to the next plant.

But endosymbionts can also decrease the vectorial capacity of their hosts as explained in the following example. The mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae) is native to Australia and regularly causes dengue outbreaks in North Queensland when travellers carrying dengue virus happen to be stung by mosquitoes. Being a vector of dengue, *A. aegypti* might then transmit the virus to local inhabitants. Record outbreaks occurring in the past 50 years motivated researchers to develop a *Wolbachia* based control strategy of dengue. A project, directed by Prof. Scott O'Neill, funded by the Foundation for the National Institutes of Health through Grand Challenges in Global Health Initiative of the Bill & Melinda Gates Foundation, led to the first releases of *wA. aegypti* in 2011 and the first citywide trial in Townsville in 2014. The technology spread around the world as *wA. aegypti* was released in Rio de Janeiro in 2014 after dengue re-emerged in Brazil in 1981 with 3.2 million cases and 800 deaths. The modified mosquito was also released in Indonesia in 2014 and in Vietnam in 2015 (<http://www.eliminatedengue.com>, consulted on the 13.1.16).

Recent work showed that it is possible to select naturally occurring *Wolbachia* strains, grow it on cell lines and inject it to a new species (Figure 2.7.). The vectorial capacity of *A. aegypti* was successfully diminished after introducing a self-perpetuating strain of *Wolbachia* through laboratory manipulations, in Australia (Ritchie 2014). The strain of *Wolbachia* chosen for controlling dengue carrying *A. aegypti* is called *wMELPop* or « popcorn » as over-replication in its host cells will lead to its premature death (McMeniman et al. 2009). From an epidemiological point of view, shortening the lifespan of a disease vector will reduce its vectoring capacity. After showing that *wMELPop* was reducing its host lifespan researchers had the surprise to show that it contributed to reduce mosquitoes' fitness by lowering their

fecundity (Yeap et al. 2011) and reducing their egg longevity (McMeniman and O'Neill 2010). In addition wMELPop also induced what was called the « bendy proboscis » symptom where females cannot penetrate human skin to feed on blood, thereby diminishing blood feeding (Turley et al. 2009). Finally the most promising result of Prof. Scott O'Neill's team was to show that wMELPop also was inhibiting the transmission of dengue virus. *Wolbachia* infected *A. aegypti* had six times less dengue virus than wild uninfected *A. aegypti*, a trait contributing to the reduced virus vectoring capacity of *wA. aegypti* (Moreira et al. 2009).

However, these encouraging results suggested that wMELPop might not be the best *Wolbachia* strain candidate to control *A. aegypti* because of its major impact on its host fitness. Indeed a too reduced fitness would reduce the propagation of the beneficial endosymbiont in wild population, through cytoplasmic incompatibility. To address these concerns, Walker and colleagues tested the effect of wMEL strain of *Wolbachia* and could prove that wMEL also blocked dengue, even if not as thoroughly as wMELPop (Walker et al. 2011). The wMEL strain of *Wolbachia* was then selected for the eliminate dengue project (<http://www.eliminatedengue.com>, consulted on the 8.1.2016). These concerns about *Wolbachia*'s capacity to spread into the target population is an example of question to be raised when assessing the use of CRISPR/Cas9 gene drive technology to modify insect populations (see chapter 3).

2.2.3. Technological constraints

The endosymbiont *Wolbachia* cannot be cultured on classical bacterial growth media (Murphy et al. 2010). It can however be extracted from insect hosts and cultured in insect cell lines. For example *Wolbachia* obtained from *Drosophila simulans* Sturtevant (Diptera: Drosophilidae) could be cultured in *A. albopictus* cell lines (Dobson et al. 2002), and *Wolbachia* obtained from *Ephesia kuehniella* Zeller (Lepidoptera: Pyralidae), from *Cadra cautella* Walker (Lepidoptera: Pyralidae) and *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) were successfully grown in cell lines from *A. albopictus* and *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) showing that *Wolbachia* can be cultured in cell lines of an taxonomically distant insect species (Furukawa et al. 2008) (Figure 2.7).

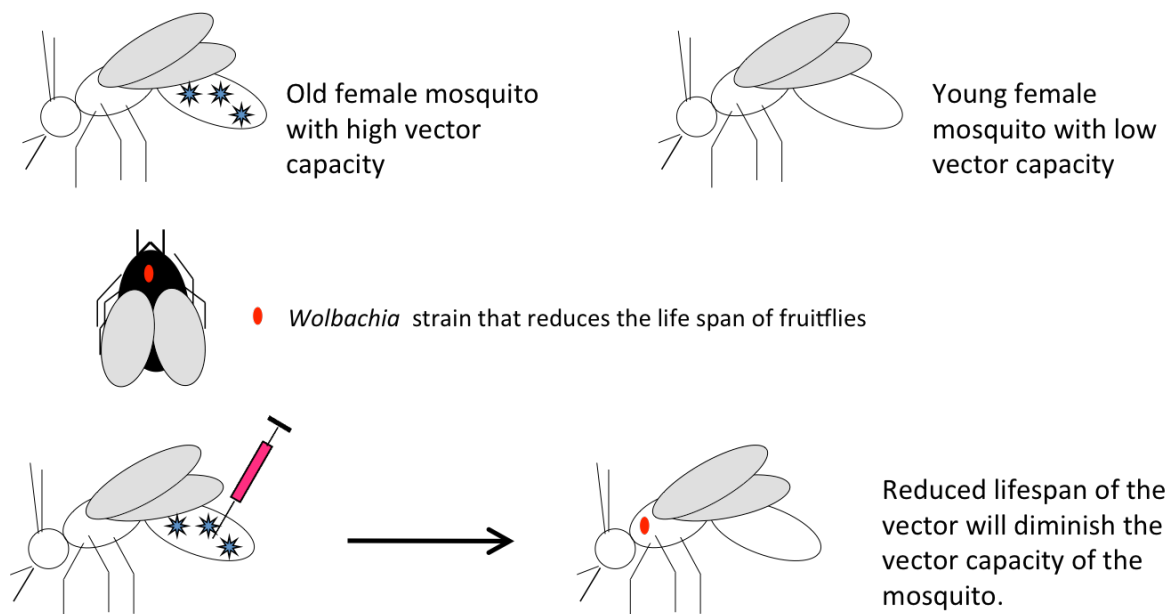


Figure 2.7. : Microinjection of *Wolbachia* in the virus vector *Aedes aegypti*

However, transfer to a novel host by microinjection is technically very challenging and unpredictable and therefore extremely time-consuming as 10'000 microinjections were required to obtain 2 infected lines of mosquitoes (McMeniman et al. 2008). However, McMeniman could micro-inject *Wolbachia* bacterias originating from a *D. melanogaster* laboratory culture to embryos of *A. aegypti* after being maintained on an *A. albopictus* cell line for two and a half years (McMeniman et al. 2008, 2009). Artificial transfer of *Wolbachia* to a new insect species is reversible. Insects can be cured from *Wolbachia* infections by antibiotic treatments (Rifampicin or tetracycline) (Kyei-Poku et al. 2003) or by heat treatments (Van Opijnen and Breeuwer 1999) (Murphy et al. 2010).

2.2.4. Australian risk assessment and legal framework

A trial release was done in Australia in 2011, during the wet season, prior to releases in Indonesia and Vietnam. The government requested a risk assessment. “The risk analysis was evaluated against the end point (adverse hazard which we do not want to occur) that the proposed release would result in more harm than that expected to be caused by naturally occurring *A. aegypti* (“Cause More Harm”)”. The risk of this event occurring was estimated with a time frame of 30 years” (Murphy et al. 2010).

The novelty of the technology encouraged the authors of this risk analysis to seek expert advice. The process was divided in 5 distinct stages. In stage one potential hazard were listed and organized in maps showing the relationships among them. Fifty different hazards were identified (see annexes 1 and 2). In stage two, the ecological interactions between *A. aegypti* and the natural environment were evaluated, in a specialized workshop. From an environmental point of view, reduced *A. aegypti* populations were feared to reduce ecosystem services such as pollination (by males who feed on nectar and pollen) or food source for predators. It also appeared that a lowered population of *A. aegypti* could leave a vacant ecological niche for other invasive mosquitoes in Australia, with cascading environmental impacts. However, the magnitude of these hazards was thought to be negligible because *A. aegypti* is an anthropophilic species, needing human infrastructure such as water containers to survive and because the biomass represented by its whole population was not considered to be large enough to be of significance in nutriment flows in the food web. In stage three, mosquito and community experts convened to model the relationship between the identified hazards and assign failure likelihoods for each hazard. As a result, 30 nodes were identified and evaluated. After expert email consultation (stage four), the risk evaluation team could estimate a failure likelihood of 77.8% for “Cause More Harm”. Finally the objective of stage five was to reduce uncertainty and calculate risk through expert consultation (Murphy et al. 2010).

The environmental risk assessment procedure undertaken in Australia showed a 12.5 % likelihood that « cause more harm » of some magnitude may be realized within 30 years of the release. This is considered a low probability. There was no indication of high environmental risk. Detailed analysis revealed that « perceptions » was the sub model with the highest likelihood score, meaning that there is a likelihood that the release of *Wolbachia Ae. aegypti* will lead to a widespread perception that the threat of dengue has been eliminated permanently (Murphy et al. 2011). In contrast, « ecology » (the likelihood that the release of *Wolbachia Ae. aegypti* leads to adverse ecological impacts), « mosquito management efficacy » (the likelihood that the efficacy of mosquito management and control efforts will be compromised due to the introduction of *Wolbachia Ae. Aegypti*), « avoidance strategy » (the likelihood that people will change their normal mosquito avoidance strategies because of the presence of *Wolbachia Ae. Aegypti*), the « standard public health » (the likelihood that the standard of public health overall will be worse as a result of the release of *Wolbachia Ae. Aegypti*) and « economic effects » (the likelihood that the introduction of *Wolbachia Ae. aegypti* will result in adverse economic impacts) were all considered as low likelihood hazards. « Monitoring » (the probability of having a sufficient system in place to monitor *Wolbachia Ae. aegypti* to ensure that the concept of no harm is measurable) and « *Wolbachia* fitness » (the likelihood that a genetic change in *Wolbachia* will cause a fitness change in *Ae. aegypti*) obtained low likelihood hazard and « ecological niche » (the likelihood that *Wolbachia Ae. aegypti* will change its ecological niche from being a predominantly domestic species to a broader or alternative niche), « density » (the likelihood that the average density of *Wolbachia Ae. aegypti* (e.g. average numbers per household) will be higher than would occur for naturally occurring *Ae. Aegypti*) and « need for control » (the likelihood that the release of *Wolbachia Ae. aegypti* will result in the need for increased levels of mosquito control) obtained very low likelihood hazards. These results highlight the need of interdisciplinary research teams to evaluate the risk associated with such technologies (see chapter 1 for an example of interdisciplinary research) (Murphy et al. 2011 and see annexe 1 for a precise description of the hazard cited).

Aedes aegypti containing *Wolbachia* is currently released in Vietnam since 2015. The aims of this project taking place on Tri Nguyen Island, and in the metropolitan Province of Khan Hoa on the south central coast of Vietnam are to demonstrate i) the establishment of *Wolbachia* infection and invasion in natural mosquito populations, ii) that anti-dengue properties of *Wolbachia* are present in mosquitoes in field conditions and iii) to the community and other countries that a release is not harmful (Truong et al. 2011). Vietnamese researchers conducted the same environmental risk assessment as the Australian team and reached the conclusion that “there only is a negligible risk that the release of *A. aegypti* containing *Wolbachia* will result in more harm than what is currently caused by naturally occurring *A. aegypti* over a 30-year timeframe” (Truong et al. 2011). A close look on their result shows that every risk identified (in general very similar to those identified by the Australian team) was evaluated with a very low or negligible risk of causing harm (Truong et al. 2011).

2.3. Regulatory framework

A paper by De Barro and colleagues entitled « The proposed release of the yellow fever mosquito, *Aedes aegypti* containing a naturally occurring strain of *Wolbachia pipientis*, a question of regulatory responsibility » (De Barro et al. 2011) describes the consultation among scientists developing this new *Wolbachia*-based technology to fight against dengue and regulators. The team led by Scott O'Neill proactively contacted regulators from different services from the Australian government to investigate potential implication their science may have for policy legislation and regulations. As mentioned by De Barro and colleagues « this case does raise a new precedent and it is an example of how a new science driven technology might challenge existing regulatory processes » (De Barro et al. 2011).

Introducing a new biological control agent into the environment is regulated by several laws as mentioned in chapter 1, focusing on classical biological control. However, some situation can represent what De Barro and colleagues describe as a « regulatory no man's land ». The fact that the proposed technology involved a native mosquito *Aedes aegypti* and a native endosymbiont, *Wolbachia pipientis* was actually a complication as it did not fall under the jurisprudence of the four pieces of regulation governing the release of species into Australia: the Quarantine Act 1908, the Biological Control Act 1984, the Environment Protection and Biodiversity Conservation Act 1999 and the gene technology Act 2000. Moreover, as highlighted by De Barro and colleagues, the health department in Australia regulates disease vectors such as mosquito even if this department has no direct responsibilities for the above-mentioned acts.

The technology involving the mosquito carrying *Wolbachia*, *wA. Aegypti* was initially considered as a biological control agent, as modified mosquitoes would be released into the environment with the prospect that they would establish and be self-perpetuating. The biological control act regulates the introduction of new species in the environment. As both *A. aegypti* and *Wolbachia* were already present in Australia, this act law was not regarded as relevant, even if the new combination of a mosquito and an endosymbiont was new to Australia (De Barro et al. 2011).

The Gene technology act was then seen as a possible regulation for the introduction of *wA. aegypti* in the field even if it was soon rejected because « the current interpretation of gene technology is a process that involves recombinant DNA methodologies to introduce DNA into the organism. A key element is that in GMOs the chromosomal content of an organism is changed in an irreversible manner; this is not the case for *Wolbachia*-infected mosquitoes as infections can be lost from the host (e.g. Keller et al. 2004). As a consequence, the process by which *A. aegypti* was infected with *Wolbachia*, and the way in which the *Wolbachia* was prepared for transfer into *A. aegypti* did not involve gene technology. As a result *wA. aegypti* was not considered as a GMO ».

Facing a complicated situation, the team consulted the Primary Industries Ministerial Council (PIMC) which mission is to « 1. Develop, implement and review policies and strategies for achieving agreed national approaches to the development of sustainable primary and related food industries, 2. Actively liaise with other Ministerial Councils and other bodies on matters relevant to the activities of the council and, 3. Direct the work of and consider matters submitted by the standing committee » (http://www.mincos.gov.au/about_pimc, consulted the 8.1.16; De Barro et al. 2011). The consultation resulted in the identification of a regulatory solution that was to consider *wA. aegypti* as a veterinary chemical product, regulated under the Agriculture and Veterinary Chemicals Code Act 1994 (AgVet Code).

In the Veterinary Chemicals Code Act, a veterinary chemical product is defined as « a substance that is used for application to an animal by any means, as a way of directly or indirectly ; modifying the physiology of the animal ; so as to alter its natural development or reproductive capacity » ([http://www.comlaw.gov.au/ComLaw/Legislation/ActCompilation1.nsf/0/6D3FCE09FD5175C1CA257788008025FE/\\$file/AgrVetChemCode1994_WD02.pdf](http://www.comlaw.gov.au/ComLaw/Legislation/ActCompilation1.nsf/0/6D3FCE09FD5175C1CA257788008025FE/$file/AgrVetChemCode1994_WD02.pdf) consulted on the 8.1.16; see pp. 37–38; De Barro et al. 2011). As explained in De Barro and colleagues' paper « The advice concluded that *Wolbachia* was a “substance” in accordance with the definition contained at section 3 of the AgVet Code as it was either ‘an organism’, ‘part of an organism’, ‘material that is produced from an organism’ or ‘matter whose production involves the use of an organism’. The injection of *Wolbachia* into the mosquito complied with “application... by any means”. Furthermore, *A. aegypti* was an “animal” within both the ordinary meaning of that term and within the definition of “animal” contained at section 3 of the AgVet Code which expands on, rather than confines, that ordinary meaning, “animal means any animal (other than a human being), whether vertebrate or not, and whether a food producing species or not, and includes:... (c) any other prescribed form of animal life, whether prescribed by reference to a species or in any other way.” (De Barro et al. 2011)

Although the outcome of this consultation process led to the identification of a legal framework compatible with the particularities of the new technology, some concerns about the level of rigour involved in the handling of such cases linked to biosecurity, and the legal binding power of the proposed solution were raised (Marshall 2010). The internal assessments and the independent environmental risk assessment done on the Australian release of *wA. aegypti* proved the « very high level of scrutiny with regard to biosafety and clearly demonstrates that simply because a technology is beyond the scope of regulation covering GMOs, that the focus on biosafety is no less rigorous » (De Barro et al. 2011).

Here, in a similar way, we will analyse the following Swiss federal Acts potentially mobilized to regulate the hypothetical introduction of *Aedes albopictus* modified with *Wolbachia*, to achieve biological control of the disease vector to evaluate their purposes, area of validity and definitions: Ordinance on the Handling of Organisms in the Environment (Release Ordinance, RO) and the Federal Act on Non-Human Gene Technology (Gene Technology Act, GTA) (see annexes 3 and 4).

« The purpose of the release ordinance act is to protect human beings, animals and the environment, as well as biological diversity and the sustainable use thereof, from hazards or impairment caused by handling organisms, their metabolic products and wastes » (Release Ordinance, RO). « This Ordinance regulates the handling of organisms, their metabolic products and wastes in the environment, in particular the handling of genetically modified, pathogenic or alien organisms » (Swiss Federal Council 2008).

