Haut Conseil des Biotechnologies

SCIENTIFIC COMMITTEE

SCIENTIFIC OPINION

in response to the referral of 12 October 2015 concerning use of genetically modified mosquitoes for vector control[1]

Paris, 31 May 2017

On 12 October 2015 the High Council for Biotechnology (HCB) was asked by the French Minister for the Environment to provide guidance on the use of genetically modified mosquitoes for control of populations of mosquitoes transmitting pathogens responsible for disease.

To address this referral, the HCB Scientific Committee[2] set up a working group[3] of experts, selected for their expertise in the subjects required, under the dual auspices of HCB and the National Centre for Vector Expertise (CNEV).

This opinion was prepared under the chairmanship of Jean-Christophe Pagès on the basis of the report by this working group. It was adopted electronically and forwarded to the competent authorities on 31 May 2017 and published on the HCB website on 7 June 2017.[4]

[1] The referral is reproduced in Appendix 1.

[2] The composition of the HCB Scientific Committee and the procedure for preparing the opinion are given in Appendix 2.

[3] The composition of the Scientific Committee working group and its working methods are given in Appendix 3.

[4] Suggested citation: HCB Scientific Committee (2017). Scientific Opinion of the High Council for Biotechnology concerning use of genetically modified mosquitoes for vector control in response to the referral of 12 October 2015 (Ref. HCB-2017.06.07). (Paris, HCB), 142 pp. Available online: http://www.hautconseildesbiotechnologies.fr.

Erratum on 7 October 2019. In the original text p. 91 "…offspring not inheriting the transgene will be viable and will carry half the genetic material of the laboratory strain…", "half" should read "a quarter of".

Keywords: Mosquitoes, mosquito-borne diseases, vector control, GMO, gene drive, SIT, *Wolbachia*, risk assessment.

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ABBREVIATIONS

Anses: French Agency for Food, Environmental and Occupational Health and Safety

Bti: *Bacillus thuringiensis israelensis*

Cas9: CRISPR-associated protein 9

CHIKV: Chikungunya virus

CI: Cytoplasmic incompatibility

CNEV: National Centre for Vector Expertise

CRISPR: Clustered regularly interspaced short palindromic repeats

DENV: Dengue virus

ECDC: European Centre for Disease Prevention and Control

EFSA: European Food Safety Authority

EID: Interdepartmental Mosquito Control Organisation

FAO: Food and Agriculture Organization of the United Nations

GD: Gene drive

GM: Genetically modified

GMO: Genetically modified organism

gRNA: Guide RNA

HCB: High Council for Biotechnology

IAEA: International Atomic Energy Agency

IIT: Incompatible insect technique

IIT-SIT: Incompatible insect technique combined with low-dose irradiation

InVS: French Institute for Public Health Surveillance

IVM: Integrated vector management

LMO: Living modified organism

MA: Marketing authorisation

na: Not applicable

NHEJ: Non-homologous end joining

PI: Pathogen interference

RIDL: Release of insects carrying a dominant lethal gene

SIT: Sterile insect technique

WG: Working group

WHO: World Health Organization

WNV: West Nile virus

YFV: Yellow fever virus

ZIKV: Zika virus

GLOSSARY

Allee threshold: Density threshold below which a vector population declines without vector control (Suckling et al., 2012).

Allochthonous: A species is considered allochthonous to a given area if it does not originate there, as opposed to an autochthonous species.

Anthropophilic: A mosquito is said to be anthropophilic if it prefers humans over other animals as a blood meal source (McGraw and O'Neill, 2013).

Arbovirus: Arthropod-borne virus.

Arthropods: Phylum of invertebrate animals. Arthropods (from the Greek *arthron,* joint, and *podos*, foot) are characterised by a jointed, segmented body – with the segments having appendages that are themselves jointed – covered by a rigid cuticle. In most cases consisting of chitin, this cuticle acts as an exoskeleton, which is renewed by moulting. Arthropods include the hexapod subphylum (including insects), crustaceans, chelicerates (including arachnids) and myriapods.

Autochthonous: A species is considered autochthonous if it originates in the area concerned, in contrast to an allochthonous or exotic species; autochthonous cases are cases of a disease that a vertebrate host has contracted in the area concerned, as opposed to an imported case (CNEV).

Auto-dissemination: Principle of attracting an insect to a trap (ovitrap or resting trap in the case of mosquitoes) where it will be contaminated with a biocide that it will itself carry to its mates and breeding sites.

Blood-feeding: Refers to animals that feed on the blood of other living animals; refers to parasites or insects that are vectors of parasitic diseases and feed on blood (*Larousse*, '*hématophage*').

Breeding sites: Sites where eggs are laid and larvae and pupae develop. May be natural or artificial (EID Mediterranée).

CRISPR-Cas9: Bacterial enzymatic system consisting of an endonuclease (Cas9) and RNA molecules (CRISPR, tracr) that define the complementary DNA sequence to be recognised and cut by Cas9. Adapted as a tool of molecular biology, this easily programmable system can be used to make targeted modifications (point mutations or transgene insertions) at specific sites in a genome.

Cytoplasmic incompatibility: A phenomenon that results in certain sperm being unable to form viable zygotes with certain eggs (Alphey, 2014); the failure of embryo development as the result of a *Wolbachia*-infected male mating with an uninfected female (McGraw and O'Neill, 2013).

Development stages of vector control techniques: Phase 1: Experiments in controlled laboratory conditions; Phase 2: Experiments in semi-field conditions (contained experimental systems placed in the environment) together with site characterisation, pilot site selection and entomological and epidemiological data-gathering; Phase 3: Experiments in field conditions, on the scale of one or more villages in rural areas or one or more neighbourhoods in urban areas, using comparison with control sites; Phase 4: Area-wide operational releases in the field in actual conditions of use including the relevant conventional vector control methods.

Endemic disease: An infectious disease that is continually present in a population or region (*Larousse*, '*endémie*').

Endemo-epidemic disease: An endemic infectious disease is usually latent, with only a few cases breaking out intermittently. However, it may occur sporadically in a more concentrated fashion, giving rise to endemo-epidemic disease (*Larousse médical*, '*endémo-épidémie*').

Endophagic: In entomology, a blood-feeding arthropod is said to be endophagic if it takes its blood meals inside its host's habitat (French Academy of Medicine, *Dictionnaire médical, 'endophage'*).

Endophilic: In entomology, an arthropod is said to be endophilic if its resting places are inside its host's habitat, such as inside dwellings, cowsheds or pigsties, henhouses, burrows, etc. (French Academy of Medicine, *Dictionnaire médical, 'endophile'*).

Enzootic: A disease regularly affecting animals in a particular geographical area or at a particular season.

Epidemic: Development and rapid spread of a contagious, and usually infectious, disease within a population (*Larousse*, '*épidémie*'). In the context of vector control, the term 'epidemic' refers to spread of a vector-borne disease rather than a 'contagious' disease.

Epizootic: An epidemic affecting animals (*Larousse*, '*épizootie*').

Eradication of a species: One objective of a vector control strategy. Logically global, eradication of a species means the disappearance of all individuals of the species worldwide. This concept must be distinguished from population elimination.

Exophagic: In entomology, a blood-feeding arthropod is said to be exophagic if it takes its blood meals outside its host's habitat (French Academy of Medicine, *Dictionnaire médical, 'exophage'*).

Exophilic: An arthropod is said to be exophilic if it lives outdoors (French Academy of Medicine, *Dictionnaire médical, 'exophile'*).

Fitness: In evolutionary biology, the fitness of an individual or gene describes its relative ability to reproduce, as defined by the change in frequency of the individual's genotype (or the gene) from one generation to the next. It is often estimated as life-time reproductive success (number of offspring of an individual or copy number of a gene) (Lambrechts et al., 2008).

Gene drive: Increasing the odds that a genetic element will be inherited beyond the natural inheritance of 50% described in Mendel's laws, resulting in the spread of this genetic element in a population.

Genetically modified organism: An organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (from the definition in Directive 2001/18/EC; see WG report, Appendix 13, for full definition).

Hemizygous: A diploid cell or organism is said to be hemizygous for a gene if it is present in a single copy, without a corresponding homologous chromosome (this is the case for most genes on X or Y sex chromosomes but also for a gene cassette introduced *de novo* into the genome). If a diploid organism carries two different alleles of the same gene at a given locus (an allele on each of the homologous chromosomes), it is said to be heterozygous for this gene; if it carries two identical alleles, it is said to be homozygous. A transgenic diploid individual carrying one copy of a transgene is hemizygous. It will be homozygous once the transgene has been acquired by both homologous chromosomes.

Homing endonuclease: A protein able to recognise and cleave specific DNA sequences of 12-40 nucleotides, allowing the gene from which it is derived to insert itself (Belfort and Roberts, 1997).

Horizontal gene transfer: Any process in which an organism incorporates genetic material from another organism into its genome without being the offspring of that organism (EFSA, 2013). In other words, horizontal gene transfer results in incorporation into a genome of an organism of a given species of genetic material from an organism of another species.

Incompatible insect technique: Vector control technique using *Wolbachia*'s ability to induce cytoplasmic incompatibility and consisting in release of *Wolbachia*-infected males to sterilise local females. The cytoplasmic incompatibility of males with females is expressed if the males are infected with *Wolbachia* when the females are not or if they are infected with a *Wolbachia* strain incompatible with that infecting the females (M. Weill).

Insects: Class of invertebrate animals belonging to the subphylum Hexapoda (characterised by three pairs of legs) of the phylum Arthropoda. Insects can be winged or unwinged. Winged insects comprise over 25 different orders, including Diptera (characterised by a single pair of wings), which encompasses mosquitoes.

Integrated vector management (IVG): A rational decision-making process to optimise the use of resources for vector control (WHO, 2012). Integrated management implies a thorough understanding of the target population's ecology, the efficacy and interactions of control methods, and the medium- and long-term economic and environmental consequences of control.

Mosquitoes (Diptera: Culicidae): Insect family of the suborder Nematocera of the order Diptera. The mosquito family has over 3500 species and is split into three subfamilies: Anophelinae, Culicinae and Toxorhynchitinae. There are 112 mosquito genera, of which the best-known are *Anopheles* (of the subfamily Anophelinae), *Aedes* and *Culex* (of the subfamily Culicinae) (http://mosquito-taxonomic-inventory.info/).

Nuisance arthropod: In zoology, a nuisance arthropod is an arthropod that bites but does not transmit disease. The concept of nuisance covers discomfort, loss of blood, inflammation due to the bite, and allergic or dermatological consequences of contact with an arthropod (Duvallet et al., 2011).

Pathogen interference: Phenomenon whereby *Wolbachia* can reduce the vector competence of some mosquitoes for some viruses and other parasites (Moreira et al., 2009).

Penetrance: Proportion of individuals of a given genotype showing the phenotype expected of this genotype.

Population elimination: One objective of a vector control strategy. Logically local, population elimination means the disappearance, in a given area, of an isolated population of vectors. This concept must be distinguished from eradication of a species.

Population modification: Vector control strategy intended to reduce the inherent ability of individual vectors in a population to transmit a given pathogen (after WHO (2014), 'population replacement'). The aim is mainly to reduce a vector population's vector competence without necessarily altering the size of the population, as opposed to population reduction strategies.

Population reduction: Vector control strategy intended to reduce the size of a vector population below the threshold required for transmission of a pathogen (after WHO (2014), 'population suppression') without affecting the vector competence of the remaining individuals, as opposed to population modification strategies.

Reservoir: Living organism that hosts and ensures the continued survival of a pathogen that can be transmitted to humans (*Larousse*, '*réservoir*').

Resting places: In entomology, places where adults rest, either after having taken a blood meal in the case of blood-feeding arthropods, or after mating.

Satyrism: Sterilisation of females of one species by males of another species as a result of unproductive interspecific mating. This phenomenon may be natural – it has been observed between *Aedes albopictus* and *Aedes aegypti* in the environment (Lima-Camara et al., 2013) and between tick species such as *Amblyomma variegatum* and *Amblyomma hebraeum* – or artificially encouraged by releases of males of one species into the habitat of the other target species. The latter is referred to as satyrisation, which is the use of the satyrism phenomenon for vector control. Satyrisation has been successfully tested in tsetse flies.

Self-limiting: A vector control technique is said to be self-limiting if its effects are limited in space and time unless application of the technique is maintained. For control techniques involving release of

modified insects, the modification will disappear from the population unless it is reintroduced by regular releases of modified insects.

Self-sustaining: A vector control technique is said to be self-sustaining if its effects spread across space and last over time without needing any maintenance. For control techniques involving release of modified insects, the modification's persistence and spread will depend on the balance between the dynamics of this spread and any selective disadvantage that it confers on the modified individuals.

Semi-field conditions: Contained experimental systems (cages in greenhouses) placed in the environment.

Sexing: Separating individuals according to sex.

Sterile insect: An insect that does not produce viable offspring; an insect that, as a result of a specific treatment, is unable to reproduce (FAO, 2017).

Sterile insect technique: Method of pest control using area-wide inundative release of sterile insects to reduce reproduction in a field population of the same species (FAO, 2017).

Sylvatic: A sylvatic disease or virus occurs in wild animals in forests (Vasilakis et al., 2011).

Sympatric: Related species are said to be sympatric if they live in the same region but do not cross-breed, generally owing to genetic factors (*Larousse*, '*sympatrique*').

Transgenic: An organism is said to be transgenic if it carries a transgene, i.e. a sequence inserted into its genome using molecular biology techniques.

Transinfection: Transfer of a bacterial or viral infection from one host to another by microinjection (McGraw and O'Neill, 2013). *Wolbachia* transinfection: artificial infection of a new host by injection of *Wolbachia* either into adult females or into the egg cytoplasm (Hughes and Rasgon, 2014).

Transposon: Mobile genetic element that has the ability to replicate and spread in a genome (Tu and Coates, 2004).

Tsetse flies (*Glossina*): Diptera of the suborder Brachycera, vectors of trypanosomiasis in humans (sleeping sickness) and in animals (disease causing anaemia in livestock).

Vector: An organism that can mediate the transmission of a pathogen between hosts (Alphey, 2014).

Vector competence: An individual vector's physiological ability to host a pathogen and become infectious (Alphey, 2014). An insect's ability to become infected from a blood meal on an infected host, to allow development of the pathogen and to facilitate its transmission to a new host (Tran et al., 2005). Vector competence depends mainly on genetic factors, gut microbiota, and other factors that are still not properly understood.

Vector control strategy: Choice of a vector control technique or combination of techniques to achieve an objective such as reduction, elimination or modification of a vector population, or eradication of a vector species, each of these objectives being defined separately in this glossary. The strategy also includes the procedure used to apply the technique or combination of techniques.

Vector system: Complex biological system consisting of three components – the pathogen, the vector arthropod (usually several species) and the vertebrate host (usually several species) – and all the interrelationships between them (Rodhain, 2015).

Vectorial capacity: Efficiency with which a vector population can transmit a pathogen (Alphey, 2014). Sum of intrinsic and extrinsic factors, including vector competence, longevity, aggressiveness and abundance (Rodhain and Perez, 1985). Expresses the number of infections that a vector will produce from one infectious case (Fontenille et al., 2009).

Vertical gene transfer: Any process in which a gene is passed to offspring (EFSA, 2013). In other words, vertical gene transfer results in transmission of genes from an organism to its offspring.

Vertical transmission: Transmission of a pathogen to the offspring of an infected female.

Wolbachia: *Wolbachia* (Alphaproteobacteria) are very frequently occurring, maternally inherited, intracellular arthropod bacteria that manipulate their hosts' reproduction in various ways (male-killing, feminisation, parthenogenesis induction, and cytoplasmic incompatibility, used in vector control). All these manipulations of a host's reproduction facilitate production of *Wolbachia*-carrying females, the only sex that transmits these bacteria, and swift spread of *Wolbachia* in host populations (M. Weill).