Organisms are defined as « cellular or non-cellular biological entities capable of replication or of transferring genetic material. Mixtures and articles and products containing such entities are also regarded as organisms » and microorganisms as « microbiological entities, in particular bacteria, algae, fungi, protozoa, viruses and viroids; cell cultures, parasites, prions and biologically active genetic material are also regarded as microorganisms ». The mosquito *Aedes albopictus* and *Wolbachia* fit to these definitions.

The invasive status of *A. albopictus* is less clear. The release ordinance state that an « alien organisms means organisms of a species, sub-species or lower taxonomic level that as a species, do not occur in Switzerland, the other EFTA and EU member states (not including overseas areas) in their wild form » (Swiss Federal Council 2008). The fact that *A. albopictus* is an invasive species established on part of the Swiss territory (in Ticino) and that will probably establish itself in the rest of the country raises questions. *Wolbachia pipientis*, infecting 66% of insect species is also already present in Switzerland. However this bacteria is an obligate endosymbiont, which means that it cannot live or be cultures outside of insect tissues, meaning that *Wolbachia* is present in Switzerland, but obligatorily in association with other insects.

However, *Aedes albopictus* can be considered as an invasive alien organism as an « invasive alien organisms is an organisms of which it is known or should be assumed that they will spread in Switzerland and could achieve such a high population density that biological diversity or the sustainable use thereof could be impaired or human beings, animals and the environment could be endangered » (Release Ordinance, RO). In contrast, *Wolbachia* cannot be considered as an invasive alien organism as it is already present in Switzerland.

Probably the best framework justifying the mobilisation of the release ordinance to regulate potential releases of *wA. albopictus* in Switzerland is summarized by the following extract « handling of organisms in the environment means any deliberate activity using organisms that takes place outside a contained system, in particular culturing, processing, multiplication, modification, experimental release, marketing, transport, storage or disposal » (Swiss Federal Council 2008). Injecting *Wolbachia* in *A. albopictus* and releasing *wA. Albopictus* in the environment (in experimental releases) fits to the definition of the « handling definition ».

To rule out the interpretation of *wA. albopictus* as a genetically modified organism, we will refer to the Federal Act on Non-Human Gene Technology (Swiss Federal Council (2008) that defines a GMO as « Genetically modified organism means organisms in which the genetic material has been altered in a way that does not occur under natural conditions by crossing or

natural recombination » (Swiss Federal Council (2008). *Wolbachia* microinjections cannot be considered as a method altering the genetic material of *A. albopictus* nor *Wolbachia* that does not occur under natural conditions. *wA. albopictus* could therefore not be considered as a GMO by Swiss regulators.

To summarize *wA. albopictus* would then be considered as «an invasive alien species » being « handled outside a contained system » and in particular « experimentally released » and as an organism under the Swiss regulation and its evaluation hence suffers no regulatory gap. Biosafety measures could then be taken and be legally binding in the event of development of a *Wolbachia* based control strategy of *A. albopictus*. A risk assessment procedure for *wA. albopictus* should follow the work of Murphy and colleagues (2010) who proposed a series of questions to be answered to evaluate the risk of causing harm to the environment, the people or the economy.

2.4. Interaction between arthropod symbionts and biological control strategies

Clear links between biological control and *Wolbachia*-based control strategies are documented in the literature. These links should be considered when selecting a control strategy against a crop pest or a disease vector. Endosymbionts can infect many beneficial arthropod species and may dramatically affect the outcome of a biological control programme (Zindel et al. 2011) (see chapter 1 on biological control). By manipulating their host's biology, they have the potential to dramatically affect all phases of a biological control program, from the rearing of a biological control agent to its establishment in the field (Zindel et al. 2011). As described in Zindel et al. 2011, endosymbionts can spread in their host's population by protecting their host against natural enemies and thereby interfere with biological control strategies (see chapter 1). Some endosymbionts have been shown to protect their hosts from predators, macroparasites (Olsen et al. 2001; Harcombe and Hoffmann 2004; Chiel et al. 2009a), bacteria and viruses (Davidson et al. 2001; Lopanik, et al. 2004; Brownlie and Johnson 2009) and to even to be involved in pesticide-resistance mechanisms (Kontsedalov et al. 2008). Such ES-mediated resistance to pathogens or chemicals can give an organism a substantial advantage over non-resistant conspecifics in an environment where biotic or abiotic antagonists are present. The mechanisms underlying the various types of resistance are often unknown even if, in some cases, the ES produces toxic compounds that affect its host's enemies (Gil-Turnes, et al. 1989; Kellner 2002; Oliver et al. 2009). In other cases, ES-induced behavioural changes in the host (Haine et al. 2005; Rigaud and Haine 2005), such as deterrence effects that protect the prey from its predator (Davidson et al. 2001; Lopanik et al. 2004), have been suggested or observed.

2.5. Questions raised

Environmental risk assessment

We suggest asking the questions formulated by the Australian ERA listed in annexes 1 and 2 to evaluate the environmental risks posed by the hypothetical introduction of *wA. albopictus* in Switzerland.

Cost-risk-benefit analysis

Wolbachia based control strategy is a long-term and expensive endeavour. Evaluating the alternative methods to control *A. albopictus* should enable regulators to decide whether engaging in such technology is worth it.

1. Is *A. albopictus* a major health threat, justifying such expensive endeavour?
2. What are the alternatives to reduce the vectoring capacity of *A. albopictus* (pesticides, water body management, use of mosquito nets, change of habits, better control of trades material such as used tires at the borders, gene drives)?
3. Could *Wolbachia*-based control strategy affect biological control program?

International management of A. albopictus

3. How will neighbouring countries handle the tiger mosquito?
4. Can technologies adopted outside Switzerland affect the decision process of Swiss regulatory authorities?

Biology of Aedes albopictus

5. How much effort should be put into a precise monitoring of *A. albopictus*?
6. *Aedes albopictus* is considered as an invasive alien species in Switzerland. Will this status hold when it will be naturalized and established in the whole country?
7. Will a status change (invasive alien species to native species) change the legal aspects?
8. Which endosymbionts infect *A. albopictus* in its European invasive range?
9. What strains of *Wolbachia* infect *A. albopictus* in its European invasive range?
10. Is *A. aegypti* an anthropophilic species, needing human infrastructure such as water containers to survive? Will its ecology change?
11. Endosymbiont may provide ES-mediated resistance to pathogens or chemicals. Which pathogens could affect *A. albopictus* in its invaded geographical range?

General question.

The majority of insect species harbour obligate or facultative symbionts, forming what is considered as multi-species organisms consisting of several species having to function as a unique organism. Regulation of microorganisms (bacteria, virus, fungi etc.) differ widely from the regulation of macroorganisms (arthropods, nematodes, etc.)

12. How should multi-species organism regulated?

*Could *wA. albopictus* considered as a GMO?*

In Australia, *wA. albopictus* was not considered as a GMO since the process by which *A. aegypti* was infected with *Wolbachia*, and the way in which the *Wolbachia* was prepared for transfer into *A. aegypti* did not involve gene technology.

13. Could *wA. albopictus* considered as a GMO in Switzerland?

Need for an interdisciplinary approach?

The Australian environmental risk assessment revealed that “perceptions” was the sub model with the highest likelihood score, meaning that there is a likelihood that the release of *Wolbachia Ae. aegypti* will lead to a widespread perception that the threat of dengue has been eliminated permanently (Murphy et al. 2011)

14. Which disciplines should be mobilized to evaluate what perception actually means (economy, psychology, anthropology, sociology)?

Individual travellers or migrant population infected by dengue or chikungunya viruses, arriving in Switzerland could favour the outbreak of these diseases in the presence of the vector *A. albopictus*.

15. How should medical service prepare them for such eventuality?

Chapter 3

A quantum leap in biotechnology: RNA guided endonucleases

Nicola Schoenenberger & Alexandre Aebi

Keywords: recombinant DNA, gene targeting, genome editing, disruptive innovation, democratization of biotechnology, product vs. process.

3.1. CRISPR-cas9

The ability to target or to cleave the double stranded DNA helix at a specific sequence is highly relevant to biotechnology as it allows to create insertions or deletions, to add or to remove desired sequences, to regulate transcription, to tag or to modify the conformation of DNA at a chosen location within the genome. Several systems have been so far discovered and employed for these purposes but one of these, the CRISPR-Cas9 complex, which is a simple two component structure consisting in a RNA guide molecule used to specifically target DNA sequences and an endonuclease enzyme able to cut DNA at a target site, has recently become the most promising tool for genome editing (Jinek et al. 2012). Because of the extreme versatility and ease of use of the CRISPR technology, it has been surprisingly efficient to change virtually any DNA sequence of nearly any species including humans, it is already often considered the major breakthrough in biotechnology since the discovery of PCR (Ledford 2015). It is likely to produce applications in all branches of biotechnology (Doudna and Charpentier 2014) and has the potential to overthrow or disrupt current technology (Newson and Wrigley 2015).

Like many other tools in biotechnology, CRISPR-Cas derive from the never-ending genetic conflict at the interface between parasites and their hosts and is inspired by nature. Amongst a variety of defense mechanisms that Archaea and Bacteria have evolved against invading foreign genetic elements such as viruses and plasmids, there are the so called CRISPR or *Clustered Regularly Interspaced Short Palindromic Repeats* - Cas or *CRISPR associated systems*. They involve the integration of short fragments of the invader's DNA into the host chromosome and provide a form of acquired and inheritable immunity like *Wolbachia* (see chapter 2) (Barrangou et al. 2007). In other words, the CRISPR loci provide a memory of infection by maintaining a genetic record of previously encountered parasites. Transcription of these loci produces RNAs that are able to guide endonucleases to complementary nucleic acid targets, specifically cleaving (or silencing) plasmid and bacteriophage double stranded DNA and thus providing bacteria and Archaea with sequence specific resistance (Wiedenheft et al. 2012).

The production of double stranded breaks at any desired location in the genome, i.e. an efficient gene targeting system, was first achieved with zinc finger nucleases (ZNFs) and later by transcription activator-like effector nucleases (TALENs). Both are non-natural chimeric proteins composed of specific DNA-binding domains coupled to endonucleases. Their use implies labor-intensive design and synthesis of genes that encode specific DNA binding domains for every new target, and they have to be used in pairs, one for each strand (Golic 2013). It was immediately clear that the CRISPR system had the huge advantage to be much simpler to design and cheaper to synthesize than ZFNs or TALENs. Zinc fingers cost US\$ 5'000 or more to order, because the DNA binding proteins are difficult to engineer. In contrast CRISPR cost only around 30 US\$, involving only the design and ordering of a guide RNA (gRNA), since the other component (i.e. the Cas9 endonuclease) is available off-the-shelf (Ledford 2015). The implications of this effective and cheap targeting mechanism have triggered a radical change in biology (van Erp et al. 2015) and the CRISPR technology is now quickly replacing ZFN's and other genome editing techniques. Moreover, the rapid adoption of the CRISPR-Cas9 technology is accelerated by the availability of open source resources such as addgene (www.addgene.org), which provides CRISPR reagents, and online forums (like e.g. www.genome-engineering.org) providing protocols, computational tools and a support

platform (Hsu et al. 2014). In fact, between 2013 and summer 2015 the web of science database registered 1415 scientific papers with CRISPR-Cas9 in the title or the abstract (Quétier 2016). However, the CRISPR field is still young and it might take years until commercial applications will appear.

3.2. How does CRISPR-cas9 work?

Docking of the Cas9 endonuclease on the genome occurs by matching (through Watson-Crick base-pairing) of 20-nucleotide sequences of the gRNA with the target DNA sequence. In presence of an adjacent Protospacer Adjacent Motif sequence (or PAM sequence), Cas9 cleaves DNA producing double stranded breaks at the target site (Doudna & Charpentier 2014) (Figure 3.1). In the natural prokaryote defense system, PAM is an antigenic signature that allows the organism to avoid self-targeting by differentiating own DNA of the inherited CRISPR loci (lacking the PAM) and foreign DNA of the invading parasitic bacteriophage or plasmid (Wilkinson and Wiedenheft 2014).

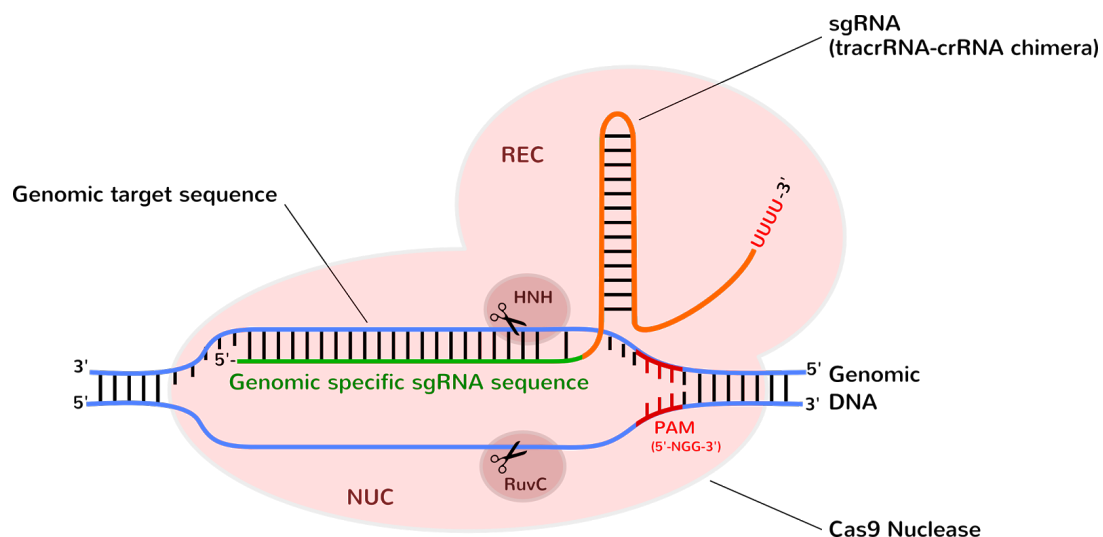


Figure 3.1. The CRISPR-Cas9 nuclease programmed with a single guide RNA (sgRNA). Upon binding with the DNA target sequence, the sgRNA guide specifically targets a short DNA sequence-tag (PAM). The complex then unzips DNA and undergoes structural rearrangement leading to double-strand cleavage. (Source: www.transfections.blogspot.ch, accessed 18.12.2015).

These lesions are then repaired by the cellular repair machinery through two possible mechanisms: the Non Homologous End Joining (NHEJ) mechanism, which is error prone and often results in small insertions or deletions called indels, or by Homology Directed Repairs (HDR), an often not very efficient process involving a template DNA with sequences homologous to the flanking regions of the break which is used by the cell as an instruction to repair the broken strands (Figure XX). Thus, using endonucleases in combination with donor DNA provides a method to add desired foreign DNA encoding new functions to precise locations, or to specifically excise fragments of DNA. Moreover, the modular feature of CRISPR-Cas9 and the short 20 nucleotide length of the targeting sequence allow targeting and cleaving of multiple target sites simultaneously (multiplexing), or the excision of large

genomic segments located between two cleavage sites, by the use of different gRNA at the same time (Cong et al. 2013). Due to the high transformation rates, mutants can be detected simply by molecular screening, and there is no need to employ selectable marker genes (Golic 2013), the presence of which might be undesirable in practical applications, e.g. in the case of antibiotic resistance genes.

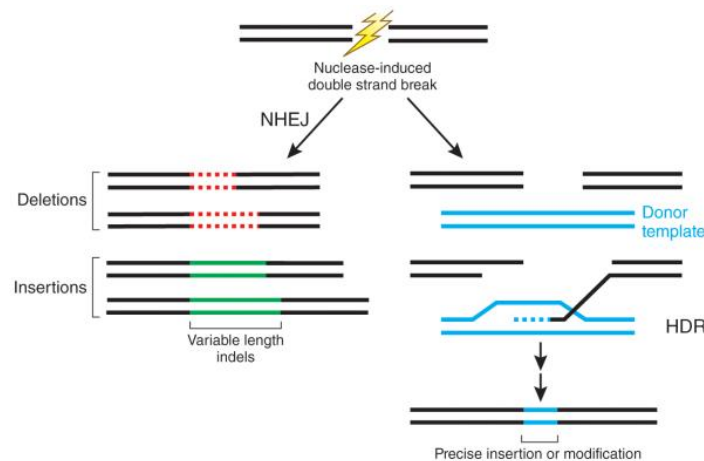


Figure 3.2. Nuclease induced genome editing through the cellular DNA repair mechanisms Non Homologous End Joining (NHEJ) and Homology Directed Repair (HDR) (source: Sander and Joung 2014).

3.3. Applications

Applications of CRISPR-Cas9 encompass almost every industry in the biotech sector, from agriculture and food industry to the biomedical sector, the research tools market, materials and biofuels (Table 3.1.) (Hsu et al. 2014, van Erp et al. 2015). It is difficult to estimate the value of the emerging CRISPR-Cas9 market, but it is likely to be huge, as suggested by the pace at which companies have raised capital since 2013 (e.g. \$ 600 million in the gene therapy sector), given that the first granted patent for the use of the technology in eukaryotic cells was issued in April 2015 (van Erp et al. 2015). In agriculture the technology has advanced beyond the laboratory into product development and even onto the market, making gene editing closer to transforming agricultural markets than human medical markets (Glorikian 2015). From a juridical point of view trial battles over the ownership of the CRISPR-Cas9 technology will take years to reach a decision (Regalado 2014).