Zoo-anthropophilic: In entomology, a blood-feeding arthropod is said to be zoo-anthropophilic if it obtains its blood meals from both human beings and animals. The degree of anthropophilia is then determined by estimating the frequency of its meals on human beings compared with those on animals.

Zoonosis: Any disease or infection that is naturally transmissible from vertebrate animals to humans and vice versa (WHO).

SUMMARY[5]

On 12 October 2015 the High Council for Biotechnology (HCB) was asked by the Minister for the Environment to provide guidance on the use of genetically modified (GM) mosquitoes for vector control of populations of pathogen-transmitting mosquitoes. The HCB Scientific Committee has prepared its opinion on the basis of a report by a working group of experts selected for their expertise in the subjects required.[6]

A **review** of the current position regarding mosquito-borne diseases (dengue, chikungunya, Zika virus disease, yellow fever, West Nile fever, malaria and lymphatic filariasis) in metropolitan and overseas France revealed the **lack of therapeutic treatments and vaccines** for most of these diseases and the **limitations of current vector control methods** for populations of mosquitoes transmitting the pathogens responsible for these diseases. To supplement the research being carried out in the medical field, it appears **essential to explore techniques that can substitute for or complement existing vector control methods**.

The Scientific Committee's opinion describes **emerging vector control techniques using GM mosquitoes**, the current state of research into and development of these techniques and the outcomes of initial experiments worldwide. To date, **only one technique has been developed to an operational level:** Oxitec's **RIDL technique**, which sets out to reduce a mosquito population by repeated mass releases of sterilising transgenic males. Two other techniques at an earlier stage of research and development are based on **gene drive**, seeking to spread a genetic trait in a wild population, either to make the mosquitoes incapable of transmitting pathogens (gene drive for population modification) or to eliminate the population by spreading sterility (gene drive for population elimination).

To identify the specific features of these vector control techniques using GM mosquitoes, a **cross-cutting assessment of all existing and emerging techniques** has been conducted with respect to possible objectives, efficacy and sustainability, technical constraints and environmental and health risks. Consideration has been given to both conventional vector control techniques (chemical, biological, physical and environmental) and emerging techniques based on release of mosquitoes, whether GM (RIDL and the different gene drive techniques) or non-GM, irradiated (standard sterile insect technique (SIT)) or carrying *Wolbachia*[7] (incompatible insect technique (IIT) and spread of pathogen interference (PI)). For many of the points considered, the techniques using GM mosquitoes do not constitute a homogeneous group distinct from other techniques but have characteristics in common with subsets of these other techniques.

[5] This summary shall not replace the full analysis contained in this opinion.

[6] The working group was placed under the dual auspices of HCB and the National Centre for Vector Expertise (CNEV).

[7] Intracellular bacteria naturally common in arthropods and endowed with specific properties (cytoplasmic incompatibility (CI) and pathogen interference (PI)) that can be used for vector control.

Techniques using GM mosquitoes are all based on mosquito release. **Techniques based on release of mosquitoes**, whether GM or not, have in common:

- Unprecedented **specificity of action** for vector control, confined to the species of mosquito released and any interfertile species. This specificity has the advantage of **reducing the direct impact of vector control on health and the environment**. It does, however, entail **as many interventions as there are non-interfertile vector mosquito species to be targeted**;
- Issues associated with **potential persistence and invasiveness** of the mosquitoes released and of the modifications that they carry, raising different questions according to the technique's objective (population reduction or population modification, see below). In the case of population reduction, possible residual fertility in released males will have to be considered;
- Issues associated with potential **release of females** (biting capacity, vector competence, fertility, etc.), whether accidental or intentional;
- Efficacy depending on the **field competitiveness** of released mosquitoes in comparison with wild mosquitoes (requiring appropriate rearing conditions amongst other factors);
- **Higher efficacy at low densities** of target mosquitoes, **requiring combination of these techniques with conventional vector control methods** that are effective at high densities, such as biocides, or else use of these techniques outside the seasons when endemic mosquito populations explode or prior to establishment of invasive species;
- An **action time of several months** to achieve the intended objective, entailing **use other than in a health emergency** and as part of integrated vector management with techniques having an almost immediate effect and useable in a crisis situation, such as biocides;
- **Technical and logistical constraints** specific to mosquito rearing and release, with variations according to the **biology of the mosquito species** concerned (adaptation of protocols) and the **vector control strategies** of which mosquito release techniques form part (scale of rearing, need for and duration of maintenance, need to separate males and females).

Techniques can be distinguished according to **possible objective** (population reduction or population modification). Whether or not they use mosquito release, and whether or not the released mosquitoes are GM, **population reduction techniques** have in common:

- An **environmental impact associated with the reduction of target mosquito population density and depending on the target species' role in the ecosystem**. This impact varies according to, amongst other factors, whether the relevant species is autochthonous or invasive, whether its habitat is urban or natural, whether specialist predators exist, the extent to which the population is reduced (simple reduction, local elimination, or eradication of the species[8]), the duration of a technique's effects (depending, amongst other things, on how isolated the treated area is) and the specificity of the technique (techniques involving

[8] The objective of eradicating a species, which would be a specific feature of gene drive techniques for elimination, is theoretical at this stage.

mosquito release being the most specific, see above). Environmental impact will therefore require appropriate assessment according to the technique used, the target species and the region to be treated.

- The potential for **unintended replacement of the target population by the population of another vector species**, which increases the more the target population is reduced and the more this reduction persists over time.
- **Loss of immunity in previously exposed human populations**, to be weighed against the benefit from disappearance of the disease for a time.

Whether or not they use GM mosquitoes, **population modification techniques** have in common:

- **Less of an impact, in principle, with regard to environmental and health risks**, since they should not affect the density of mosquito populations. An assessment of the risks associated with the induced modification is still necessary.
- **Persistence and varying invasiveness of the modifications induced**, with the need to consider the evolution and long-term effects of the factors responsible for these modifications (*Wolbachia*, transgenes), including their potential for transfer to other species.

Two other subsets can be distinguished: self-limiting techniques (with effects that are limited in space and time unless application of the technique is maintained) and self-sustaining techniques (whose effects spread across space and last over time without calling for any maintenance).

Self-limiting techniques, whether or not they make use of mosquito release, and whether or not the released mosquitoes are GM, have in common:

- the advantage of being **controllable and adjustable** in the light of monitoring data,
- the drawback of **calling for demanding maintenance in the long term**.

Self-sustaining techniques, whether or not they use GM mosquitoes, have in common:

- the advantage of **not calling for maintenance** or large-scale infrastructure,
- the drawback of being **fairly inflexible, or even without the possibility of control** (e.g. of intended spread within a species).

There is a continuum of techniques between these two extremes. Moreover, self-limiting or self-sustaining characteristics may vary for a given technique depending on the control strategy of which it forms part (population reduction or population elimination) and the properties of the *Wolbachia* strains used.

With regard to their evolving aspects in terms of **loss of efficacy over time** through (1) development of resistance to their modes of action, (2) development of behavioural resistance, or (3) functional drift, and the consequences of potential evolution in terms of environmental and health risks, the techniques are less easy to classify into sets, since these developments result from the specific operating mechanisms of each technique.

The Scientific Committee nevertheless notes that **techniques operating through a genetic target**, whether they use GM mosquitoes or not, may lead to **development of resistance to their modes of action**. This is the case for certain biocides (whose efficacy has been compromised for whole populations of resistant mosquitoes) and for gene drive techniques (which would limit their invasiveness at present, given their current stage of development). The other techniques, including the RIDL technique, SIT, IIT and other conventional vector control techniques, have mechanisms of action that do not operate through genetic targets or else involve too many targets for such resistance to develop easily. Moreover, given the characteristics specific to each technique, **development of behavioural resistance** is conceivable for the RIDL technique and *Wolbachia*-mediated techniques but unlikely for gene drive techniques. Lastly, the **risk of functional drift** is a possibility for RIDL and for gene drive techniques as well as for *Wolbachia*-mediated techniques. The sustainability of the different techniques must be assessed in the light of these three possible types of loss of efficacy.