Industry sector	Product/application	Company
Food	Yogurt, cheese	Danisco (DuPont)
	Crops	Dow Agrosiences
	Livestock	Recombinetics
	Crops	Cellectis Plant Sciences
Laboratory	Research tools	System Biosciences
	Expression systems	Sigma-Aldrich

Sublicensing Medical	Research tools	GE Healthcare
	Animal models	Sage
	Research tools	ThermoFisher
	Animal models	Taconic
	Agricultural, Industrial, Biological research	Caribou
	Pharmaceuticals	Novartis
	<i>In vitro</i> applications only	Collectis
	Target validation	AstraZeneca
	Therapeutics	Crispr Therapeutics
	Monogenic diseases	Sangamo Biosciences
	Therapeutics	Intellia
	Therapeutics	Editas

Table 3.1. Industry of CRISPR-Cas9 based applications (Source: van Erp et al. 2015).

3.4. Biological risks

Risks associated with any genetically engineered organism are case-specific and cannot be overruled by across-organism generalization (Dolezel et al. 2009). Risks linked to genome editing technologies will depend on their outputs, i.e. where and how the genome is modified, which genes are engineered and which intended or accidental properties were gained by the modified organisms, and, in case of hypothetical environmental releases, on the whole organism or population in its receiving environment. Although risks need to be assessed on a case-by-case principle and are product specific (i.e. depend on the concrete outcome) rather than process specific (i.e. depending on the employed technology), a few general considerations can be made about risks related to the CRISPR-Cas9 technology (see chapter 2 on *Wolbachia* and Annex 2 for a list of hazards identified during the environmental risk assessment of this natural gene-drive).

CRISPR-Cas9 requires homology between the target sequence and the gRNA. However, it allows a few mismatches in the 20 bases long target sequence, which may generate cleavages outside the aimed sequence. Off-target effects may be difficult to identify and may entail the disruption of genes or the alteration of their expression, including pleiotropic effects or epigenetic alterations, with consequences that are difficult to predict. The toxicity and long-term consequences of off-target mutations represent the biggest actual concern of the CRISPR systems, and the extent of their occurrence is an important consideration for purposes of risk assessment. In fact, a mismatch of 1-2 bases between the spacer region of the gRNA (20 bases) and the target genome will generally result in engagement of CRISPR-Cas9 and consequent DNA cleavage at a frequency that is comparable to the intended target sequence (with full complementarity between the gRNA and the genomic DNA to be cleaved). Even 3 mismatches between gRNA and DNA can still produce double stranded breaks and result in off-target mutations (reviewed in Golic 2013). Moreover, Cas9 can remain bound to DNA even by a short sequence of complementary DNA without necessarily inducing cleavage (Hsu et al. 2014), possibly leading to the alteration of DNA function in the transfected cells. On the other

hand, off-target mutations may be scanned before commercial release by whole genome sequencing or other unbiased ways of detecting double stranded breaks and may be removed by recombination at meiosis provided they are not too close to the target mutation, as a biosafety measure. It is interesting to note that such security switch cannot be developed for a technology based on *Wolbachia* (see chapter 2). Technology to achieve higher efficiency and specificity are rapidly being developed, i.e. by the use of two Cas nicking enzymes driven by a pair of gRNA's, each one breaking a single DNA strand. This method allows to double the number of bases of the target sequence recognition entailing a reduction of off-target breaks by 50 to 1500 times. High-fidelity variants of CRISPR-Cas9 with no detectable genome wide off-target mutations are also being developed, e.g. in human cells (Kleinstiver et al 2016). Sequencing of the genome of most crop plants and model plants, coupled to bioinformatic tools and PAM databases, allow to define the best candidate sequences for the design of gRNA's or to predict the number and likeliness of off target cleavages (Quétier 2016). In perspective, off-target breaks may be increasingly minimized and easier to detect, but will likely not be totally eliminated.

The democratization of genome editing through CRISPR-Cas9 leads to a further consideration to be made in relation to biological risks. The disruptive nature of the application of this technology could have unexpected and undesirable outcomes involving potential hazards. In fact, CRISPR-Cas9 has the potential to conquer "garage biotechnologists" or "biohackers" and make their movement grow in popularity. These do-it-yourself biologists practice biotechnology as a hobby, e.g. by creating organisms that glow in the dark, using the same methods as traditional research institutions, but generally with lack of oversight by professionals or governments (Ledford 2010). Do-it-yourself CRISPR genome editing kits aimed at bringing genetic engineering to one's kitchen bench already exist, and cost as little as US\$ 130 (www.gizmag.com/home-crispr-gene-editing-kit/40362/). Uniform supervision of the phenomenon is impossible and the aficionados of do-it-yourself biotechnology argue that the best way to mitigate potential hazards such as accidents or evil intentions (the "dual use" problem) is to encourage a culture of transparency and increased education (Boustead 2008). In other words, "where external oversight is perceived to be ineffective or intrusive" self-governance might be a suitable approach to governance of genome editing (Newson and Wrigley 2015).

3.5. Regulation

The question whether organisms carrying edited genomes achieved by the use of CRISPR-Cas9 will fall under GMO regulation or not, is similar (if not the same) as with organisms engineered with ZFNs and TALENs. In fact, if CRISPR-Cas9 is used to induce mutations at a precise location without the addition of template DNA, NHEJ repair mechanism will produce single nucleotide polymorphisms or indels. Advisory bodies in Europe suggest that plants carrying indels of up to 20 nucleotides do not give rise to a recombinant nucleic acid and shall not be considered GMOs, as any deliberate alteration of less than 20 nucleotides cannot be distinguished with sufficient certainty from an incidental occurrence of the sequence. Moreover, they cannot be differentiated from genetic modifications arising from conventional mutagenesis achieved by the use of chemical compounds or physical irradiation (some of these techniques have been in use since the 1930s), or natural mutation (NTWG 2012, ZKBS

2012). In most countries including Switzerland, organisms which have been genetically modified by mutation breeding are not regulated, whereas in a few countries such as Canada mutagenized crops face the same regulations and testing as crops obtained by genetic engineering. Recently, the Swedish Board of Agriculture has stated that some plants in which the genome has been edited by CRISPR-Cas9 do not fall under the European GMO definition, and therefore are exonerated from the regulatory regime. The logic is that with genome editing technologies, a process that is used to produce GMOs, it is also possible to create plants that are identical in all aspects to plants that are created by classical mutagenesis, just containing indels, and thus are not considered to be GMOs. This can create a paradox with the hypothetical existence of two identical plants where the cultivation of one is forbidden because regulated as a GMO, while the other can be grown with no restrictions because considered conventionally bred (UPSC 2015). On the other hand, the Netherlands Commission on Genetic Modification (COGEM) considers that within the current EU legal framework, CRISPR-Cas9 applications fall under the GMO legislation (COGEM 2014).

In the US, the exemption from the GMO regulation saves the seed industry 5.5 years and 35 Mio. USD costs related to the preparation of a time-consuming dossier for bringing a product on the market. The US Department of Agriculture (USDA) already ruled that some mutations made by ZFNs and TALEN do not fall under their regulatory authority and the position on CRISPR-Cas will likely follow in near future (van Erp et al. 2015). Compared to the older forms of genetic modification, the fact that these mutations do not carry the status of genetically modified organism, or GMO, which is a controversial label, positions its products as the non-GMO alternative (Glorikian 2015).

In general, GMO legislation was written on the basis of the biotechnology state of the art that was developed and used twenty years ago, but science delivers innovations at an impressive speed and administrative bodies take much longer to elaborate regulations (COGEM 2014). To overcome this problem a suggested approach that is becoming popular is to favor a product-based regulation, i.e. concentrating on engineered traits, rather than a process-based one, i.e. depending on the methodology employed, which appears to be more realistic particularly when the induced change leaves no detectable trace of the process used (e.g. CRISPR-Cas vs. classical mutagenesis) (Ricroch and Hénard-Damave 2015). In any case, edited genomes with the insertion or foreign template DNA should be regulated as GMO's, whatever the origin of the template DNA (Quétier 2016).

3.6. Uncertainties

Genetic manipulation with CRISPR-cas9 is a very new field in biology, and papers on the development and use of the technology are published almost weekly in high-ranking journals. As such, it intrinsically bears high levels of uncertainties. At present the debate on biological risks mostly focuses on the phenomenon of off-target activity, i.e. the binding of the CRISPR-cas9 complex to mismatched DNA sequences, with or without DNA cleavage. In fact, the dimension of off-target effects and the importance of the structure and state of chromatin on the cleavage activity of Cas9 are poorly understood. The mechanism by which CRISPR-Cas9 engages on the DNA are complex, as PAM is not the only sequence critical for Cas9 targeting but also other sequences adjacent to PAM may elicit engagement (Wilkinson and Wiedenheft

2014). The mechanism of Cas9 to initiate DNA strand separation are unknown, moreover CRISPR-Cas9 can bind to DNA and remain catalytically inactive in presence of sequences only partially homologous with the gRNA. We do not know if these interactions are transient or long lived and what their effects could be (Doudna and Charpentier 2014). Cas9 can be used for a number of functions other than double stranded DNA breaks and genome editing, such as transcriptional modulation, epigenetic control or marking of genomic loci (Hsu et al. 2014), raising the question of unforeseen effects. On the other hand, some researchers consider that the real risk of off-target activity remains doubtful, as they tend to show that off-target effects are rare and most of the detected indels after CRISPR-Cas9 editing of stem cells are assignable to cell culturing and do not result from the specific genome editing event (reviewed in COGEM 2014). Recently discovered proteins that can inhibit CRISPR-Cas systems will, without a doubt, provide tools to better understand these systems by experimental manipulations (Bondy-Denomy et al. 2015)

Cellular repair mechanisms of broken DNA strands remain to be understood in detail and the pathways leading to NHEJ rather than HDR are rather mysterious. The efficiency of HDR, the pathway allowing integration of foreign genetic material is low. Current research objectives are to increase the frequency of HDR in spite of NHEJ in order to promote specificity of insertions of genetic material (Doudna and Charpentier 2014).

In animal breeding, another challenge is genetic mosaicism, i.e. the presence of cells with different genotypes in one and the same organism, occurring particularly when CRISPR is injected into zygotes with the aim to create fully edited organisms (Hsu et al. 2014). Genetic mosaics originate when a nuclease does not cut both copies of the target sequence in an embryo or when the cell starts dividing before complete editing is achieved (Lanphier et al. 2015). We ignore potential impacts of mosaicism on biosafety.

Long-term implications remain unclear. To elucidate them will be paramount to characterize biological risks, as once genome editing is done, it will be most likely permanent. To summarize, the words of Bo Huang University California San Francisco seem to fit well: "There is a mentality that as long as it works, we don't have to understand how or why it works" (cited in Ledford 2015).

A list of questions raised was put together at the end of the case studies on genetically modified mosquitoes (chapter 5) and herbicide susceptibility restoration in wild plants (chapter 6).

Chapter 4

Gene drives

Nicola Schoenenberger & Alexandre Aebi

Keywords: selfish DNA, CRISPR-Cas9, biased inheritance, ecological engineering, biosafety

The existence of selfish genetic elements able to increase the chances to be inherited has been known for a long time (e.g. the bacterium *Wolbachia* is a naturally occurring selfish genetic element spreading in a population via cytoplasmic incompatibility; See chapter 2) and it has been proposed that such elements might provide the basis to design gene drives able to spread engineered genes through populations. Such genes may bias inheritance by cutting the corresponding allele on the homologous chromosome, which induces the cell to use them as a template when it repairs the break by the HDR mechanism. In contrast, the propagation of *Wolbachia* in the target population relies on the fitness advantage provided by the endosymbiont to its host (See *Wolbachia* chapter 2) This results in the duplication of the gene thus converting heterozygotes into homozygotes for the given locus, and creating a gene drive element that spreads through populations (Esvelt et al. 2014). Until recently attempts to produce practical models or applications of gene drives by the engineering of homing endonuclease genes (HEG, which are selfish genetic elements capable of inserting copies of itself into homologous chromosomes) or the use of ZFNs or TALENs were hindered by targeting and stability problems (Simoni et al 2014). The use of the CRISPR-Cas9 system to build RNA guided gene drives, allows to overcome these problems and seems to be the ultimate tool to produce autocatalytic mutations that spread from the chromosome of origin to the homologous chromosome at high efficiency (97% in *Drosophila melanogaster* and >99% in *Saccharomyces cerevisiae* over successive generations) in what has been creatively called the "Mutagenic Chain Reaction" (Figure 4.1.)(Ganz and Bier 2015, DiCarlo et al. 2015). The high efficiency and likely evolutionary stability of CRISPR-CAS9 based gene drives, indicate the suitability of the technology for eventual real-world applications.

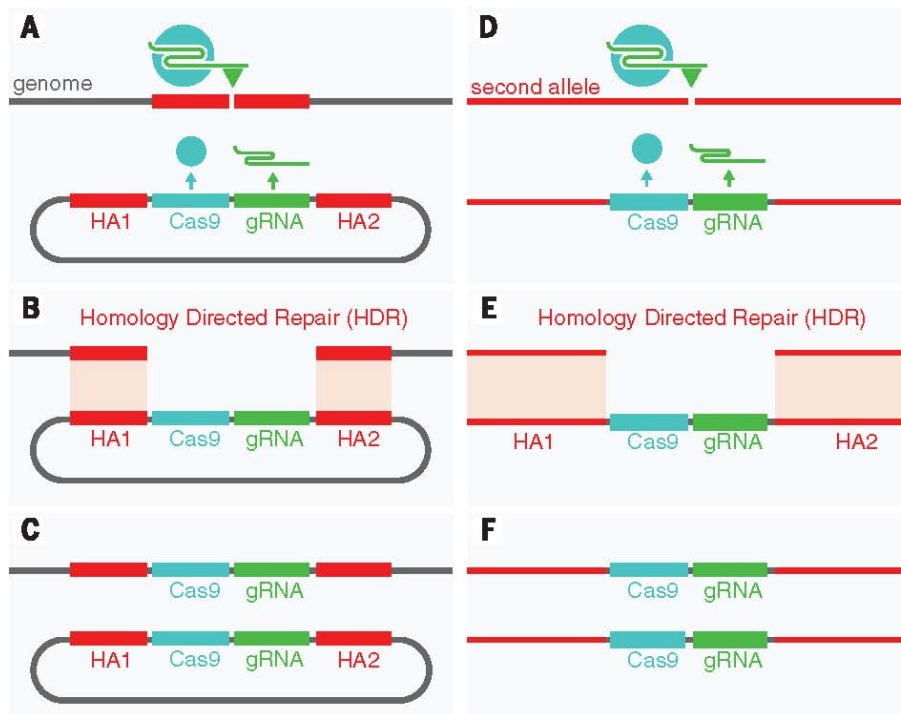


Figure 4.1. Scheme of a CRISPR-Cas based gene drive. (A) A plasmid inserts the Cas9-gRNA sequence into the targeted locus via HDR [(B) and (C)]. (D) The inserted sequence expresses both Cas9 and the gRNA, leading to cleavage (D) and HDR mediated insertion of the sequence into the second allele, thereby rendering the mutation homozygous [(E) and (F)]. HA1 and HA2 denote the two homology arms that directly flank the gRNA directed cut site necessary for first integration of plasmid DNA (Source: Ganz and Bier 2015)

By inserting multiple gRNA sequences aiming at different regions of the target gene into the Cas9-gRNA sequence, the cutting frequency of a targeted gene can be increased, and more efficient CRISPR-Cas9 based gene drives can be created. The competition between HDR and NHEJ pathways might be manipulated in favor of HDR, the pathway that is essential to create the drive, by exploiting the ability of Cas9 to modulate gene expression. The NHEJ pathway would not lead to the copying of the drive but would lead to indels. The target sequence would thus be changed and the gRNA would not be able to recognize it any more, in other words the NHEJ pathway would ultimately lead to resistance against the drive. The copying step of the gene drive may occur immediately after fertilization or later during development in the germ line cells that are precursors of the gametes, leaving somatic tissue heterozygous for the drive (Esvelt et al. 2014). Important limitations of the system are the facts that gene drives will only function in sexually reproducing species (which excludes all prokaryotes, protists and other clonally reproducing groups) and a drive will take several generations until it affects a population (Oye et al. 2014). The time required to spread to all members of a population depends on the number of modified individuals released, the impact of the drive on fitness, the efficiency of homing (i.e. the ability for the drive to be inserted into the receiving DNA filament), the generation time and the reproductive ecology or breeding system of the species (like for *Wolbachia* bacteria, see chapter 2) (Esvelt et al. 2014). On the other hand high efficiencies achieved with CRISPR-Cas9 based gene drives in fruit fly (Ganz and Bier 2015) and yeast (DiCarlo et al. 2015), suggest that RNA-guided gene drives might work in several of the sexually reproducing species that have already been engineered with CRISPR-Cas9.

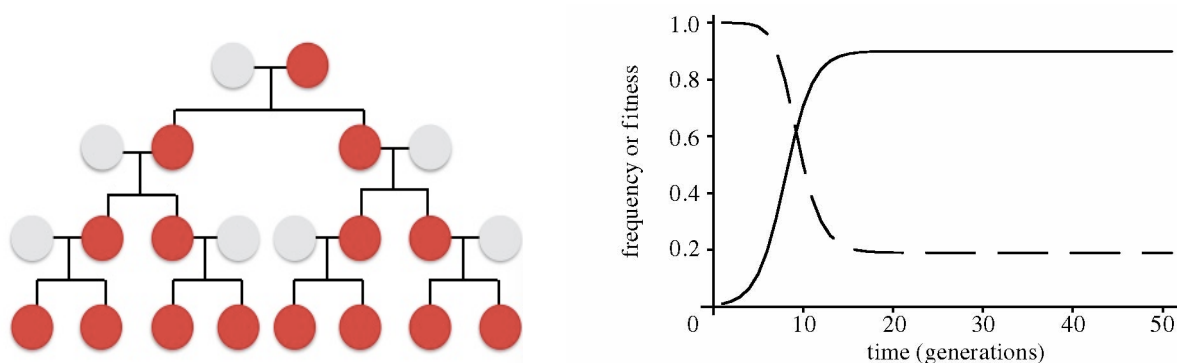


Figure 4.2. Left: genealogical tree of a gene drive where "red" is transmitted to 100% of the progeny (Source: www.igtrcn.org). Right: if a drive containing a recessive lethal gene (or knockout) is released into 1% of an idealized randomly mating population, then it may take only 12 generations for the load (expressed as the fraction of dying zygotes) to reach its equilibrium (with 4/5 of the zygotes dying)(Source Burt: 2003)

The use of gene drives aims first of all at addressing several major environmental or health problems such as vector-borne diseases, the rise of pesticide resistance or invasive alien species (see following case studies in chapters 5 and 6). The idea is to engineer gene drives that reduce fitness of the individuals carrying the drive or that are lethal to their progeny. Such a gene drive may disrupt a native gene or carry a transgene, and if it were inserted into the germ line of a species, it would theoretically allow the efficient population-wide delivery of the genetic modification even if the number of individuals released were a small proportion

of the target population. Approximately all offspring of the released individuals will inherit the modification, which will then spread exponentially through the population. In fact, if the gene drive represents a lethal genetic load capable of eradicating a population, it can be imposed in less than 20 generations (Burt 2003) (see figure 4.2.). However, it will likely take months to years to learn how to insert a gene drive into the germ line of a species and to optimize its function and several practical issues need to be overcome before gene drives will be considered for any application (see chapters 5 and 6). Gene drives might be applied in a modular and combined way, e.g. immunizing drives could be designed to protect native populations of a species from a suppression drive which aims to eradicate the invasive presence in other biogeographical areas. Precision drives may target only subpopulations of a given species. Sensitizing drives could be used to render target populations sensitive to particular chemicals, that can be used as pesticides once the population is made sensitive (Figure 4.2). However, as biological systems are complex many of these examples will be probably turn out to be shortfalls (Esvelt et al. 2014).

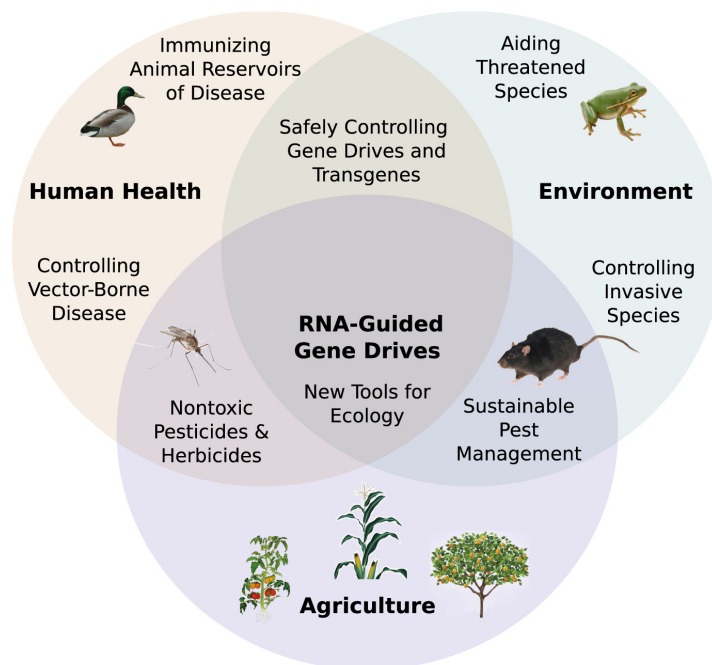


Figure 4.2. Potential applications of RNA-guided gene drives. Clockwise from left. Disease vectors such as malarial mosquitoes might be engineered to resist pathogen acquisition or eliminated with a suppression drive. Wild populations that serve as reservoirs for human viruses could be immunized. Drives might spread protective genes through threatened or soon-to-be-threatened species such as amphibians facing the expansion of chytrid fungus. Invasive species might be locally controlled or eradicated. Sensitizing drives could restore herbicide sensitivity in resistant weeds. Gene drives could test ecological hypotheses concerning gene flow, sex ratios, speciation, and evolution. Technical requirements for these applications vary with the drive type required (Source: Esvelt et al. 2014).

4.1. Risks

This highly invasive technology bears substantial risks, particularly in case of unintended releases of experimental gene drives into the environment, as it has the potential to actively spread unwanted genes across individuals and populations at high speed (Ganz and Bier 2015). Evaluating the risk of unintended laboratory escape needs a case-by-case approach. Scientists experimenting with CRISPR-Cas9 which are not working with gene drives should consider the risk of unwillingly creating a gene drive if they are delivering a *cas9* gene and a gRNA coding DNA sequence which might be inserted into the hosts genome too close to the site targeted by the gRNA (DiCarlo 2015). However, if gRNA or a plasmid coding for it is injected into a transgenic embryo expressing Cas9, the possibility to accidentally generate a gene drive is ruled out as sequences coding for the gRNA are not inserted into the hosts genome, and will therefore not end up close to Cas9 genes or in the cleavage site (Akbari et al 2015).

Altering or eliminating an entire population may have important and irreversible consequences like effects on higher trophic levels along the food chain, the emergence of other pests or resistances, or other ecosystemic effects (see chapter 2 on *Wolbachia*). A gRNA could mutate and spread drives to target other regions of the genome with unpredictable effects. Consequences of releasing functional gene drives would be carried by local or even global communities (Esvelt et al. 2014). Thus, the decision to release gene drives needs to be done collectively (Di Carlo et al. 2015). The chapter 1 on biological control and Nicola Schönenberger's work on genetically modified plants spreading along Swiss railways (Schönenberger and D'Andrea 2012) highlight the need of harmonized regulations to regulate the introduction of biological control agents or organisms carrying gene drives in the environment.

Similarly to biosafety research in other fields, environmental risk assessment of gene drives will have to consider the effect on the target species in its ecosystem, eventual gene flow to non target sexually compatible species, eventually horizontal gene flow or direct toxicity of the Cas9 enzyme or of any other altered metabolite (Oye et al. 2014).

The ease-of-use and accessibility of CRISPR-Cas9 may rise concern over potentially malicious use of gene drive applications (dual use) and leads scientists to claim for a precautionary approach. Analogous to the classification of instructions on how to make nuclear weapons, instructions on engineering gene drives into the germ line may not be disclosed, claims David Gurwitz (2014).

4.2. Risk management

Stringent containment measures are needed during laboratory experimentation and tests on drives capable of spreading into the environment should be avoided as much as possible. Experts agree that any single confinement measure may fail, and recommend that any future studies on RNA-guided gene drives shall use a combination of stringent biosafety measures suffering from independent vulnerabilities in order to make their combination multiplicative

in terms of safety. Measures that are not vulnerable to human error need to be included, e.g. the use of forms of molecular confinement in combination with ecological confinement like the performing of research in places where wild populations of the modified species do not exist or where the species would not survive local environmental conditions. Other confinement strategies include reproductive barriers such as the use of laboratory model organisms that are unable to breed with their wild counterparts, physical barriers like the ones used in biosafety level laboratories for research on pathogens (Akbari et al. 2015), and the training of personnel and adherence to standard operating procedures (Benedict et al. 2008).

For laboratory model systems, simple methods of molecular containment have been described and used, i.e. the targeting of synthetic sequences in model organisms that are not present in wild populations, or the physical separation of drive components (e.g. by adding gRNA at each generation). As a possible safeguard to counteract unwanted releases or adverse ecological effects it has been suggested that during testing of standard drives, reversal or corrective drives able to restore the original phenotype should be simultaneously designed and tested (DiCarlo et al. 2015). However, the genotype would be irreversibly altered, as sequences encoding Cas9 and the gRNA would remain in the "corrected" genomes. Even immunizing drives can be imagined, which are drives that spread into a population altering target sites of other, unwanted drives (Esvelt et al. 2014).

In any case, long-term studies to evaluate the effect of a gene drive on a target population and ecosystem would need to be carried out. Environmental samples of investigated species may be monitored and screened for the presence of the drive. Moreover, the potential for dual use should be assessed (Oye et al. 2014)

4.3. Uncertainties and open questions

One of the most challenging conditions for a gene drive to occur, is to make sure that the cleaved sequence is repaired through HDR, and thus the drive copied, rather than through the competing NHEJ repair mechanism. The frequency of HDR greatly varies across species, cell types, developmental stages, and even the phase of the cell cycle. The efficiency of copying of an endonuclease gene drive may vary as much as from 2% up to 99% (reviewed in Esvelt et al. 2014, DiCarlo et al. 2015). Whenever indels alter the target sequence, which may occur after a cleavage is repaired by NHEJ, the allele becomes resistant to the drive. Resistance may evolve fast and resistant alleles become quickly abundant (Esvelt et al. 2014). Resistance might be hindered by designing drives that target multiple sites (multiplexing) in the same gene, in order to lower the probability that a resistant allele will survive long enough to accumulate mutations on all the cutting sites (Burt 2003). In very large target populations it may be necessary to release several successive drives each targeting multiple sites to overwhelm resistance as it emerges (Esvelt et al. 2014).

Besides stability of a drive, also fitness costs, naturally evolving resistances, or horizontal transmission remain open questions (Burt 2003). Also molecular factors such as homing efficiency, cleavage, and specificity must be considered. Off-target cutting may severely reduce fitness of the organism. Also the exact phenotype of the organism carrying the drive needs to be elucidated. In fact, scientists have minimal experience in engineering biological systems for evolutionary robustness (Oye et al. 2014).

Although it is clear that regulatory changes regarding biosafety of gene drives lack behind the speed of research (Ledford 2015), the National Research Council of the National Academy of Sciences has recently formed a panel to review and discuss possible field releases of organisms carrying gene drives relying on genome editing techniques. In particular the committee shall:

- assess environmental and other hazards to target and non-target organisms
- identify key scientific techniques for reducing risks and consider appropriate mitigation strategies, such as reversal drives
- examine the roles and adequacy of institutional biosafety committees, regulatory authorities, and international frameworks
- discuss relevant legal, social, or ethical considerations in selecting sites for field releases
- provide general guidance on responsible practices in gene drive research from laboratory to release

(Source: <https://www8.nationalacademies.org/cp/projectview.aspx?key=49717>, accessed on the 13.1.2016)

A list of questions raised was put together at the end of the case studies on genetically modified mosquitoes (chapter 5) and herbicide susceptibility restoration in wild plants (chapter 6).

Chapter 5

Could gene drives be used against the tiger mosquito *Aedes albopictus* in Switzerland?

Nicola Schoenenberger & Alexandre Aebi

Keywords: pest management strategy, sex ratio bias, population replacement, population suppression, environmental risk assessment, *Aedes albopictus*.

5.1. The model system

The tiger Mosquito *Aedes albopictus* Skuse, its European distribution, invasion dynamics and the health hazards it poses as a vector for human infectious diseases, are described in chapter 2. To date no gene drive has been engineered into *Aedes albopictus* to our knowledge, and most efforts in genetically modifying vector mosquitos with the aim of lowering health hazards have focused on work with malaria vector mosquitoes or arbovirus-transmitting species other than the tiger mosquito, such as *Aedes aegypti*, *Anopheles gambiae* or *Anopheles stephensi*.

Different strategies based on genetic engineering may be employed to avoid the spread of epidemic pathogens by mosquitoes. A classical pest management strategy called the sterile insect technique (SIT) is being developed and tested in the field to control *Aedes aegypti* (Harris et al. 2012). It consists in the inundative release of millions to billions of sterile GM-male mosquitoes, which do not produce viable offspring when they mate with wild females with the aim of suppressing populations to levels that do not support pathogen transmission. Field releases of GM-male mosquitoes developed by the UK company Oxitec have occurred in the Cayman Islands, Malaysia, Brazil and Panama. However, the efficiency of SIT by the use of Oxitec's patented OX513A GM-*Aedes aegypti* strain in suppressing wild mosquito populations or reducing Dengue fever still remains to be approved scientifically, and Panama, Malaysia and the Cayman Islands stopped releases after preliminary trials (GeneWatch UK 2015). A further approach relies on the idea to disrupt essential genes for female fertility thus reducing the reproductive ability of the insect. They base on the release of insects carrying dominant lethal genes (RIDL) that are sex-specifically expressed, i.e. only in females. The inheritance of the dominant gene would cause only males to survive leading to the collapse of the population over time (Franz et al. 2014).

Vector mosquitoes may be rendered resistant against a pathogen by manipulating genes, which are related to vector competence, i.e. the genetic capability to serve as a host for the development of the pathogen. Such anti-pathogen effector genes may then be spread through populations in what is called the "population replacement strategy" by the use of gene drives (Benedict et al. 2008). Genes conferring immunity against the parasitic protozoan *Plasmodium falciparum* have been recently described in the malaria vector mosquito *Anopheles gambiae* (Li et al. 2015). In the arbovirus transmitting and easy-to-manipulate *Aedes aegypti* (the principal yellow fever vector) targeted gene disruption has been achieved by the use of ZFNs, TALENs and CRISPR-Cas9, opening the perspective of understanding and manipulating vector competence genes (Dong et al. 2015) or engineering gene drives. In fact, this close relative of the tiger mosquito has become an important model to investigate the molecular base of interactions between vectors and pathogens.

Gene drive mechanisms seem to represent the more promising strategies as they insure the quick spread and maintenance of a trait at high frequency across a target population by releasing relatively few individuals only once (Akbari et al. 2013). The so-called Y drive, which is a sex ratio biasing drive, represents a likely candidate (Burt 2014). It is based on the idea to engineer the Y chromosome with a homing endonuclease gene (HEG) that cleaves and destroys the X chromosome during male meiosis, e. g. by targeting conserved and highly repeated sequences in it, causing sperm to contain principally Y chromosomes and thus the offspring of a genetically engineered male with a wild type female would be essentially male. Females would progressively disappear, fewer eggs would be laid at each generation and less females biting and transmitting the disease would be present in the environment, until the

population crashes or becomes extinct (Figure 5.1). Such a system has been developed in *Anopheles gambiae*, where shredding of the paternal X chromosome resulted in fully fertile mosquito lines with extreme male biased sex ratios producing >95% male offspring. At the next generation the produced males again generated sperm carrying mainly the Y chromosome making the effect self-sustaining (Galizi et al 2014). Other suppression drives may be achieved through an approach involving genes that produce infertility only in a homozygous state. A population would crash or become extinct due to the accumulated load of recessive lethal mutations (Burt 2003).



Figure 5.1. Schematic representation of the population level consequences of distorting sex ratios using a gene drive system (Source: www.igtrcn.org).

Highly efficient CRISPR-Cas9 based gene drives that disrupt essential genes for female fertility have been achieved in the malaria vector *Anopheles gambiae* (Hammond et al. 2015). Also in the Asian malaria vector *Anopheles stephensi* a CRISPR-Cas9 mediated gene drive system capable to spread functional parasite resistance genes into target mosquito populations has been produced (Gantz et al. 2015). Although we were unable to find any record of effective gene drives engineered into the tiger mosquito *A. albopictus*, the available strategies aimed at avoiding pathogen spread in other mosquito species and the high pace of research show that it is conceivable that such applications will arise in the future and there might be the desire to use them to try to mitigate the risk of epidemic spread of arboviruses by the tiger mosquito, possibly even in Europe. However, development of an efficient and safe application that would be legally employable and socially accepted will certainly take a long time as technical, regulatory and public acceptance issues may need to be overcome.

5.2. Environmental Risks

The aim of an environmental risk assessment (ERA) is to estimate the probability and magnitude of changes in species and ecosystems and may specifically focus on the likelihood that undesirable or adverse effects arise, i.e. risks. Altering or eliminating an entire population may have important and irreversible consequences. Potential effects on the target species in its ecosystem, the eventuality of gene flow to sexually compatible non-target species, horizontal gene flow and evolutionary consequences need to be assessed (Benedict et al. 2008). In a given ecosystem, the engineered organism may represent a resource for a predator, a consumer, a competitor or a disease vector controlling other species, including non-humans (David et al. 2013). Even getting rid of disease-carrying mosquitoes might have consequences (Hesman 2015) (see environmental hazards listed in the chapter on *Wolbachia* based control strategy, Chapter 2). A long-term reduction of insect abundance can entail important consequences for interacting species or provoke cascading effects, like the disruption of food webs or other functional traits, which can negatively impact communities and ecosystems. Bats, birds, fish, spiders and aquatic arthropods that eat insects could lose a valuable food supply, blood-feeding insects being particularly protein rich. Environmental risk assessment of gene drives will also have to consider the effect on the emergence of other pests or resistances (Oye et al. 2014). In fact, the displacement of populations of one mosquito species might favour outbreaks of competitor species (David et al. 2013). In North America, competition by invasive tiger mosquito can reduce established *Aedes aegypti* populations (Juliano and Lounibos 2005). However, tiger mosquito does not seem to hybridise with other related *Aedes* species (Leahy and Craig 1967) (see chapter xxx on biological control and hybridization risks).