Thus the Scientific Committee assessment shows that, for many of the points considered, techniques using GM mosquitoes do not have specific features associated with their GM character but are comparable to other vector control techniques in different subsets depending on the aspects considered. Apart from these similarities, **gene drive techniques are distinguished by their unique invasive potential to date**. While the technical limitations of current gene drive developments are likely to interrupt the spread of gene drive cassettes, assessment of improved applications in the future will have to take into account the particular invasive potential of these techniques and the uncertainties associated with their evolution in the environment, given the speciation events and genome rearrangements found in some cases of natural gene drive.

The HCB Scientific Committee has considered **risk assessment criteria for GM mosquitoes**. While each new dossier must be assessed on a case-by-case basis, the HCB Scientific Committee has not in principle identified any particular environmental risks that could not be covered by the general criteria listed in Directive 2001/18/EC on deliberate release of GMOs in the European Union. It nevertheless notes that **gene drive** has given rise to some **radically new issues**, given the **deliberate invasiveness** of the desired modification, which in theory has the potential to reach all individuals of a species in the environment, whether to eradicate or to modify it. By design, release is not limited in space or time. Risk assessment for gene drive must therefore be adapted to this change in scale and possible objectives. The Scientific Committee has concluded that **the criteria listed in Directive 2001/18/EC, officially applicable to environmental risk assessment for release of GM mosquitoes in the European Union, are scientifically relevant and in principle sufficient for assessment of the risks associated with use of GM mosquitoes for vector control**. As provided for in the case-by-case approach of the directive, **the specific information required for assessment of GM mosquitoes for gene drive must be determined and outlined.** Furthermore, whatever the regulatory status of insects artificially infected with *Wolbachia* bacteria in the European Union, the Scientific Committee believes that assessment using criteria adapted from Directive 2001/18/EC would be relevant.

Lastly, the HCB Scientific Committee has identified **some of the benefits and limitations of use of GM mosquitoes on French territory** for the purposes of vector control.

Apart from the molecular mechanisms at work and some of the specific features ensuing, **the RIDL technique seems closely related to SIT and IIT**. All three techniques could be tested step by step on a precautionary basis for the purpose of contributing to vector control in French territories, depending on the vectors concerned,[9] in combination with the conventional techniques currently used for integrated vector management. **Employing IIT, SIT or the RIDL technique would in particular help reduce insecticide use**. In addition to a lesser risk of exposure for humans and ecosystems, lower insecticide use owing to use of techniques based on mosquito release would preserve insecticide efficacy by lessening pressure for selection of resistance. This would thus enable **insecticide use to be reserved specifically for epidemics and public health emergencies**.

The two gene drive approaches discussed in this opinion are still at an **early stage of development**. Current research is concerned with, amongst other things, reducing evolution of resistance to gene drive, developing a gene drive mechanism whose spread would be limited, and designing tools able to reverse an existing gene drive. Research is also under way into procedures for assessing the long-term effects of gene drive on ecosystems. **At this time the Scientific Committee believes that it is premature to contemplate deployment of gene drive in the environment**. As for the objective of population modification, the alternative approach using *Wolbachia*-mediated spread of PI is already being tested in the field even though PI mechanisms are still not properly understood.

The choice between different vector control techniques or combinations of techniques should therefore be informed by the intended objective, by vector biology and behaviour and by the epidemiological, environmental and socio-economic context, including available human and financial resources. Through this guidance on emerging vector control techniques based on release of mosquitoes, whether GM or not, this opinion should **add to the information on new options available to the public authorities to inform decision-making for an integrated vector management approach**. Practical integration of these options into the range of vector control tools currently used, depending on the specific contexts in the different French territories, would call for additional knowledge to complement the expertise of HCB.

[9] The Scientific Committee notes that, while the RIDL technique is the furthest advanced in terms of development, it could only be used to control *Aedes aegypti* at present; for the other vectors, SIT and IIT, possibly combined with low doses of sterilising irradiation (SIT-IIT), could be envisaged.

1. Introduction

1.1. Referral

On 12 October 2015 the High Council for Biotechnology (HCB) was asked by the Minister for the Environment to provide guidance on the use of genetically modified mosquitoes for vector control. This referral is reproduced in Appendix 1.

Recognising the limitations of current methods of controlling mosquitoes carrying pathogens that cause disease (methods based mainly on use of chemical insecticides), the minister notes that the government must look closely at all the available options, including use of mosquito populations with a modified genetic make-up.

This being so, the minister requests guidance from HCB on use of genetically modified mosquitoes for vector control. Under the terms of the referral, this guidance should specifically set out to:

1) Review the current position regarding research into and marketing of genetically modified mosquitoes, together with production methods, highlighting their specific features in comparison with methods already employed;
2) Detail the criteria applying to health and environmental risk assessments of these mosquitoes nationally (including French overseas departments, regions and collectivities), at the European level and internationally;
3) Review the outcomes of initial experiments and use of genetically modified mosquitoes worldwide;
4) State the potential risks and benefits of using genetically modified mosquitoes for France, including overseas territories, particularly from the social, economic and ethical angles.

1.2. Scope

HCB has defined the scope of its work in response to the referral:

- Of the insect vectors of pathogens, only mosquitoes in the strict sense of the term have been taken into account, i.e. insects of the Culicidae family. Some examples are nevertheless drawn from insects of other families owing to the scale of their socio-economic and/or health impact, or if they are considered relevant to development of vector control strategies using insects with a modified genetic make-up;
- All methods of vector control (chemical, biological, genetic, physical and environmental), both current and under development, have been taken into account to provide comparative guidance that is as informative as possible in response to the questions in the referral, and particularly the question relating to risks and benefits of using genetically modified mosquitoes, which cannot be addressed out of context;
- The referral has been considered at the national level (including French overseas territories), the European level and internationally;
- The HCB Scientific Committee has dealt with the technical and scientific aspects of the referral, while the HCB Economic, Ethical and Social Committee has considered its social, economic and ethical aspects. The Scientific Committee has also addressed the regulatory framework for control techniques because it influences how they are assessed and how assessment criteria for the associated risks are defined.

1.3. Process

The HCB Scientific Committee has dealt with this referral in three stages:

1) By setting up an expert working group[10] that used its combined expertise to draw up a report[11] covering the various technical and scientific aspects of the questions raised by the referral and providing background information on use of vector control methods on French territories;

2) By discussing the working group's report (WG report) at Scientific Committee meetings and through a number of exchanges between the Scientific Committee and the working group for the purpose of clarifying the report and preparing an opinion;[12]

3) By preparing a draft opinion focused on the referral's questions, produced using the WG report and further information collected from the scientific literature, from actors in the field of vector control and from working group members consulted in their capacity as experts when necessary. The draft was discussed at Scientific Committee meetings and amended in the light of these discussions and after verification by experts in the relevant fields. The finalised opinion was adopted by the committee on 31 May 2017.[13]

Consequently, this Scientific Committee opinion is based on the WG report and makes extensive reference to it. The report is available on the HCB website.

1.4. Opinion structure

In answering the referral questions, the opinion follows the structure set out below:

- An introductory Section 1 describes the referral and how it has been addressed by the HCB Scientific Committee;
- Section 2 gives background and outlines the basics of vector control, which is treated at greater length in the WG report;
- Section 3 answers Questions 1 and 3 of the referral, giving an account of vector control techniques that use GM mosquitoes, taking stock of the current position regarding research into and development of these techniques, including outcomes of initial experiments worldwide, and reviewing how they are marketed;
- Section 4 completes the answer to Question 1 of the referral, highlighting the specific features of vector control techniques using GM mosquitoes in comparison with other control methods, including not only techniques already employed but also other emerging techniques, in terms of possible objectives, efficacy and sustainability, technical constraints and environmental and health risks;
- Section 5 answers Question 2 of the referral by suggesting risk assessment criteria for GM mosquitoes in the light of the regulatory framework applicable to French territories;

[10] The Scientific Committee working group's composition and working methods, including a seminar open to members of both HCB committees and a hearing of representatives from the British firm Oxitec, are to be found in Appendix 3.