In human population, regular exposure to a pathogen favours acquired immunity, and vector suppression or replacement can result in a reduction of such immunity allowing resurgence of the disease. Moreover, reduction in transmission may result in loss of social immunity (or herd immunity), the phenomenon for which few individuals that are not immune are protected by the fact that a large proportion of the population is immune and thus avoid further spread of the disease. For example recent resurgence of Dengue fever in Singapore, where it had been suppressed for 15 years, was partially attributed to loss of social immunity (Kyle and Harris 2008). Pathogen or vector evolutions are important issues, and the most unwanted outcome would be to select for parasites that are more virulent than their predecessors. This might occur when an engineered increase of the mosquito's immune system against the pathogen triggers increased immunosuppression induced by the pathogen. Even poor capacity of a vector to transmit a disease might select for higher virulence resulting in more severe disease (David et al. 2013). In fact, models of different GM mosquito strategies show that with reduced vector density and transmission, virulence of the Dengue virus may decrease or even increase (Medlock et al. 2009).

In the case of mosquitoes carrying CRISPR-Cas9 based gene drives, direct toxicity of the Cas9 enzyme or of any other altered metabolite may be an issue (Oye et al. 2014), as sometimes Cas9 appears to be toxic to mosquitoes increasing embryo lethality (Gantz et al. 2015, Hesman 2015). Moreover, even if sex ratio biasing gene drives are only transmitted by males, the presence of GM-Females can not be ruled out and the hazards of allergic responses of humans to any novel protein injected through mosquito bites need to be assessed (Reeves et al. 2012).

Risk assessments of GM-mosquitos engineered with a gene drive to replace populations should include testing for adverse effects and take into consideration following aspects (Benedict et al. 2008, McNaughton 2012):

- Increase in the incidence of non target pathogens (the tiger mosquito can transmit up to 26 arbovirus species (see chapter 2 on *Wolbachia*), alteration in blood feeding or oviposition behaviour
- Alteration in the virulence of the pathogen
- Alteration in the phenotype, in population dynamics and life traits (longevity, mating behaviour, sex ratio)
- Alteration of the fitness
- Alteration of the immunogenic profile of salivary gland proteins (an indication of changes in vector competence)
- Testing of the persistence of the transgenes in the environment and of the potential for horizontal gene transfer, in particular to microorganisms that live in close contact with the mosquitoes
- Testing for hybridization to closely related species (see chapter 1 on hybridization risks associated to biological control)
- Evolutionary stability of the gene drive. Occurrence of gene silencing, off-target effects, insurgence of resistance against the gene drive or evolution of the drive towards new target sites in the genome, accumulation of different drives over time. Gain of function or loss of function
- Predicted rate of spread into the local vector population, importance of human mediated accidental dispersal
- Understanding of the socio-political context, community expectations and requirements for engagement and authorization

Instruments for environmental monitoring and remediation should be made available like:

- Assays to detect GM material for monitoring purposes
- Methods to test for vertical and horizontal gene transfers
- Strategies for remediation of adverse effects for target and non target organisms
- Possibility to eradicate the GE-mosquito species if adverse effects arise (e.g. with insecticides)

To conclude, for a solid ERA, sound ecological knowledge and information on ecosystem functions are required (David et al 2013). ERAs based on generic considerations of GM insects are often of limited scientific value, as they should be developed on a case-by-case basis and with a stepwise approach (Figure 5.1). Pre-release publications of a complete ERA should also include public consultations (see chapter 2 on *Wolbachia*). A credible EIA of GM-insects requires a multi disciplinary approach in consultation with unbiased molecular biologists, medical entomologists, immunologists, ecologists and social scientists. Reeves et al. (2012) provide a checklist that allows non-specialists to assess the scientific quality of ERAs and regulatory release approvals.

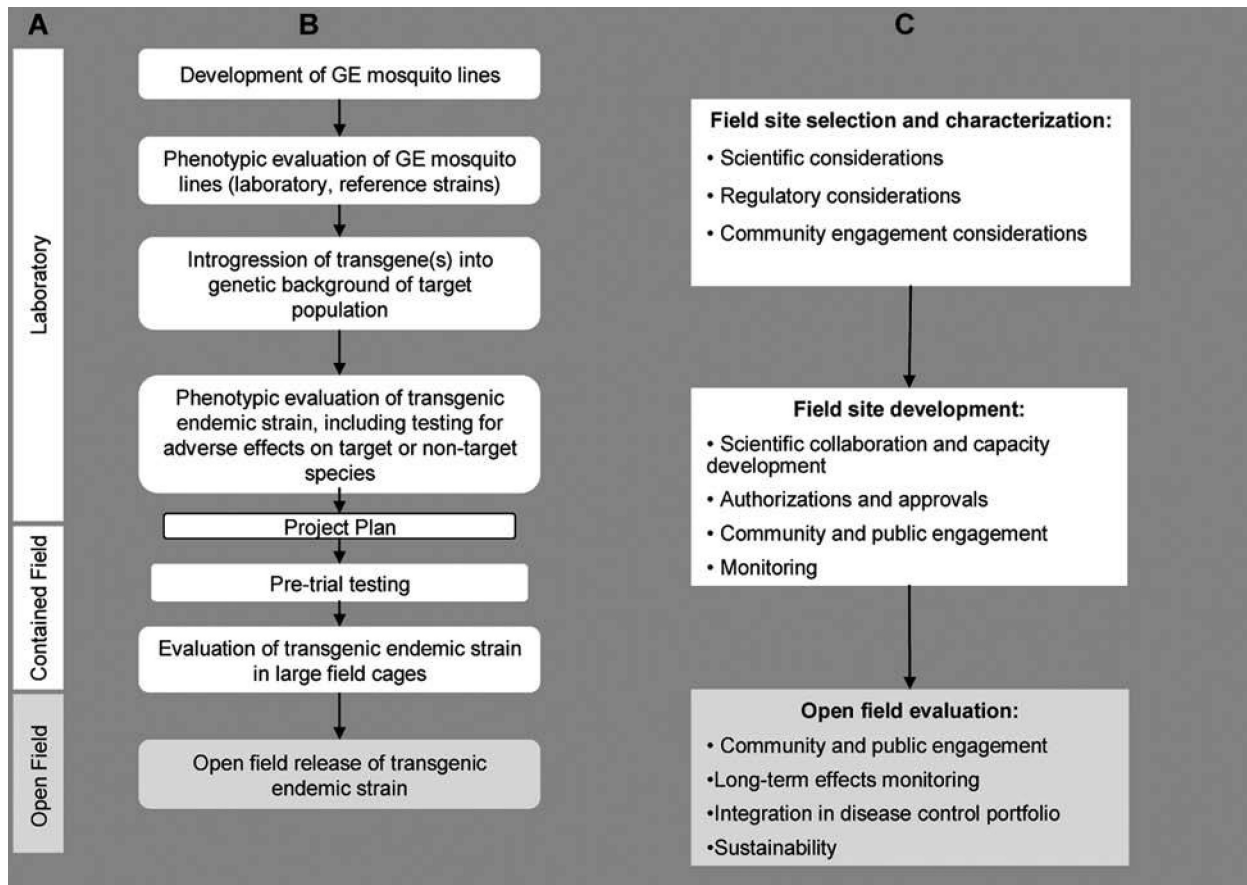


Figure 5.2. An integrated stepwise pathway for the testing of GM mosquitoes, from the laboratory to open field releases (Source: Benedict et al. 2008)

In the hypothetical case of a Swiss release of a tiger mosquito containing a gene drive, several of the previously illustrated aspects, like e.g. the ecosystemic effects or pathogen evolution, would hardly apply, as tiger mosquito is an invasive alien species exclusively restricted to anthropogenic habitats, which just recently arrived in Switzerland (in 2003) where infectious diseases transmitted by mosquitoes are absent (Wymann et al. 2008).

5.3. Uncertainties and open questions

It is impossible to predict if a tiger mosquito strain engineered with a gene drive system to counter spread of diseases will ever be developed or released in nature. The technology is at it's beginning, the unknowns are numerous (see chapter 3 on gene drives), and we are far from any real world application. At present in Switzerland the tiger mosquito has only stably colonised the urban areas of Canton Ticino at low altitude where control measures are implemented. Thanks to insecticide treatments (mostly products for biological control based on *Bacillus thuringiensis israelensis*) in small water bodies as e.g. gullies, the mosquito spreads northwards only slowly and the population densities remained unchanged in the last few years (GLZ 2014). These intensive efforts aim at keeping the population densities below a threshold level in order to avoid the risk of a Dengue or Chikungunya epidemic. However, if tiger mosquito populations were to reach densities at which they might become a threat for

human health in Switzerland, many experts considering it quite probable (Elias et al. 2015, see chapter 2), if costs for prevention or control measures by the implementation of novel regulations (e. g. the obligation to control tiger mosquito) or the large scale use of insecticides become politically unbearable and alternative technologies based on genetic engineering become available then a scenario with the intentional spread of a gene drive might become more realistic.

So far the complexity of interactions governing the population dynamics of mosquitoes transmitting diseases in the environment is under investigated and several open questions remain before efficient disease management strategies based on genetically engineered species may be applied. Moreover, the knowledge gaps in mosquito ecology are striking, which makes all ecological impact predictions of GM-mosquitoes uncertain (David et al. 2013). An important knowledge gap is the pattern of effective mosquito reproduction, which is fundamental for the success of a gene drive in population replacement or suppression endeavours. All we know is that almost certainly mosquito reproduction is not random, which is exactly what most modelling studies assume, but might rather be assortative - certain phenotypes mating preferentially with one another (Scott et al. 2002). An issue could be the evolution of behavioural resistance that would need to be appropriately managed such as the case observed in a SIT program against the melon fly in Japan where wild females started to reject sterile males in courtships (Koyama et al. 2004). The size of a mosquito population and its genetic structure are essential to assess the spread dynamic of a transgene. Such study should be made in extensive but confined field trials, e.g. in caged containment facilities or even on small islands, and should be performed before any nation or continent-wide environmental release. Such large-scale experiments would enable a stepwise environmental risk assessment approach (Figure 5.2. above). That was the case for the evaluation of the hazards posed by *A. aegypti* containing *Wolbachia*, in Australia (see chapter 2). The outcome of mosquito control by the use of gene drives, which might be a more or less transient population suppression or elimination, depend on the interplay between genetics, i.e. the characteristics and efficiency of the gene drive, and ecological factors, such as competition or life history traits of the species, including generation overlap and generation time (Alphey and Bonsall 2014). Another issue is the often observed substantial reduction of fitness in genetically engineered and lab reared mosquitoes if compared to their wild counterparts, as genetic engineering of new traits is generally linked to metabolic cost (Burt 2014, Franz et al. 2014), challenging the reproductive success of released individuals.

Resistances of the pathogen against anti-pathogen effector genes or resistance of the mosquito against a gene drive may quickly evolve and deserve to be thoroughly researched. In fact, resistance alleles co-evolving with naturally existing sex distorter systems, can limit artificial gene drives distorting sex ratio through rapid insurgence of resistance in female mosquitos (Galizi et al. 2014). We also need to know the extent to which a population has to be reduced or replaced in order to produce the desired health outcomes; in fact dengue researchers don't have a reliable measure for assessing disease risk in *Aedes aegypti* (Scott et al. 2002).

If an efficient gene drive were released in the wild it might eventually be able to spread at the speed predicted by Burt (2003), who stated that a gene drive could be imposed in less than 20 generations in a population. In fact, in the 1950s a selfish DNA transposon called the P element spread itself worldwide without any human intervention and invaded the genome of all wild *Drosophila melanogaster* fruit flies in just a few decades (Engels 1996). This indicates that if an efficient gene drive will ever be released or accidentally escape a research facility somewhere in the world, it might reach Switzerland before any authority will be able assess

and decide over such a release. *Wolbachia* also showed a rapid spread of a cytoplasmic incompatibility inducing strain in fruit flies in California, where a spread of 100 kilometres per year was measured (Turelli and Hofmann (1991)). A similar case occurred with the release of the parasitoid *Torymus sinensis* in Italy, a biological control organism of the chestnut gall wasp that quickly reached Switzerland before an environmental risk assessment could be performed and before the authorities may have been able to decide over an eventual release (see chapter 1). However, by the use of gene drives suppression of sub-populations with limited gene flow between them might be achievable through threshold-dependent drive mechanisms, e. g. when a transgene bearing chromosome undergoes frequency dependent fitness changes, i.e. affecting the hosts fitness negatively only if exceeds a certain frequency in the population. In that case population suppression is initiated only after a threshold frequency of the transgenes in the population has been reached. This could be achieved for instance with a system where two maternally expressed toxins, located on separate chromosomes are each linked to a zygotic antidote able to rescue the lethality of the other toxin. These systems might be desirable in several regulatory and social contexts, as they allow local population suppression, but in case of gene flow to a neighbouring population the transgene would be lost by dilution (Akbari et al. 2013).

5.4. Regulatory gaps

In Switzerland according to the Federal Act on Non-Human Gene Technology (Federal Assembly of the Swiss Confederation 2003), "genetically modified organism means an organisms in which the genetic material has been altered in a way that does not occur under natural conditions by crossing or natural recombination". A tiger mosquito strain engineered with a gene drive, by the use of either HEG or CRISPR-Cas9, which is distorting sex ratio, altering vector competence or carrying a lethal mutation aimed at suppressing or replacing the Swiss tiger mosquito population, would most probably fall under the definition of a GMO. Moreover following the Ordinance on the Handling of Organisms in the Environment (Swiss Federal Council 2008) "the handling of genetically modified organisms in the environment must be carried out in such a manner that it neither endangers human beings, animals and the environment nor impairs biological diversity or the sustainable use thereof, and in particular so that [...] the genetically modified organisms cannot spread or multiply in an uncontrolled way in the environment", which would virtually forbid the release of such an engineered tiger mosquitoes into the environment.

To our knowledge, the only environmental releases of GM mosquitoes done so far concern Oxytec's GM-*Aedes aegypti* SIT strains released in the Cayman Islands, Malaysia, Brazil and Panama (GeneWatch UK 2015). Perhaps unsurprisingly the Cayman Islands remain one of the few areas in the world without any enacted specific legislation regulating the release of living GMOs in the environment. The Malaysian National Biosafety Board, on the other hand, approved the field releases in Malaysia, and the decision was taken with a substantial degree of public and political consultation. However, a risk assessment report by the Malaysian Genetic Advisory Committee highlights key references to support its conclusions that are either scientifically deficient or not publicly available (Reeves et al. 2012).

5.4. Questions raised

Alternative strategies

A classical pest management strategy called the sterile insect technique (SIT) is being developed and tested in the field to control *Aedes aegypti* (Harris et al. 2012). Sterile insect technique is considered to be the only way to eradicate an invasive species. It can be achieved with radiation or cytoplasmic incompatibility inducing *Wolbachia*.

1. Which technology should be chosen to control the tiger mosquito?
2. Which technology will efficiently control the tiger mosquito while not causing harm to the environment?

Environmental risk assessment

Potential effects on the target species in its ecosystem, the eventuality of gene flow to sexually compatible non-target species, horizontal gene flow and evolutionary consequences need to be assessed. Most questioned coined for the environmental risk assessment of *wA. aegypti* could be used when assessing the biosafety of gene drives as *Wolbachia* can be considered as a natural gene drive (see chapter 2). In North America, competition by invasive tiger mosquito can reduce established *Aedes aegypti* populations.

3. Should the pioneering work with *Wolbachia*-based control strategy be a model for the risk assessment of gene drive strategy?
4. By eradicating one disease vector, could we favour the establishment of a new disease vector?
5. 3. Even if the tiger mosquito does not seem to hybridise with other related *Aedes* species (Leahy and Craig 1967). What would happen if a mosquito species carrying a gene drive hybridizes with a native mosquito species? (see chapter 1 on biological control and hybridization risks).

Patents

The patented OX513A Genetically-modified *Aedes aegypti* strain was used in Panama, Malaysia and the Cayman Islands (GeneWatch UK 2015).

6. Whom will belong the intellectual property of a genetically modified insect in Switzerland?
7. Can a firm earn money by developing a technology to diminish a public health concern?
8. Can life be patented?

Environmental impact

The use of anti-pathogen effector genes may be spread through populations in what is called the "population replacement strategy" by the use of gene drives (Benedict et al. 2008).

9. Can introducing another gene drive be the solution to reduce the impact of a first gene drive?

Risk of creating a gene drive inadvertently

Aedes aegypti, a close relative of the tiger mosquito has become an important model to investigate the molecular base of interactions between vectors and pathogens. Scientists are using gene drives for research purposes.

10. How can we mitigate the risk of inadvertently creating a gene drive and releasing it in the environment?
11. Should the use of CRISPR Cas9 (even for fundamental research purposes) be limited to laboratory with a certain biosecurity level?

Health

In human population, regular exposure to a pathogen favours acquired immunity, and vector suppression or replacement can result in a reduction of such immunity allowing resurgence of the disease. Moreover, reduction in transmission may result in loss of social immunity (or herd immunity), the phenomenon for which few individuals that are not immune are protected by the fact that a large proportion of the population is immune and thus avoid further spread of the disease.

12. How certain can we be that gene drive will not cause herd immunity?

Cost-risk-benefit assessment

At present in Switzerland the tiger mosquito has only stably colonised the urban areas of Canton Ticino at low altitude where control measures are implemented. Thanks to insecticide treatments (mostly products for biological control based on *Bacillus thuringiensis israelensis*) in small water bodies as e.g. gullies, the mosquito spreads northwards only slowly and the population densities remained unchanged in the last few years (GLZ 2014).