[11] The Scientific Committee working group's report is available on the HCB website at: http://www.hautconseildesbiotechnologies.fr/sites/www.hautconseildesbiotechnologies.fr/files/file_fields/2017/05/31/rapportgtcshcbcnevadopte170523.pdf.

[12] The working group's work and the report in progress were discussed by the Scientific Committee at meetings on 24 March, 28 April and 22 June 2016. Briefer updates on the report's progress were provided at meetings on 13 July and 27 September. The report was presented at the meetings of 27 October and 15 December prior to finalisation and was discussed on 26 January 2017 with a view to preparing a Scientific Committee opinion.

[13] The draft Scientific Committee opinion was discussed at the meetings of 25 February, 28 March, 27 April and 24 May 2017. It was adopted electronically on 31 May 2017.

- Section 6 answers Question 4 of the referral in terms of the benefits and limitations of vector control techniques using GM mosquitoes in comparison with other control techniques as regards the technical and scientific aspects, since the Economic, Ethical and Social Committee will complete this exploration for the social, economic and ethical aspects.

2. Background

2.1. Mosquito-borne diseases

Vector-borne diseases are infectious diseases transmitted by vectors. These vectors are mainly blood-feeding arthropods, the majority of which are insects – including mosquitoes, but also flies and midges (sandflies, biting midges, black flies, etc.), fleas, lice and bugs – as well as arachnids such as acarids, including ticks.

- Global burden of mosquito-borne diseases

According to WHO, vector-borne diseases account for over 17% of all infectious diseases, causing more than one million deaths annually worldwide. Of the diseases transmitted by mosquitoes, parasitic diseases such as malaria and lymphatic filariasis contribute significantly to infection-related morbidity and mortality globally, even if the trend is downwards; viral diseases are more diverse, with more frequent epidemics and territorial encroachment, but their impact is less (WG report, Table 1).

- Situation in France

Of the various diseases specifically carried by mosquitoes, we shall here focus on the most significant in terms of global impact on human and animal health and/or their presence in metropolitan and overseas France,[14] namely malaria, lymphatic filariasis, dengue, chikungunya, yellow fever, West Nile fever and the Zika virus disease. For each, the WG report describes the disease, the infectious agent, the vector, the global burden, global epidemiology and presence on French territories (WG report, Sections 2.1.1 and 2.1.2, with arbovirus transmission cycles and the distribution range of these diseases shown in Appendices 8 and 9 respectively), information that is summarised in the boxes below on mosquito-borne diseases in France.

These diseases mainly affect human health. The veterinary impact of mosquito-borne diseases in the strict sense of the term is less (animal health is more affected by pathogens carried by ticks, biting midges and sandflies); it is discussed at greater length in Appendix 6 of the WG report.

[14] Overseas France encompasses territories in the Pacific (French Polynesia, New Caledonia, Wallis and Futuna, and Clipperton Island), the Indian Ocean (Réunion, Mayotte and some islands belonging to the French Southern and Antarctic Lands (TAAF)), and America, with the French West Indies (Martinique, Guadeloupe, Saint Martin and Saint Barthélémy) in the Caribbean, French Guiana in South America, and Saint Pierre and Miquelon in North America. Apart from Saint Pierre and Miquelon, the TAAF and Clipperton Island, all these territories are concerned by vector-borne diseases. Appendix 5 of the working group's report provides further information on the geographical situation and legal status of the various French overseas territories.

Mosquito-borne diseases in France: 1. Arboviral diseases

Arboviral diseases are viral diseases caused by arboviruses, i.e. arthropod-borne viruses. Over 500 arboviruses have been listed, with just under half being transmitted by mosquitoes and a hundred or so infecting human beings. The arboviral diseases present on French territory are described below.

Dengue

Dengue results from an infection by any of the four serotypes of a dengue flavivirus (DENV, an RNA virus), transmitted mainly by *Aedes aegypti* mosquitoes but also by other vector *Aedes* such as *Aedes albopictus*. In most cases the disease is mild, although very often temporarily incapacitating owing to a syndrome of fever, vomiting, and joint and muscle pain. More rarely, serious haemorrhagic forms (which can result in death) have been recorded. Since 2010, continental France has been affected by a series of outbreaks which, associated with adaptation of *Ae. albopictus* to local conditions, could lead to the emergence of epidemics. It has been calculated that some 3.9 billion people in 128 countries are exposed to infection by the dengue virus (Brady et al., 2012). According to a recent estimate there are 390 million cases of dengue per year, of which 96 million have clinical manifestations (Bhatt et al., 2013). Dengue is endemo-epidemic in French territories in the Caribbean (French West Indies) and South America (French Guiana), the Indian Ocean (Réunion and Mayotte) and the Pacific (French Polynesia, New Caledonia (epidemic outbreak in 2017), and Wallis and Futuna).

Zika virus disease

The Zika virus (ZIKV, an RNA virus) is a flavivirus transmitted to humans by mosquitoes of the genus *Aedes*, mainly *Ae. aegypti*, and possibly *Ae. albopictus* and other species of *Aedes* in the urban cycle. It can also be transmitted sexually, *in utero* if the mother is infected, and through blood transfusion. Previous infection by DENV could facilitate infection by the Zika virus. Most infections are asymptomatic. However, the impact of foetal infection on brain development, potentially causing microcephaly, and the development of Guillain-Barré syndrome in some Zika-infected patients, make the emergence of this disease an important public health issue. The first major epidemic took place in French Polynesia in 2013-14 and New Caledonia in 2014. The French West Indies and French Guiana were affected in 2016 in the wake of the 2015-16 epidemic in Brazil. The virus's various modes of transmission allow it to be transmitted from cases imported into continental Europe regardless of the progress of the actual vector (*Ae. albopictus*).

Chikungunya

Chikungunya (CHIKV, an RNA virus) is caused by an alphavirus transmitted to humans epidemically by mosquitoes of the genus *Aedes*, mainly *aegypti* and *albopictus*. There are currently three genotypes circulating worldwide. Infection usually produces symptoms, with attacks of debilitating muscle and joint pains. Forms leading to severe liver or neurological damage have also been recorded. The virus is regularly found in epidemic outbreaks not only in Asia but also in Africa and on the American continent. The virus's ability to adapt, through mutation of its envelope genes to a second host (*Ae. albopictus*) in a different biotope, contributes to the spread of CHIKV epidemics. An epidemic wave occurred first in Réunion in 2005-2006 and then in Mayotte in 2006. Cases have been recorded in metropolitan France since 2010. In 2013, autochthonous cases of CHIKV were detected on the island of Saint Martin and spread to all the Caribbean islands, resulting in a major epidemic in 2014.

Yellow fever

The yellow fever virus (YFV, an RNA virus) is a flavivirus. It is transmitted by *Ae. aegypti* or *Ae. albopictus* in urban areas. There are two transmission cycles: urban and sylvatic (enzootic in primates). The human disease occurs when humans enter the sylvatic cycle (less commonly a primate may take the virus into an urban environment). The disease often presents with few symptoms, manifesting merely as an influenza-like illness, but it may be associated with the onset of jaundice, as well as serious kidney damage and haemorrhagic fever with a high death rate. YFV is rife mainly in Africa and to a lesser extent in South America. In French territories, only French Guiana is affected; the last human case was reported there in 1998, as vaccination is compulsory in this region.

West Nile fever

The West Nile virus (WNV, an RNA virus) is a flavivirus of which three genotypes are linked to human infection. WNV is transmitted to humans by mosquito bites, with over 60 species involved. Mosquitoes of the genus *Culex* (mainly *restuans* and *pipiens*) are the vector between birds, the main reservoir hosts, and humans. The disease is usually associated with few symptoms; under 1% of persons infected develop the severe clinical form with neuroinvasive damage, leading to death in 20% of cases. The virus is present on all continents and has been associated with a string of epidemics. WNV resurfaces regularly in bird populations, raising concerns about development of new epidemics among humans. After a first epidemic in 1962, outbreaks have been rare in metropolitan France, where the disease is found mainly in horses. Its detection triggers a series of preventive measures in the field of public health. The virus is circulating in Guadeloupe, although no human cases have been recorded.