13. Is the health threat of dengue or chikungunya epidemics severe enough to justify the expenses needed to develop a safe gene drive to reduce the population of the tiger mosquito?

Mosquito ecology

The knowledge gaps in mosquito ecology are striking, which makes all ecological impact predictions of GM-mosquitoes uncertain (David et al. 2013). Knowledge on the size of a mosquito population and its genetic structure are essential to assess the spread dynamic of a transgene. Such study should be made in extensive but confined field trials, e.g. in caged containment facilities or even on small islands, and should be performed before any nation or continent-wide environmental release.

14. Should the ecology of *A. albopictus* be studied in prevision of the development of a gene drive or a Wolbachia based control strategy?

Internationally harmonized regulation

If an efficient gene drive will ever be released or accidentally escape a research facility in central Europe, it might reach Switzerland before any authority will be able assess and decide over such a release.

15. Should international regulation for gene drive be harmonized in Europe?

Need for an interdisciplinary approach?

The Australian environmental risk assessment revealed that “perceptions” was the sub model with the highest likelihood score, meaning that there is a likelihood that the release of *Wolbachia Ae. aegypti* will lead to a widespread perception that the threat of dengue has been eliminated permanently (Murphy et al. 2011)

16. Which disciplines should be mobilized to evaluate what perception actually means (economy, psychology, anthropology, sociology)?

Chapter 6

Could gene drives be used to eradicate invasive alien plants and reverse herbicide resistance in Switzerland?

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Keywords: Herbicide resistance, pesticide use, invasiveness, sensitizing gene drives, suppression gene drives.

6.1. The model system

Invasive alien plants (IAP) are species that are not native to a specific region which have the ability to spread to an extent to cause damage to biodiversity, economy or human and animal health. They have been introduced by human activity, intentionally or accidentally, across biogeographical barriers such as oceans or mountain ridges since the end of the Middle Ages (Kowarik 2010). Globalization and economic growth are important drivers of biological invasions and invasive alien species profit from elements of global change, like climate change or increased nitrogen deposition (Dukes and Mooney 1999). The amount of new IAP species and magnitude of their impacts is destined to increase in the future (Essl et al. 2011). Economic damage caused by invasive alien organisms can be huge. In Europe the estimated costs of managements measures and losses due to damage are estimated to reach 10-12 billion Euros per year (Nentwig 2011). The Swiss Black List of invasive alien plants registers 41 plant species that are threatening biodiversity and ecosystem functions, human and animal health or economic activities such as agriculture, forestry or tourism (Info Flora 2014).

Agricultural weeds are alien or native plant species that colonize and persist in agro ecosystems. Weeds reduce farm productivity by invading crops, smothering pastures and may harm livestock as some can be toxic to animals or provoke injuries. They aggressively compete for water, nutrients and sunlight, and are one of the principal factors for crop yield losses and poor crop quality around the world. In most farming systems they are controlled with herbicides. In fact, herbicides dominate the world market for pesticides, and repeated use of the same active compounds may more or less quickly lead to the emergence of weeds having acquired herbicide resistance (HR) that can be transmitted to the next generation (Libman et al. 2004). The incidence of HR agricultural weeds increases constantly and to day 461 unique cases (species x site of action of the herbicide) concerning 247 species are known to have evolved globally (Figure 6.1.) (ISHRW 2016).

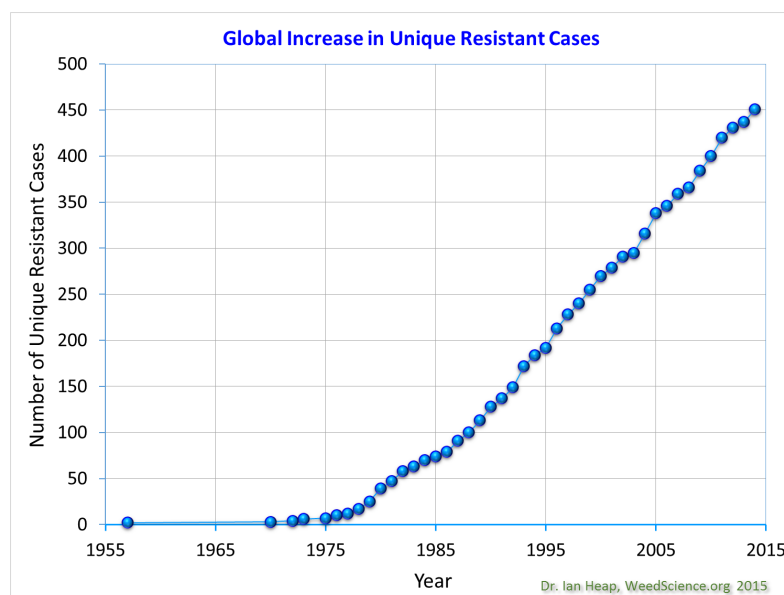


Figure 6.1. Global increase of HR weeds in agricultural systems. Unique resistant cases refers to species x site of action of the herbicide (source: www.weedscience.org, accessed 5.1.2016)

With the rise of HR genetically engineered crops in 1996, despite the often-repeated claims that their use reduces pesticide use, herbicide spray has dramatically increased in the United States, and so has general pesticide use. Thanks to GM crops it became possible for farmers to

use the broad-spectrum herbicide glyphosate in ways that were previously impossible. The impacts are substantial with an ever growing number and geographical spread of glyphosate resistant weeds, which in turn is increasing the use of even more herbicides (Benbrook 2012). In fact, the first glyphosate resistant weed (*Lolium rigidum* Gaudin) emerged in 1996 in Australia, and the number of new glyphosate resistant species is constantly increasing (Figure 6.2).

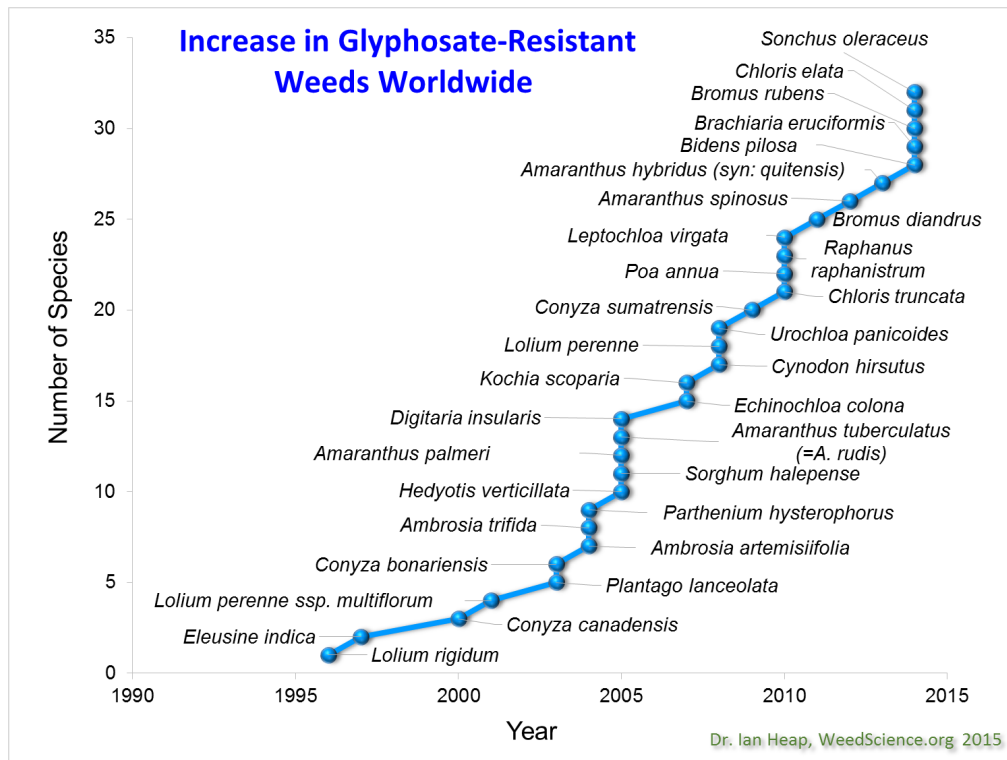


Figure 6.2. Worldwide increase in glyphosate resistant weed species since the first release of GM-HR crops (source: www.weedscience.org, accessed 5.1.2016)

In Switzerland 17 known unique cases of herbicide resistance have appeared in agricultural weeds in the last 40 years (Table 6.1). However, the situation in Switzerland is much less problematic than in other areas of Europe or the world (Delabays et al. 2004). Different factors explain the low incidence of herbicide resistant weeds in Switzerland: a highly regulated agricultural system with an important share of the farms practicing integrated pest management or organic farming, diversified crop rotation schemes (and alternating application of different types of herbicides) and small agricultural plots with refugia that are not submitted to herbicide pressure (Delabays et al. 2004). Moreover, triazine based herbicides such as atrazine were forbidden in Switzerland because of groundwater pollution hazards (FOEN 2013). Thus, HRs against this compound, developed between 1970 and 1990 are now irrelevant as resistances have probably been lost in some if not most of the species over time. On the other hand the emergence of glyphosate resistance in *Lolium multiflorum* Lam., a widely cultivated perennial grass species that reproduces both sexually and asexually shows that HR still is an issue in Switzerland.

Species	Year of detection	Tolerated herbicide
<i>Chenopodium album</i>	1977	Triazine
<i>Amaranthus blitum</i>	1978	Triazine
<i>Amaranthus hybridus</i>	1982	Triazine
<i>Amaranthus retroflexus</i>	1982	Triazine
<i>Chenopodium polyspermum</i>	1982	Triazine
<i>Conyza canadensis</i>	1982	Triazine
<i>Senecio vulgaris</i>	1982	Triazine
<i>Solanum nigrum</i>	1983	Triazine
<i>Amaranthus bouchonii</i>	1986	Triazine
<i>Chenopodium ficifolium</i>	1986	Triazine
<i>Poa annua</i>	1986	Triazine
<i>Stellaria media</i>	1986	Triazine
<i>Senecio vulgaris</i>	1987	Linuron
<i>Galinsoga ciliata</i>	1991	Triazine
<i>Apera spica-venti</i>	1994	Isoproturon
<i>Alopecurus myosuroides</i>	1997	isoproturon
<i>Lolium multiflorum</i>	2011	Glyphosate

Table 6.1. Year of appearance of HR agricultural weed species in Switzerland (source: Delabays et al. 2004, ISHRW 2016).

It has been hypothesized that the use of engineered gene drives could address the issues of environmental and economic damage due to IAP and HR in agricultural weeds. The use of suppression drives may help to eradicate invasive species or sensitizing drives could replace mutated alleles that confer HR in weeds with their ancestral equivalents to restore susceptibility to herbicides (Esvelt et al. 2014, Oye et al. 2014). However, we were not able to find any published work proving the principle that engineered gene drives in plants may work. On the other hand, CRISPR-Cas9 applications promise to change agricultural research (Doudna and Charpentier 2014) and have quickly become the preferred genome editing tool of plant scientists particularly in the development of new crop varieties (Schaeffer and Nakata 2015). For instance in rice an almost 50% transformation efficiency was achieved with the technology, when targeting 11 different genes in a proof of principle study. The genetic changes were stably inherited from the first generation and often found in homozygous state, without new mutations or reversions nor significant off target mutations, as revealed by whole genome sequencing. Genetic modification of crop plants will be much easier than it was with other technologies (Zhang et al. 2014). However, practical implementation of the CRISPR-Cas9 technology can be challenging and deserves proper planning and technical developments, like the tailoring or development of new species-specific vectors to deliver DNA, or methods to deliver RNA or proteins into cells or protoplasts (Schaeffer and Nakata 2015).

6.2. Environmental risks

As it was described previously (see chapter 5) the development of GMOs needs to follow a stepwise approach and environmental risks must be assessed on a case-by-case basis. The key questions raised by Benedict et al. (2008) for mosquitoes are also valid for plants. Potential

effects on the target species in its ecosystem, the possibility of gene flow to sexually compatible non-target species, and evolutionary consequences need to be evaluated (we suggest using the environmental risk assessment applied to *Wolbachia*-based control strategy as described in chapter 2).

Hybridization is a very common phenomenon in plants. In fact, it has been an important force in generating angiosperm species diversity (Soltis and Soltis 2009), and hybridization between species or between disparate source populations may serve as a stimulus for the evolution of invasiveness (Ellstrand and Schierenbeck 2000). Moreover, several if not most domesticated plants exchange genes with their wild relatives at least somewhere in the world and many important weeds are close relatives to the crops whose fields they invade forming so called crop-weed complexes (Ellstrand et al. 1999). In Switzerland at least 12 species of pigweed (*Amaranthus* spp.) are known to grow spontaneously (Info Flora 2016), and HR evolved in four of them (Table 6.1). In the case of a gene drive engineered into one of these species, particular attention should be put on the probability and outcomes of hybridization, as *Amaranthus* spp. frequently form hybrids in Switzerland. The same is true for several other weeds that occur in Switzerland (Aeschimann and Burdet 1994). If ever an invasive alien plant should be suppressed by the use of a gene drive, the probability of propagules spreading to the area where the plant originated and the effects this might have on native populations should be evaluated. In fact one of the primordial characteristics of an invasive plant is the ability to exploit human activity to spread over long distances (Davis 2010). The detection of feral GM oilseed rape spreading along railways in Switzerland, a country that does not import nor cultivate transgenic oilseed rape, may serve as an example to illustrate the possibility of human mediated long distance dispersal of a GMO (Schoenenberger and D'Andrea 2012). As it is the case for several agricultural weeds, the GM seeds arrived as a contaminant of an imported agricultural commodity, in this case durum wheat from Canada (Schulze et al. 2015). This indicates that if an efficient gene drive will ever be released somewhere in the world, the potential exists for it to reach Switzerland before any authority will be able decide over such a release.

6.3. Limitations and uncertainties

An important limitation intrinsically linked to the idea of gene drives is the condition of sexual reproduction and short generation time needed for a drive to spread. In other words gene drives cannot affect species reproducing clonally and are limited in affecting long-lived species. Even species relying on both sexual and asexual reproduction might be skewed towards clonality in presence of a drive in order to avoid suppression (Esvelt et al. 2014).

Agricultural weeds are generally annual species as they synchronize with the cropping cycle. Annual species (therophytes) are by definition reproducing sexually through seeds. However, several weed species are predominately self-fertilized, as it is the case e.g. of *Conyza canadensis* (L.) Cronquist, which would hinder a gene drive to spread through populations as the drive would not be able to affect descendants of other individuals in the population. Thus, gene drives would only work in species with appropriate breeding systems.

Like hybridization, polyploidy, i.e. the doubling of the chromosome number during evolution, is also a very common phenomenon in plants. Perhaps all angiosperms have undergone at least one round of polyploidization in their evolution. Polyploidy is often the direct consequence of hybridization between plants sharing similar genomes, which simply double their chromosomes in order to restore fertility, ending up with multiple sets of chromosomes that are homeologous, i.e. partially homologous as a result of ancestral homology (Soltis and Soltis 2009). For instance *Chenopodium album* L., a species that developed HR in Switzerland (Table 6.1), can be diploid ($2x=2n=18$), tetraploid ($2x=4n=36$) or even hexaploid ($2x=6n=54$). The hexaploid type was issued through hybridization of different taxonomic entities and is thus considered an allopolyploid (Ohri 2015). Extensive genomic mapping and functional genetic studies would need to be made in order to engineer a gene drive conferring susceptibility to a herbicide that would work. The question whether the drive should be engineered on just one set of chromosomes, on two or even all three sets, i.e. in a part or all the homeologous loci should be addressed. On the other hand, weedy and polyploid forms of *Chenopodium album* are characterized by a high level of self-fertilization, which would hinder a gene drive to spread efficiently through populations (Ohri 2015). Moreover, in the case of triazine herbicides, the mutation conferring resistance is located on the chloroplast genome and is thus almost exclusively maternally inherited (Reith and Straus 1987), which would impede a gene drive approach. In fact in order to be affected by gene drives, the target gene should be on the nuclear genome, in order to spread both through male and female gametes. That would be the case e.g. for the acetolactate synthase (ALS) gene, which is located in the nuclear genome and whose mutations confer resistance to sulfonylurea herbicides (Delabays et al. 2004).

Amongst the 41 IAPs listed in the Swiss Black List only 6 species fulfil the basic conditions needed for gene drives to function efficiently, i.e. they both reproduce exclusively sexually and display a short generation time (*Abutilon theophrasti* Medik., *Ambrosia artemisiifolia* L., *Bunias orientalis* L., *Echinocystis lobata* Torr. & A. Gray, *Impatiens glandulifera* Royle and *Sicyos angulatus* L.). The remaining 35 species, and amongst them several of the most severe IAPs like *Reynoutria japonica* Houtt. and *Ailanthus altissima* Swingle, are either long lived or are able to reproduce asexually (Info Flora 2014).

Allison Snow is a plant population ecologist at Ohio State University and a pioneer on environmental risk assessments of GMOs. Her words may be cited as a summary : "All of the benefits and drawbacks to gene drives are just so hypothetical right now". She doubts, for instance, the suggestion that weeds could be gene-drive engineered to eliminate herbicide resistance. "These early predictions are rosy and it is far too soon to say what will happen if such engineered weeds are ever released" (Hesman 2015).