Mosquito-borne diseases in France: 2. Parasitic diseases

Parasitic diseases are diseases caused by parasites. Two parasitic diseases are caused by parasites transmitted by mosquitoes: malaria and lymphatic filariasis. Both are present on French territory.

Malaria

Malaria is still one of the deadliest infectious human diseases globally, with 212 million cases and 429,000 deaths in 2015, even though incidence has dropped by 41% and mortality by 62% since 2000, particularly due to vector control. It is caused by a protozoan of the genus *Plasmodium*, an obligate intracellular parasite transmitted by mosquitoes of the genus *Anopheles*, with the species depending on region. Major vectors are *Anopheles gambiae* and *An. funestus* in Africa, *An. stephensi*, *An. culicifacies* and *An. dirus* on the Indian subcontinent, and *An. quadrimaculatus*, *An. hermsi*, *An. albimanus* and *An. darlingi* in the Americas. Of the 156 species of *Plasmodium*, at least five cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. *P. falciparum*, which is responsible for the severest clinical forms of the disease, is also the commonest of the *Plasmodium* species, prevalent in Africa and present in Asia and South America. *P. vivax, P. ovale* and *P. malariae* are associated with mild forms that are sometimes recurrent owing to persistence of the parasite in the liver. *P. knowlesi* infections are uncommon. The clinical symptoms of malaria include headaches, muscle pain and digestive disorders. In attacks of malaria, shaking is followed by fever and then by heavy sweating, reflecting the parasite's cycle in the red blood cells that leads to their destruction. The most serious form of the disease, cerebral malaria, can be fatal; it is found only after infection by *P. falciparum*. Since disappearing from metropolitan France (last cases in Corsica in 1970), malaria has occurred mainly in cases that have been imported, when patients return from malaria-infected countries and, more rarely, when the mosquitoes themselves are imported (resulting in so-called airport malaria). Malaria is still endemic in French Guiana and Mayotte.

Lymphatic filariasis

Filariasis is caused by infection by filarial worms, which are microscopic worms of the phylum Nematoda. In humans, three species of filarial worms are responsible for lymphatic filariasis: *Wuchereria bancrofti* (90% of cases), *Brugia malayi* and *Brugia timori*, each transmitted by different vectors, with features specific to each area, owing to evolutionary adaptation. The main vectors are *Anopheles* (*An. gambiae*, for example) and *Culex*. The worms' reproductive cycle limits the impact of the mosquitoes' high vectorial capacity. In humans, lymphatic filariasis has acute and chronic manifestations, but at least half of all patients infected have no symptoms. The acute phase presents with lymphadenopathy associated with inflammation and fever. The presence of microfilarial worms in the blood impairs the lymphatic vessels, although there are no external signs, producing a chronic phase. In this phase, obstruction of the lymphatic vessels blocks circulation of the lymph and leads to lymphoedema. The distal end of the limbs affected may swell excessively and remain swollen. Pulmonary forms, the pulmonary eosinophilia syndrome, are found in Asia. In French territories, the agent is *W. bancrofti*, carried by *Aedes* in the Pacific and *Anopheles* in the Indian Ocean, while *Culex quinquefasciatus* may be a vector in urban areas. In Mayotte, regarded as one of the world's major focal areas some 40 years ago, filariasis has receded considerably, although its prevalence has not been measured exactly. In Réunion, anti-malaria campaigns and a higher standard of living have no doubt contributed substantially to a dramatic decline in the disease, which disappeared in the 1970s.

- Vector mosquitoes considered in the opinion

The mosquito species responsible for transmitting the above-mentioned diseases in the various French territories are mostly *Aedes albopictus*, *Aedes aegypti* and various species of *Culex* and *Anopheles* (WG report: Sections 2.1.1 and 2.1.2, Tables 3 and 6). Since there are over 3500 species of mosquito, HCB has focused its discussion on control of populations of these vector mosquito species present in the various French territories.

- Other modes of transmission

It should be noted that with some of these diseases their pathogens may be spread by modes other than vector transmission: blood transfusion[15] can be responsible for secondary cases (Dodd et al., 2015), but its impact remains low compared with the number of mosquito-borne cases (Gray and Webb, 2014). Sexual and congenital transmission is also possible for the Zika virus (Foy et al., 2011; Weaver et al., 2016). The impact of these modes of transmission is hard to gauge from the available literature.

[15] Preventive measures have been taken by transfusion management bodies, i.e. the French Blood Agency (EFS) in France, which reports to the General Directorate for Health. Depending on the virus and its biological characteristics, these measures include systematic screening of donations, temporary exclusion of donors returning from areas with high prevalence, and decontamination of products whenever possible.

- Diseases transmitted by other vectors

Lastly, a number of vector-borne diseases transmitted by vectors other than mosquitoes are also worth mentioning here because of the scale of their socio-economic and/or health impact, particularly for animal health: in both temperate and tropical zones, diseases transmitted by ticks and biting midges are highly significant with regard to animal health. This is the case for bluetongue, for example: the major epizootic in 2006-2010 led to economic losses totalling one billion euros in France.[16] It is also the case for the Schmallenberg virus, whose rapid spread from the Netherlands in 2011 resulted in several thousand focal areas in France.[17] Both these arboviral diseases are carried by biting midges. These diseases and their vectors will be not considered in this opinion, since they are outside the scope of the referral, which focuses on mosquitoes. It should nevertheless be borne in mind that they raise the same vector control issues.

2.2. Control methods for vector-borne infectious diseases

Control methods for vector-borne infectious diseases include therapeutic treatments and preventive measures, among which is vector control.

2.2.1. Therapeutic treatments

Of the diseases considered, only malaria and lymphatic filariasis have commercially available therapeutic treatments, for which strategies must nevertheless be put in place to limit the risk of resistance developing and to manage the associated consequences (WHO, 2015). A treatment for chikungunya is in the early clinical stages. No conclusive treatments are in prospect for the other diseases (WG report, Section 2.2.1).

2.2.2. Preventive measures

2.2.2.1. Preventive treatment

For malaria, some of the molecules used curatively for therapeutic treatment can also be used preventively (WHO, 2015). However, this approach is mostly confined to time-limited exposure. Thus preventive treatment for malaria is prescribed only for people living outside areas where it is endemic and who are exposed for short periods; it is not used for autochthonous populations owing to the side effects that might result from chronic use and to possible development of resistance in the parasite. There are currently no preventive treatments for arboviral diseases.

2.2.2.2. Vaccines

Only two of these diseases have commercially available vaccines: yellow fever, since the 1940s, and dengue, for which a vaccine came on the market in 2016. Since the vaccine strain currently used for yellow fever is an attenuated virus strain not without adverse side effects, an alternative vaccine has been developed from an inactivated strain; this vaccine is not yet on the market (Monath et al., 2011; Pereira et al., 2015). Either way, it should be noted that the number of vaccine doses available at any one time remains a concern in the event of an epidemic outbreak (Green, 2016; Kupferschmidt, 2016). Vaccines for the other diseases are still under development (WG report, Section 2.2.2).

In this context, vector control is a vital method of prevention, complementing the preventive treatment and vaccines currently available and needed to mitigate the lack of therapeutic treatments for most of these diseases.

[16] https://www.anses.fr/fr/content/la-fi%C3%A8vre-catarrhale-ovine-fco-ou-bluetongue.

[17] http://agriculture.gouv.fr/maladies-animales-le-virus-de-schmallenberg.

2.2.2.3. Vector control: definition, goals and strategies

In its broadest sense, vector control covers control of and protection against blood-feeding arthropods – vectors of human and animal vertebrate pathogens – as well as their surveillance (France's National Centre for Vector Expertise).

The overall objective of vector control is to reduce mortality and morbidity from vector-borne diseases. It has the following specific goals:

- For individuals: protection against infective arthropod bites;
- For communities: prevention of or reduction in the intensity of pathogen transmission by acting on four of the key parameters of vectorial capacity, namely vector density, vector longevity, host-vector contact and vector competence.

Control strategies may entail a variety of methods and techniques depending on the vector and the epidemiological and socio-economic context:

- Existing methods have recourse to chemical, biological, physical, environmental and social processes. They include biocide control (particularly using insecticides: adulticides and larvicides), biological control (using mosquito pathogens and predators), mechanical control (capturing vectors and providing protection against host-vector contact (using traps, mosquito nets, etc.)) and environmental control – aimed at making an environment hostile to the development of vector populations (drainage, elimination of breeding sites, etc.) and requiring a significant amount of health education and social mobilisation (see Section 4.1);
- The techniques that are now emerging or under development make use of genetic processes in the broad sense of the term.[18] These techniques include use of genetically modified mosquitoes, the subject of the referral (introduced in Section 3), and other techniques based on mosquito release (introduced in Section 4.2).

2.3. Complexity and diversity of vector systems at work

Establishing a vector control strategy by choosing a particular technique or combination of techniques calls for a proper understanding of the vector systems at work. In particular, the characteristics of the vector mosquito targeted must be taken into account, together with how the environment affects pathogen transmission.

2.3.1. Vector systems: definition and fundamentals of vector control

A vector system is a complex biological system consisting of three components – pathogen, vector arthropod (usually more than one species) and vertebrate host (usually more than one species) – and all the interrelationships between them (Rodhain, 2015). Its working cannot be separated from the environment in which it operates.

Transmission of a pathogen by a vector is a complex, active and specific process that takes place in three stages:

1. Infection of the vector during a blood meal[19] on an infective vertebrate (i.e. one in which the pathogen is present in sufficient quantity in the blood);

[18] In the field of vector control, so-called genetic control consists in employing any conditions and methods of treatment that might reduce the reproductive potential of harmful species by modifying or replacing hereditary material (Fontenille et al., 2009). This term will not be used in the remainder of this opinion.

[19] The blood meal, taken solely by female mosquitoes after they have been fertilised by males, induces egg formation and maturation (see Section 2.3.3 on life cycle of mosquitoes).

2. Pathogen development[20] in the vector organism. It is during this stage that the pathogen reaches the site in the vector organism through which it can be transmitted (salivary glands or proboscis in the mosquito) and the vector becomes infective. Once a vector has become infective it remains so for life (except in the case of filarial worms);
3. Transmission of the pathogen to another vertebrate by the infective vector. In the case of mosquitoes, pathogen transmission takes place through the saliva, which is injected by the arthropod during the blood meal. A different mechanism is at work in the case of filarial worms, which migrate through the proboscis and penetrate the skin when the mosquito bites.

The time interval between acquisition of the pathogen by the vector and the point when the latter is able to transmit it is known as the extrinsic incubation period. This is usually between 3 and 15 days for mosquito-borne pathogens, depending on the populations of pathogens, vectors and vertebrate hosts and the environment in which they interact; for a given pathogen it will depend on factors specific to the vector mosquitoes and on environmental factors (mainly temperature) (see Section 2.3.3). The extrinsic incubation period is an important aspect of epidemiology: the sum of this period and the incubation period in the vertebrate host constitutes the minimum period between onset of initial symptoms in the index case and in a secondary case. If the vector does not survive long enough for the incubation process to be completed (owing to its own lifespan, environmental conditions or a vector control technique), it will be unable to transmit the pathogen.

Only a vector that is competent for a given pathogen will be able to transmit it. Vector competence is the ability of a vector to become infected and ensure the development and transmission of a pathogen (Tran et al., 2005); it depends mainly on genetically controlled intrinsic factors (Tran et al., 2005) and may be influenced by gut microbiota (Carissimo et al., 2015; Gendrin et al., 2015; Richman et al., 1997) and other factors that are still not properly understood.

The efficiency with which a vector population transmits a pathogen is known as vectorial capacity. This concept integrates both factors intrinsic to the vector (vector competence) and extrinsic factors, which include the pathogen's development efficiency as a function of temperature, feeding behaviour (anthropophilic rates), frequency of blood meals, longevity (as a function of climate) and density of vector population (Rodhain, 2015; Rodhain and Perez, 1985). Knowledge of a mosquito population's vectorial capacity for a pathogen in a given environment can make it easier to determine, for the purposes of vector control, the critical density below which the pathogen cannot remain endemic.

2.3.2. Bio-ecological characteristics of vector mosquitoes

2.3.2.1. Mosquitoes and vector mosquitoes

The mosquito family (Diptera: Culicidae) has over 3500 species and is split into three subfamilies: Anophelinae, Culicinae and Toxorhynchitinae. There are 112 mosquito genera, of which the best-known are *Anopheles* (Anophelinae), *Aedes* and *Culex* (Culicinae) (http://mosquito-taxonomic-inventory.info/).

For this referral we shall concentrate on the characteristics of mosquito species carrying causative agents of diseases chosen because of their global impact and/or their presence in French territories, namely malaria, lymphatic filariasis, dengue, chikungunya, yellow fever, West Nile fever and Zika virus disease:

- *Anopheles* spp.: Vectors of the malaria agent *Plasmodium* spp.

[20] Pathogen development in the vector varies according to the nature of the pathogen (see Section 2.3.3 on pathogen characteristics).

Over 500 different species of the genus *Anopheles* have been recorded; 68 of them can transmit malaria, 40 as principal vectors and 28 as secondary vectors. *Anopheles gambiae s.s.*[21] is considered to be the most important vector of malaria in Africa. *Anopheles arabiensis* is a species that is morphologically indistinguishable from *An. gambiae s.s.*, but unlike the latter it is able to live away from human habitats and be a nuisance in arid zones. *Anopheles albimanus* is considered the main malaria mosquito in the New World and *Anopheles stephensi* in Asia, where there are a large number of vectors.

In the *An. gambiae s.l.* species complex, *An. gambiae s.s.* shows some cross-breeding, albeit limited, with *An. coluzzii* (Coetzee et al., 2013) and with *An. arabiensis* (Weetman et al., 2014). It should, however, be noted that on French territories the risks of gene transfer between mosquitoes belonging to these (sub)species are theoretically non-existent, since they are not known to cohabit there at present.[22]

- *Aedes aegypti* (monophyletic group[23]), commonly known as the 'yellow fever mosquito': a major vector of the YFV, DENV, CHIKV and ZIKV arboviruses.
- *Aedes albopictus* (monophyletic group), commonly known as the 'tiger mosquito': a major vector of CHIKV in the Indian Ocean region, a secondary vector of DENV and responsible for cases of autochthonous DENV and CHIKV transmission in Europe; in experiments *Ae. albopictus* has also been shown to be a competent vector for a number of other viruses, including ZIKV, but its role in transmission has not been proven in the field.
- *Culex pipiens* spp., particularly *Cx. pipiens pipiens*, a common mosquito in Europe where it is a vector for WNV, and *Cx. quinquefasciatus*, one of the vectors of lymphatic filarial worms in Mayotte. These two species cross-breed but do not cohabit on French territories.

2.3.2.2. Native ranges and biogeography

The mosquito species considered in this opinion are mapped in greater detail in the WG report (Section 2.3.2.2, Figures 1-4).

Ae. aegypti and *Ae. albopictus*, major primary or secondary vectors of arboviruses transmitted to humans, are invasive mosquito species. Following a remarkable expansion from their native ranges (Africa and Asia respectively) over the past few years, their presence has now been recorded in a substantial number of countries (Kraemer et al., 2015). However, the global distribution of *Ae. aegypti* is confined to tropical zones, whereas over the past 30 years that of *Ae. albopictus* has expanded to temperate continental regions. Particular note should be taken of the recent spread in France of *Ae. albopictus*, which became established in 2004 and was present in 30 departments of metropolitan France by 2015 (InVS).[24] This dramatic spread of *Ae. albopictus* has been made possible in particular by its adaptability to cold climates owing to its ability to enter winter diapause at the egg stage (Lacour et al., 2015).

The *Cx. pipiens* species complex also has a global distribution. While *Cx. quinquefasciatus* is found mainly in tropical regions, *Cx. p. pipiens* is present in continental temperate regions as well as some areas of tropical and Mediterranean Africa (Farajollahi et al., 2011).