6.4. Regulatory gaps

As exposed in chapter 5 an organism engineered with a gene drive would most probably fall under the definition of a GMO. As the handling of GMOs in the environment must be carried out in a manner that it cannot spread or multiply in an uncontrolled way, the release of such an engineered plant into the environment would most likely not be possible.

6.4. Questions raised

Technology

With the rise of HR genetically engineered crops in 1996, despite the often-repeated claims that their use reduces pesticide use, herbicide spray has dramatically increased in the United States, and so has general pesticide use.

1. Will gene drive reduce pesticide use?

Particular attention should be put on the probability and outcomes of hybridization, as several plant species frequently forms hybrids in Switzerland.

2. What is the probability that a gene drive crosses species barriers and spread into the environment as a consequence of hybridization between a plant carrying a gene drive and a close relative?

This indicates that if an efficient gene drive will ever be released somewhere in the world, the potential exists for it to reach Switzerland before any authority will be able to decide over such a release.

3. Will gene drive regulation be harmonized in Europe?

Polyploidy is very common in plants.

4. The question whether the drive should be engineered on just one set of chromosomes, on two or even all three sets, i.e. in a part or all the homeologous loci should be addressed.

Amongst the 41 IAPs listed in the Swiss Black List only 6 species fulfil the conditions needed for gene drives to function efficiently, i.e. they both reproduce exclusively sexually and display a short generation time (*Abutilon theophrasti* Medik., *Ambrosia artemisiifolia* L., *Bunias orientalis* L., *Echinocystis lobata* Torr. & A. Gray, *Impatiens glandulifera* Royle and *Sicyos angulatus* L.). The remaining 35 species, and amongst them several of the most severe IAPs like *Reynoutria japonica* Houtt. and *Ailanthus altissima* Swingle, are either long lived or are able to reproduce asexually (Info Flora 2014).

5. Is gene drive the best strategy to reduce the impact of HR plants?
6. What would be the alternatives?

Conclusion

The analysis of technologies involving classical biological control, *Wolbachia*-based control strategy and CRISPR-Cas9 gene drive, as means to reduce the impact of crop pest species or disease vectors all involve gene transfers into the environment. This report reviewed current knowledge on these fast-evolving topics. The amount of scientific literature available varied a lot among the subjects and is a measure of the novelty of some of these techniques. The vast literature on environmental risk assessment of biological control agent used around the world since several decades and the likelihood of hybridization between introduced and native closely related species was reviewed. The release of *Aedes aegypti* in which *Wolbachia* was artificially injected to reduce its vector capacity of the dengue and chikungunya viruses were done in Australia for the first time in 2011, followed by releases in Vietnam, Brazil and Indonesia. Only two countries published an environmental risk assessment of this rather new technology. RNA guided endonucleases such as CRISPR-Cas9 is a major breakthrough in biotechnology that was discovered and described in 2013 and then triggered an enormous interest in the scientific community as to date, 1415 scientific papers were published on the topic and articles on the development and use of the technology are published almost weekly in high ranking journals. However, even if the technology has applications in several biotech sectors and even if CRISPR-Cas9 is used by researcher for fundamental research, no insect or plant carrying CRISPR-Cas9 were ever released in the environment.

The classical biological control case study focusing on risks associated with hybridization between introduced biological control agents and native species showed that the laws regulating releases of environment in the environment is suitable to ensure biosafety but that the environmental risk assessment procedure is incomplete. No procedure describes the environmental risk assessment of potential newly formed taxonomic entity as a result of hybridization between an introduced exotic biological control agent and a native species. In such cases, two distinct biological entities would be introduced in the environment and several aspects of their biology would need to be evaluated such as geographical range, host-range, attack of non-target species for example. This case study revealed the need for an harmonized European environmental risk assessment as an organism released in France and in Italy crossed Swiss borders and established in Switzerland and the need to integrate socio-economic aspects in environmental risk assessment procedures. A biology-anthropology analysis showed that the fear of loosing traditions linked with chestnut trees was the main driver of gene introduction into the environment despite sound environmental risk.

The *Wolbachia*-based control strategy of *A. albopictus* example showed that the Swiss laws regulating releases of organisms in the environment seems perfectly suitable to ensure biosafety. The environmental risk assessment scheme developed by Australian researchers seems to be adequate to evaluate the risks even if the technology is probably to new for evaluating its long-term appropriateness. It is interesting to note that *wA. aegypti* can be considered as a natural gene-drive and that the environmental, economic and social risks identified by Australian researchers can be used for future risk assessment of endonuclease based gene drives involving CRISPR-Cas9.

The chapters on the CRISPR/Cas9 technology that could be used to control disease vectors such as mosquitoes, herbicide resistant agricultural weeds or invasive alien plants highlighted potential gaps between law texts in force in certain countries and this rapidly evolving new technology. Biotechnology regulations were written on the basis of biotechnologies developed twenty years ago and will most likely be out of date very soon. To overcome this problem, we encourage regulators to consider a product based regulation rather than a technology-based regulation.

To summarize, this work highlights the need to consider every case involving biological control, *Wolbachia*-based control strategies or future gene drive applications as unique cases, necessitating a precise and thorough evaluation or expertise. We strongly encourage regulators to advocate for case-by-case environmental risk assessment as generalizations, based on a hand full of case studies are insufficient to perform sound risk assessment. Finally, we would like to stress the importance of socio-economics aspects when evaluating the environmental risks associated with the use of technologies such as biological control or gene editing technologies.

Glossary

Admixture: the production of new genetic combinations in hybrid populations through recombination (Allendorf et al. 2001).

Anthropophilic species: a species that needs to live near humans and their infrastructures to survive (Murphy et al. 2010).

Assortative mating: phenotypes mating preferentially with one another.

Backcross: to cross (a hybrid) with one of its parents or with an individual genetically identical to one of its parents (www.thefreedictionary.com)

Biological control: the use of one or several natural enemies of a crop pest species to reduce the impact of the latter by controlling its population.

Classical biological control: the use of an exotic natural enemy to combat an exotic pest.

Conservation biological control: the use of natural enemies, already present in the environment surrounding the crop to control a crop pest species.

Deleterious genes: genes that are harmful for their carrier.

Diagnostic locus: a locus that is fixed or nearly fixed for different alleles in two hybridizing populations.

Endosymbiont: any organism that lives within the body or cells of another organism.

Obligatory -: obligatory symbionts are crucial for the functioning of vital functions of their host such as nutrition or reproduction and their hosts would not survive without them.

Facultative -: facultative endosymbionts, on the other hand, are not essential for the survival of their host.

Extrinsic outbreeding depression: outbreeding depression that results from reduced adaptation to environmental conditions (Allendorf et al. 2001).

First generation hybrid (F1): the first filial generation of offspring of distinctly different parental types (https://en.wikipedia.org/wiki/F1_hybrid, consulted the 13.1.16)

Fitness: describes the ability of an organism to survive and pass on its genes

Genetic mixing: the loss of a formerly distinct population through hybridization (Allendorf et al. 2001).

Horizontal transfer: the transfer of *Wolbachia* from one organism to another species (Murphy et al. 2010).

Hybridisation: Interbreeding of individuals from genetically distinct populations, regardless of the taxonomic status of the population (Allendorf et al. 2001).

Hybrid breakdown: Hybrid breakdown is a type of reproductive failure that appears after the F2 generation of crosses between different species or subspecies (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1470736/>).

Hybrid taxon: an independently evolving, historically stable population or group of populations possessing a unique combination of heritable characteristics derived from two or more discrete parental taxa (Allendorf et al. 2001).

Hybrid zone: an area of contact between two genetically distinct populations where hybridization occurs (Allendorf et al. 2001).

Indel: insertion or deletion of a few nucleotides resulting from the error-prone NHEJ repair after a double stranded break in the DNA.

Interbreeding: any or all of the following reproductive interactions between species: Mate (copulation with or without sperm transfer), hybridize (produce viable or sterile offspring), introgress (transfer DNA sequence between species) (Hopper et al. 2006).

Intercross: all crosses between individuals of different “species” (Allendorf et al. 2001).

Introgression: Gene flow between populations whose individuals hybridize (Allendorf et al. 2001).

Intrinsic outbreeding depression: outbreeding depression that results from genetic incompatibility between the hybridizing taxa (e.g. chromosomal rearrangements that disrupt pairing during meiosis) (Allendorf et al. 2001).

Inundative biological control: Inundative biological control is the repetitive introduction of biological control agents in a greenhouse, year after year to achieve control of a given crop pest.

Invasive species: is an organism that is not native to a specific location (an introduced species), and which has a tendency to spread to a degree believed to cause damage to the environment, human economy or human health (https://en.wikipedia.org/wiki/Invasive_species).

Maternally inherited: organelles or endosymbiont inherited from the mother to the offspring, via the cytoplasm (e.g. mitochondria or *Wolbachia*).

Outbreeding depression: a reduction in fitness in hybrid individuals relative to the parental types (Allendorf et al. 2001).

Parasitoids: an organism that develops inside or on an host, but that kill its host at the end of its development.

Polymerase chain reaction (PCR): a molecular biology method permitting the amplification of a great number of copies of a selected DNA region.

Reproductive isolation: reproductive barriers preventing the production of an offspring between two species.

Prezygotic - : reproductive isolation occurring before the fertilization.

Postzygotic - : reproductive isolation occurring after the fertilization.

Reproductive barrier: mechanisms, behaviours and physiological processes preventing the members of two different species that cross or mate from producing offspring, or which ensure that any offspring that may be produced are sterile (https://en.wikipedia.org/wiki/Reproductive_isolation).

Premating - : reproductive barriers acting before mating.

Postmating - : reproductive barriers acting after mating.

Taxon: a group of one or more populations of an organism or organisms seen by taxonomists to form a unit (<https://en.wikipedia.org/wiki/Taxon>).

Univoltine: an organism that has one generation per year.

Vector competence: the ability of the vector to support pathogen infection, replication and / or development and transmission (nearly always by bite) (Zindell et al. 2011).

Vectorial capacity: vectorial capacity includes vector competence as a factor, but is a field-derived estimate obtained through vector biting rates and vector survival, which can greatly influence disease transmission (Zindell et al. 2011).

wA. Aegypti : *A. aegypti* containing *Wolbachia* :

wA. Albopictus : *A. albopictus* containing *Wolbachia* :

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Annexe 1.

The hazard definition and their likelihood states for the ecology submodels (from Murphy et al. 2012):

Hazards compatible with an environmental risk assessment of gene drives (see chapter 4) are marked with an *. Note that most hazards would be relevant.

***Density**

What is the likelihood that the average density of *Wolbachia Ae. aegypti* (e.g. average numbers per household) will be higher than would occur for naturally occurring *Ae. aegypti*? This could be a result of changes in factors such as:

- Fecundity
- Longevity
- Population dynamics

Same (average density remains the same)

Increases (average density increases)

***Ecological Niche**

What is the likelihood that *Wolbachia Ae. aegypti* will change its ecological niche from being a predominantly domestic species to a broader or alternative niche? Niche changes could be the result of physical, biological, genetic or behavioural changes induced by *Wolbachia*.

Same (niche remains unchanged)

Other (change or broadening of niche)

***Ecology**

What is the likelihood that the release of *Wolbachia Ae. aegypti* leads to adverse ecological impacts? Possible issues include, but are not limited to:

- Reduction of larval and adult *Ae. aegypti* as a food source to predators
- Reduction in detritus removal by larval feeding
- Reduced pollination services by males feeding on flowers for energy
- Horizontal transfer of *Wolbachia*

No Impact (no ecological impacts)

Negative Impact (adverse ecological impacts occur)

***Geographic Range**

What is the likelihood that *Wolbachia Ae. aegypti* will achieve a greater potential geographic range than that of naturally occurring *Ae. aegypti*? Consider the historic range of *Ae. aegypti*, possible constraints on geographic distribution, and whether we

could discriminate between random geographic dispersal or actual adaptation. Possible drivers for this include:

- Better climatic tolerance, e.g. drought or desiccation tolerance
- Changes in host range

Same (potential geographic range would not be greater than expected)

Increases (potential geographic range would be greater than expected)

Invertebrate Transfer

What is the likelihood of horizontal transfer of *Wolbachia* from *Wolbachia Ae. aegypti* to another invertebrate species? Possible routes of transmission may include predation on the mosquito, co-feeding on a plant or animal host, parasitism of mosquito.

Unlikely (little or no chance of horizontal transfer to an invertebrate)

Possible (low to moderate chance of horizontal transfer to an invertebrate)

Likely (High chance of horizontal transfer to an invertebrate)

Vertebrate Transfer

What is the likelihood of horizontal transfer of *Wolbachia* from *Wolbachia Ae. aegypti* to a vertebrate species?

No (no chance of horizontal transfer of *Wolbachia* to a vertebrate)

Yes (chance of horizontal transfer of *Wolbachia* to a vertebrate)

***Avoidance Strategies**

What is the likelihood that people will change their normal mosquito avoidance strategies because of the presence of *Wolbachia Ae. aegypti*? Possible behavioural changes include:

- Increased social avoidance behaviour, e.g. reduced outdoor activities

- Increased household insecticide use

- Removal of breeding sites around household

No Change (avoidance strategies do not)

***Household Control**

What is the likelihood that households in areas containing *Wolbachia Ae. aegypti* will change their expenditure and effort to control mosquitoes because of perceptions about the *Wolbachia Ae. aegypti* mosquito?

Same (no change in household expenditure and control effort)

Decreased (decreased household expenditure and control effort)

***Insecticide Resistance**

What is the likelihood that *Wolbachia Ae. aegypti* will have increased insecticide resistance above the expected level for naturally occurring *Ae. aegypti*?

Same (same levels of insecticide resistance)
Increased (increased levels of insecticide resistance)

***Monitoring**

What is the probability of having a sufficient system in place to monitor *Wolbachia Ae. aegypti* to ensure that the concept of no harm is measurable?
Sufficient (sufficient monitoring will occur to measure)
Insufficient (monitoring will be insufficient to detect change)

***Mosquito Management Efficacy**

What is the likelihood that the efficacy of mosquito management and control efforts will be compromised due to the introduction of *Wolbachia Ae. aegypti*? Consider changes in:
Need for control
Emergence of insecticide resistance
Investment in future research
Household control practices

Same (no changes in efficacy of control measures)
Reduced (reduced efficacy of control measures)

***Need for Control**

What is the likelihood that the release of *Wolbachia Ae. aegypti* will result in the need for increased levels of mosquito control? Take into account whether there may be a need to apply more or greater diversity of treatments.
Same (control effort remains the same)
Increased (greater control effort required)

***Perceptions**

What is the likelihood that the release of *Wolbachia Ae. aegypti* will lead to a widespread perception that the threat of dengue has been eliminated permanently?
Same (perception of dengue threat unchanged)
Reduced (perception of dengue threat is reduced)

The hazard definition and their likelihood states for the “economic effects” submodel were (from Murphy et al. 2012):

***Economic Effects**

What is the likelihood that the introduction of *Wolbachia Ae. aegypti* will result in adverse economic impacts? Take into account:
Tourism
Real estate
Labour availability

Same (no adverse economic effects)
Worse (adverse economic effects occur)

***Health Care**

What is the likelihood that the cost of general community health care will increase over time as a result of the release of *Wolbachia Ae. aegypti*?

Same (community health costs remain unchanged)
Increased (community health costs increase)

***Labour Availability**

What is the likelihood that there is change in supply of labour (permanent and/or seasonal) in areas containing *Wolbachia Ae. aegypti* due to its presence? This includes the possibility of increased labour costs as result of labour shortage.

Same (no changes in availability and cost of labour)
Decreased (decreased labour availability and increased cost of labour)

***Real Estate**

What is the likelihood that real estate or property values will be affected in areas where *Wolbachia Ae. aegypti* is present because of its introduction?

Same (no changes in real estate values)
Decreased (real estate values decline)

***Tourism**

What is the likelihood that international and local tourism will be affected in areas containing *Wolbachia Ae. aegypti* because of its introduction?

Same (no changes in tourism)
Decreased (reduced tourism occurs)

***Host Preference**

What is the likelihood that *Wolbachia Ae. aegypti* takes a higher proportion of blood meals from humans than naturally occurring *Ae. aegypti*?

Same (percentage of meals from humans remains the same)
Increased (percentage of meals from humans increases)

***Feeding Frequency**

What is the likelihood that *Wolbachia Ae. aegypti* takes blood meals more frequently than naturally occurring *Ae. aegypti* due to physiological, behavioural or other changes?

Same (feeding rates remain the same)
Increased (feeding rates increase)

***Dengue Evolution**

What is the likelihood that the dengue virus will evolve to be transmitted more effectively?

Same (Dengue virus does not evolve to be more effective at transmission)

More Effective (Dengue virus evolves to be more effective at transmission)

***Dengue Transmission**

What is the likelihood that the rate of dengue transmission will be increased by *Wolbachia Ae. aegypti* compared with naturally occurring *Ae. aegypti*? Possible issues include:

Ae. aegypti gains increased vectorial capacity for dengue

Changes in *Ae. aegypti* feeding habits

Dengue virus mutation under selection pressure from *Wolbachia*

Same (dengue transmission rates remain the same)

Increased (dengue transmission rates increase)

***Dengue Vector Competence**

What is the likelihood that *Wolbachia Ae. aegypti* becomes a more capable vector of dengue viruses than naturally occurring *Ae. aegypti*? Possible factors include:

Reduced infection barriers to dengue virus

Increased dengue virus growth in mosquitoes

Faster development of dengue virus in *Wolbachia Ae. aegypti*

More effective transmission during feeding

Same (vector competence for dengue viruses remains the same)

Increased (vector competence for dengue viruses increases)

***Increased Biting**

What is the likelihood that *Wolbachia Ae. aegypti* takes blood meals more frequently than naturally occurring *Ae. aegypti* due to physiological, behavioural or other changes?

Same (feeding rates remain the same)

Increased (feeding rates increase)

***Host Preference**

What is the likelihood that *Wolbachia Ae. aegypti* will feed on a greater variety of host animals than naturally occurring *Ae. aegypti*?

Same (host species do not change)

Broadens (greater variety of host species)

***Mosquito Density**

What is the likelihood that the average density of *Wolbachia Ae. aegypti* (e.g. average numbers per household) will be higher than would occur for the naturally occurring *Ae. aegypti*? This could be a result of changes in factors such as:

- Fecundity
- Longevity
- Population dynamics

Same (average density remains the same)

Increased (average density increases)

***Nuisance Biting**

What is the likelihood that *Wolbachia Ae. aegypti* will result in an increased pest status of this species due to an increase in human biting events compared to naturally occurring *Ae. aegypti*? Contributing factors may include:

- Increased tendency to associate with people
- Higher mosquito densities than for naturally occurring *Ae. aegypti*
- Increased biting behaviour (require more frequent blood feeding events)
- Increased tendency to inhabit houses

Same (nuisance status remains the same)

Increased (nuisance status increases)

***Non-Dengue Vector Competence**

What is the likelihood that *Wolbachia Ae. aegypti* will become a better vector of pathogens other than dengue (including viruses, bacteria, parasites) in comparison to naturally occurring *Ae. aegypti*?

Same (vector competence for non-dengue pathogens remains the same)

Increased (vector competence for non-dengue pathogens increases)

***Other Pathogens**

What is the likelihood that the transmission rate of pathogens other than dengue virus (virus, bacteria, parasite) is increased by *Wolbachia Ae. aegypti*. This may arise due to changes in:

- Mosquito density
- Host preference
- Increased vector competence for these pathogens

Same (transmission rate of other pathogens remains the same)

Increased (transmission rate of other pathogens increases)

***Standard of Public Health**

What is the likelihood that the standard of public health overall will be worse as a result of the release of *Wolbachia Ae. aegypti*? Consider:

Arbovirus transmission rates
Nuisance biting
Any other factor affecting public health standards

Same (standard of public health does not change)
Worse (standard of public health declines)

***Wolbachia* Fitness**

What is the likelihood that a genetic change in *Wolbachia* will cause a fitness change in *Ae. aegypti*? Consider fitness to describe the ability of an organism to survive and pass on its genes, but ignore the selective mating advantage provided by Cytoplasmic Incompatibility (CI).

Same (*Wolbachia* will have no effect on *Ae. aegypti* fitness)

Increases (*Wolbachia* will increase *Ae. aegypti* fitness)

Final 50 hazards identified by Stage one workshop (W) and email solicitation from the dengue consultation group (DCG), community engagement (CE) (Adapted from Murphy et al. 2010)

Name	Description
Adverse media	Adverse media coverage (reduces community and/or regulatory and/or institutional support by raising controversy or spectre of GMO).
<i>Aedes aegypti</i> population crash	<i>Ae. aegypti</i> population density crashes, possible local extinction.
<i>Aedes aegypti</i> vectors other arbovirus	<i>Ae. aegypti</i> gains ability from <i>Wolbachia</i> to vector arboviruses that it otherwise would not be able to vector.
All serotypes in circulation	All four dengue serotypes in circulation in same geographic area at the same time.
Change in behaviour	People's behaviour changes to reduce interaction with <i>Wolbachia Ae. aegypti</i> . Includes avoidance, household insecticide use and removal of breeding sites.
Changes in <i>Ae. aegypti</i> behaviour	<i>Ae. aegypti</i> behaviour deviates from naturally occurring <i>Ae. aegypti</i> as a result of <i>Wolbachia</i> .
Changes in herd immunity	Changes in disease epidemiology that adversely affect herd immunity, e.g. result of circulating serotypes which could lead to increased incidence of DHF etc.
Community knowledge	Community have insufficient technical or incorrect knowledge of dengue, <i>Wolbachia</i> and <i>Ae. aegypti</i> to make informed decisions.
Dengue carrier(s) present	As dengue is not endemic in Australia, outbreaks require infected individuals returning from overseas (or possibly other infected area in Australia).
Dengue evolves in response to <i>Wolbachia</i>	Dengue fitness evolves in response to non- or limited transmission by <i>Ae. aegypti</i> to increase its transmission rates
Dengue vector	A dengue vector needs to be present
Ecosystem change	Release of <i>Wolbachia Ae. aegypti</i> results in ecological change
Fitter <i>Ae. aegypti</i>	<i>Wolbachia Ae. aegypti</i> is more likely to pass on genes than naturally occurring <i>Ae. aegypti</i> .
Horizontal <i>Wolbachia</i> transfer	Transfer of <i>Wolbachia</i> to other species (vertebrate or invertebrate) via predation or host feeding events.
Host biting	Host biting needs to occur
Increased biting	Increased biting or number of blood meals required by <i>Wolbachia Ae. aegypti</i> .
Increased control costs	<i>Wolbachia Ae. aegypti</i> populations will require increased or more intensive treatments.
Increased emigration	The rate of emigration from release area increases because of fear of <i>Wolbachia Ae. aegypti</i>
Increased exposure to dengue	Individuals are increasing exposed to potential dengue transmission events.
Increased geographic range	<i>Ae. aegypti</i> increases geographic distribution beyond predicted limits or at a faster than expected rate.

Increased insecticide use	More insecticide use is necessary to achieve same control.
Increased medical care	Cost of community medical care increases above expected values as a result of the release.
Insecticide resistance	<i>Wolbachia</i> provides increased <i>Ae. aegypti</i> insecticide resistance
Larger <i>Ae. aegypti</i> population	<i>Ae. Aegypti</i> population density per unit area increases permanently above current mean.
Lost income	Individual and businesses lose income through reduced real estate, tourism or employment
Lost productivity	Adverse effects on economy as less seasonal or permanent workers available in region.
Metabolic costs of <i>Wolbachia</i>	Metabolic costs of <i>Wolbachia</i> on host.
Natural increase	<i>Wolbachia Ae. aegypti</i> populations undergo a natural; increase in size because of optimal conditions.
New exotic mosquito species	New species is able to establish
New mosquito species arrives	New species arrives (but not established).
New serotype	New dengue serotype evolves
Other arboviruses present	Other arboviruses in circulation at the time.
Perception <i>Wolbachia</i> solves problem	Perception that <i>Wolbachia</i> will solve <i>Ae. aegypti</i> dengue problem.
Predation	Horizontal transfer of <i>Wolbachia</i> to predator species could occur when they feed on the mosquito.
Reduced <i>Ae. aegypti</i> fitness	<i>Wolbachia</i> incurs changes in <i>Ae. aegypti</i> biology that render it less competitive against naturally occurring conspecifics and/or other mosquito species.
Reduced control	Conflict of interest or assumption that <i>Wolbachia Ae. aegypti</i> will reduce Dengue problem, so less investment in control development or control effort.
Reduced Immigration	The rate of immigration into release area decreases because of fear of <i>Wolbachia Ae. aegypti</i> .
Reduced real estate values	Real estate values declines because of fear of <i>Wolbachia Ae. aegypti</i> in region.
Reduced seasonal workers	Reduced numbers of seasonal workers (e.g. backpackers) available because of fear of <i>Wolbachia Ae. aegypti</i> .
Reduced tourism	Tourism declines because of fear of <i>Wolbachia Ae. aegypti</i> in region.
Release site damaged	Risk of proposed release sites (e.g. Gordonvale) being damaged or unable to sustain <i>Ae. aegypti</i> population d.t. extreme event, e.g. flooding, drought, absence of people or habitat.
Social behaviour changes	Social behaviours change directly as a result of the release of <i>Wolbachia Ae. aegypti</i> .
Social vilification	Community divided over release of <i>Ae. aegypti</i> and may target those associated with supporting release.
Vacant niche	<i>Ae. aegypti</i> vacates niche for other species or is uncompetitive against new species.
<i>Wolbachia</i> failure	Risk that <i>Wolbachia</i> does not provide expected reduction in dengue vectoring or provides some other adverse effect
Worse community health	A decline in overall community health from dengue events.

Worse dengue	The overall effects of dengue (prevalence, transmission rate, severity) increase as a result of the release.
Worse ecological impacts	The release of <i>Wolbachia Ae. aegypti</i> results in worse ecological harm than would be expected by naturally occurring <i>Ae. aegypti</i> .

Ordinance on the Handling of Organisms in the Environment (Release Ordinance, RO) of 10 September 2008 (Status as of 1 June 2012)

The Swiss Federal Council,

in accordance with Article 29c paragraphs 2 and 3, 29d paragraphs 2 and 4, 29f, 38 paragraph 3, 39 paragraph 1, 41 paragraphs 2 and 3, 44 paragraph 3, 46 paragraphs 2 and 3, 48 paragraph 2 and 59b of the Environmental Protection Act of 7 October 1983¹ (EPA) with Article 11 paragraph 2, 12 paragraph 2, 14, 17 paragraphs 1, 2, 4 and 5, 19, 20 paragraphs 1-3, 24 paragraphs 2 and 3, 25 and 34 of the Gene Technology Act of 21 March 2003² (GTA) and Article 29a paragraphs 2 and 3 as well as 29d of the Epidemics Act of 18 December 1970³ as well as in implementation of Articles 8 and 19 of the Convention on Biological Diversity of 5 June 1992⁴

ordains:

Chapter 1: General Provisions

Art. 1 Purpose

The purpose of this Ordinance is to protect human beings, animals and the environment, as well as biological diversity and the sustainable use thereof, from hazards or impairment caused by handling organisms, their metabolic products and wastes.

It also aims, during the handling of genetically modified organisms, their metabolic products and wastes, to guarantee consumers' freedom of choice and protect production that does not use genetically modified organisms.

Art. 2 Scope and area of validity

This Ordinance regulates the handling of organisms, their metabolic products and wastes in the environment, in particular the handling of genetically modified, pathogenic or alien organisms.

Handling organisms in contained systems is regulated by the Containment Ordinance of 9 May 2012.

The protection of personnel working with microorganisms is regulated by the Ordinance of 25 August 1999 on Protection of Employees from Dangerous Organisms.

The marketing of pathogenic organisms:

- a. for application as plant protection products in agriculture is regulated by the Plant Protection Products Ordinance of 18 May 20054;
- b. for application as biocidal products, the Biocidal Products Ordinance of 18 May 20055.

For the marketing of alien insects, mites and nematodes for use as plant protection products in agriculture as well as for experimental releases of such organisms, the Ordinance of 18 May 2005 on Plant Protection Products applies.

This Ordinance does not apply to uses of organisms:

- a. in clinical trials on human beings;
- b. which are listed in Annexes 1 and 2 of the Plant Protection Ordinance of 28 February 20016.

Amended by Annex 5 No 10 of the Containment Ordinance of 9 May 2012, in force since 1 June 2012 (AS 2012 2777).

Art. 3 Definitions

In this Ordinance:

- a. organisms means cellular or non-cellular biological entities capable of replication or of transferring genetic material. Mixtures and articles and products containing such entities are also regarded as organisms;
- b. microorganisms means microbiological entities, in particular bacteria, algae, fungi, protozoa, viruses and viroids; cell cultures, parasites, prions and biologically active genetic material are also regarded as microorganisms;
- c. small invertebrates means arthropods, annelids, nematodes and flatworms;
- d. genetically modified organisms means organisms in which the genetic material has been altered by methods of gene technology in accordance with Annex 1, in a way that does not occur under natural conditions by crossing or natural recombination, as well as pathogenic or alien organisms that have also been genetically modified;
- e. pathogenic organisms means organisms that can cause diseases in human beings, livestock and useful plants, in wild flora or fauna or other organisms, as well as alien organisms that are also pathogenic;

f. alien organisms means organisms of a species, sub-species or lower taxonomic level that:

1. as a species, do not occur in Switzerland, the other EFTA and EU member states (not including overseas areas) in their wild form,
2. have not undergone selection for use in agriculture or horticultural production to such an extent that their viability in the wild is reduced;

g. ...

h. invasive alien organisms means alien organisms of which it is known or should be assumed that they will spread in Switzerland and could achieve such a high population density that biological diversity or the sustainable use thereof could be impaired or human beings, animals and the environment could be endangered;

i. handling of organisms in the environment means any deliberate activity using organisms that takes place outside a contained system, in particular culturing, processing, multiplication, modification, experimental release, marketing, transport, storage or disposal;

j. direct handling of organisms in the environment means handling organisms in the environment, not including the handling of therapeutic products, foodstuffs and animal feedstuffs;

k. marketing means the transfer of organisms to third parties in Switzerland for use in the environment, in particular by sale, exchange, giving as a gift, renting, lending or sending on approval, as well as their import for the use in the environment.

Transfer of organisms in order to carry out an experimental release does not count as marketing.

Federal Act on Non-Human Gene Technology (Gene Technology Act, GTA) of 21 March 2003 (Status as of 1 June 2014). The Federal Assembly of the Swiss Confederation,

based on Articles 74 paragraph 1, 118 paragraph 2 letter a and 120 paragraph 2 of the Federal Constitution¹, in implementation of the Convention of 5 June 1992 on Biological Diversity and the Cartagena Protocol of 29 January 2003 on Biosafety to the Convention on Biodiversity, and having considered the Dispatch of the Federal Council dated 1 March 2004 and the Report of the Council of States' Committee for Science, Education and Culture dated 30 April 2005,⁶

decrees:

Chapter 1: General Provisions

Art. 1 Purpose

The purpose of this Act is:

- a. to protect human beings, animals and the environment from abuses of gene technology;
- b. to serve the welfare of human beings, animals and the environment in the application of gene technology.

In particular, it shall:

- a. protect the health and safety of human beings, animals and the environment;
- b. conserve biological diversity and the fertility of the soil permanently;
- c. ensure respect for the dignity of living beings;
- d. enable freedom of choice for consumers;
- e. prevent product fraud;
- f. promote public information;
- g. take account of the significance of scientific research on gene technology for human beings, animals and the environment.

Art. 2 Precautionary and polluter-pays principles

Early precautions shall be taken to prevent hazards or harm that may be caused by genetically modified organisms.

Any person who causes measures to be taken under the provisions of this Act shall bear the costs.

Art. 3 Area of validity

This Act applies to the handling of genetically modified animals, plants and other organisms, as well as their metabolic products and wastes.

For products obtained from genetically modified organisms, only the regulations on labelling and provision of public information (Art. 17 and 18) apply.

Art. 4 Reservation of other laws

More detailed provisions in other federal laws concerning the protection of human beings, animals and the environment from hazards or harm caused by genetically modified organisms are reserved.

Art. 5 Definitions

Organisms means cellular or non-cellular biological entities capable of replication or of transferring genetic material. Mixtures, articles and products that contain such entities are also regarded as organisms.

Genetically modified organism means organisms in which the genetic material has been altered in a way that does not occur under natural conditions by crossing or natural recombination.

Harm: means any harmful effect or nuisance caused by genetically modified organisms to human beings, animals or the environment.

Handling means any activity undertaken in connection with organisms, in particular their production, experimental release, putting into circulation, import, export, keeping, use, storage, transport or disposal.

Putting into circulation means any supply of organisms to third parties in Switzerland, in particular by sale, exchange, giving as a gift, renting, lending or sending on approval, as well as their import; supply for activities in contained systems or experimental release does not count as putting into circulation.

Installations means buildings, traffic routes and other fixed installations, as well as modifications to the land. Appliances, machines, vehicles, ships and aircraft are also regarded as installations.

Art. 6 Protection of human beings, animals, environment and biological diversity

Genetically modified organisms may only be handled in such a way that they, their metabolic products or wastes:

- a. cannot endanger human beings, animals or the environment;
- b. do not harm biological diversity or the sustainable use thereof.

Genetically modified organisms may be released for experimental purposes if:

- a. the information sought cannot be obtained through experiments in contained systems;
- b. the experiment also contributes to research on the biosafety of genetically modified organisms;
- c. they do not contain genes inserted by gene technology which cause resistance to antibiotics used in human or veterinary medicine; and
- d. according to the current state of knowledge, the dispersal of these organisms and their new traits can be excluded and the principles of paragraph 1 cannot otherwise be contravened.

Genetically modified organisms lawfully intended for use in the environment may only be put into circulation if they do not contain gene technologically inserted resistance genes to antibiotics used in human or veterinary medicine, and if experiments in contained systems and field trials have shown that they:

- a. do not harm the population of protected organisms or organisms that are important for the ecosystem in question;
- b. do not lead to the unintended extinction of a species of organism;
- c. do not severely or permanently harm the material balance of the environment;

- d. do not severely or permanently harm any important functions of the ecosystem in question, and in particular the fertility of the soil;
- e. do not disperse or spread their traits in an undesired way; and
- f. do not otherwise contravene the principles of paragraph 1.

Hazards and harm must be evaluated both individually and as a whole and in terms of their interaction; connections to other hazards and harm from causes other than genetically modified organisms should also be considered.

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Dr Nicola Schoenenberger is a natural scientist specialized in botany and plant genetics. His field of activities include research, lectureship and advisory services in plant and agro ecology, risk assessment of GMOs, invasive alien species, and biodiversity and genetic resources conservation. He is a member of several governmental panels and committees on biodiversity, management of natural resources and biosafety. He is also board member of Swiss environmental NGOs.