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[21] *Anopheles gambiae s.l.* (*sensu lato*) is a complex of several cryptic or sibling species (morphologically indistinguishable), including *An. gambiae s.s* (*sensu stricto*), *An. arabiensis* and *An. coluzzii*.

[22] In Réunion, of these three (sub)species only *An. arabiensis* is present, as a residual population in the countryside and too far from the airport for hybridisation with any *An. gambiae* mosquitoes brought in by air. In Mayotte, of these three (sub)species only *An. gambiae s.s.* is present. It is not established in the airport area, and control operations are carried out around the airport.

[23] *Aedes aegypti* is a monophyletic group in that it contains only one species.

[24] http://invs.santepubliquefrance.fr/Dossiers-thematiques/Maladies-infectieuses/Maladies-a-transmission-vectorielle/Chikungunya/Donnees-epidemiologiques/France-metropolitaine/Chikungunya-dengue-et-zika-Donnees-de-la-surveillance-renforcee-en-France-metropolitaine-en-2016.

Unlike the *Aedes* and *Culex* species, which are few in number with each having a global distribution and carrying a number of diseases, *Anopheles* has many species carrying the same pathogen, *Plasmodium* spp., with different species being found in different regions of the globe. Thus each continent or subregion has a unique set of *Anopheles* vectors (Sinka et al., 2012). They do not all contribute equally to malaria transmission; we talk about major vector species and minor vector species. A species classified as a minor vector species may nevertheless be epidemiologically significant at the local level.

2.3.2.3. Vector life cycle

General characteristics

All mosquito species have a similar life cycle, with an aquatic phase and an aerial phase.

The aquatic phase comprises the larval and pupal stages: there are four successive larval stages, followed by the formation of pupae that are mobile but do not feed. Metamorphosis takes place in the pupa, which will produce a male or female winged adult. With normal aquatic development, male adults emerge before female adults, which can be fertilised as soon as they emerge. Females usually mate only once in their lives[25] and keep the sperm alive in a spermatheca.

One characteristic of adult mosquitoes is that only the females require a blood meal, which will ensure production of offspring. The males feed on sugar juices (nectar in particular). The blood meal taken by a female results in vitellogenesis and egg maturation, with eggs being laid 48 to 72 hours after the meal for most species. The period between the blood meal and the egg-laying is known as a gonotrophic cycle. Such a cycle may be longer than 72 hours if the females fail to find a suitable site for laying. Some species of *Anopheles* may also take several blood meals in the course of a single gonotrophic cycle. The eggs are laid on a humid substrate (*Aedes*) or directly on the water surface (*Culex* and *Anopheles*).

Specific characteristics

The various species of the three main mosquito genera studied here (*Aedes, Culex, Anopheles*) can be distinguished by the specific characteristics described below and summarised in Table 1. These characteristics are all-important when choosing the most appropriate control methods for the target species.

a) Mode of reproduction

   Whereas *Anopheles* and *Cx. pipiens/quinquefasciatus* mate in swarms, *Ae. aegypti* and *Ae. albopictus* frequently mate close to the vertebrate host that females require for their blood meals (Delatte et al., 2009; Ponlawat and Harrington, 2005). Swarms form at specific sites that the mosquitoes recognise by visual markers (Butail et al., 2013). Swarms are usually composed of a single species, but swarms of several species can occur side by side. These swarms consist of males, which attract females by the frequency of their wingbeat. Any female entering a swarm is immediately caught by a male. The pair falls to the ground, where copulation takes place.

b) Egg-laying

   Ae. aegypti and *Ae. albopictus* generally lay the eggs from a single batch (gonotrophic cycle) in small quantities, distributing them among different breeding sites (Reiter et al., 1995). *Culex* mosquitoes, on the other hand, lay all their eggs at once gathered into a 'raft' on the

[25] The degree of monogamy seems to vary according to species, being very high for *Ae. aegypti*, in which the females are generally refractory to a second insemination (Bargielowski et al., 2011) – although some polyandry has been reported in semi-field conditions –, while *Ae. albopictus* females seem to tolerate a significant degree of multiple inseminations (Boyer et al., 2012).

surface of one breeding site. *Anopheles* mosquitoes likewise lay all the eggs of a single gonotrophic cycle on the same site but individually.

c) Reproductive environment

Ae. aegypti and *Cx. pipiens* are urban mosquitoes, while *Anopheles* mosquitoes are largely rural. A notable exception is *An. stephensi*, which colonises urban breeding sites. However, environmental – and maybe climate – changes have enabled *An. gambiae* (a major vector in Africa) gradually to colonise the peri-urban habitats of Africa's megacities (Fossog et al., 2013).

In its native distribution in the tropics *Ae. albopictus* was associated with forest edges. The species' invasive form seems to be urban or semi-urban as well (Delatte et al., 2009; Ponlawat and Harrington, 2005).

These vector species can also be distinguished by the characteristics of their breeding sites. Thus *Ae. aegypti* will prefer to lay in clean water in small manmade receptacles such as flower-pot saucers, food tins and old tyres. *Ae. albopictus* prefers to lay in tree holes. In urbanised areas it uses breeding sites similar to *Ae. aegypti* although less artificial and closer to green spaces (F. Rodhain, public hearing at the Parliamentary Office for Scientific and Technological Assessment (OPECST), 2016).

Cx. pipiens uses breeding sites full of organic matter, which can also be very polluted. Typical examples are sewers, gutters and septic tanks.

The variety of malaria-carrying *Anopheles* is also matched by the variety of these species' breeding sites, but common features are clean water free of organic matter and exposure to sunlight. Breeding sites vary, from large stretches of water varying in depth (rice fields) to small pools of water (in ruts and tyre or animal tracks). *An. stephensi* in urban areas may also colonise part-covered deep gutters or drinking troughs.

d) Survival and longevity

The survival of adult mosquitoes depends on climate and access to resting and hibernation areas (for cold winters in temperate continental areas (overwintering) and for the dry season in subtropical zones). In high temperatures, survival will be better if humidity is also high. Outside their periods of activity (mating, hunt for blood meal and hunt for laying sites), mosquitoes look for resting places that are humid and cool by comparison with the ambient temperature.

Aedes mosquitoes are able to survive over long periods because of their eggs' resistance to desiccation. *Ae. albopictus* has the distinctive feature of being able to survive the cold season, either because its eggs enter winter diapause (Pluskota et al., 2016) or because the adults in some populations are tolerant to the cold (ECDC website). *Culex* mosquitoes are able to survive in adulthood during the cold season in carefully chosen resting places (e.g. metro, sewers, etc.). European *Anopheles* mosquitoes may spend the cold season either as adults in refuges (e.g. stables) or as larvae in deep water frozen at the surface, for example (e.g. *An. plumbeus*). Recent data have shown that *An. gambiae* adults can survive in the dry season in the Sahel, but their resting places have not been identified (Adamou et al., 2011; Lehmann et al., 2010; Yaro et al., 2012).

An adult female's lifespan therefore varies according to temperature and humidity, regardless of predators. It is usually considered to be about 30 days. For females that hibernate it may be as much as several months. Over this period they may take one or more new blood meals to replenish their reserves, but this is not followed by laying. If they are carrying pathogens, they can contaminate their hosts (known cases of winter malaria in Europe in the 19th and 20th centuries (Bruce-Chwatt and De Zulueta, 1980)).

It should be noted that a system of thermoregulation allows female adults to maintain their temperature after ingesting the vertebrate host's blood by excreting drops of urine mixed with the host's blood (a sort of transpiration) on a scale and at a rate that vary according to the species of mosquito (Lahondère and Lazzari, 2012). Any impairment of this complex mechanism could adversely affect the mosquito's survival.

e) Host preference and behavioural diversity

Ae. aegypti and *Ae. albopictus* are both highly anthropophilic, biting outdoors (exophagy) and resting either indoors (endophily) or outdoors (exophily) (Delatte et al., 2009; Ponlawat and Harrington, 2005). *Ae. aegypti* has two peaks of aggressiveness: at dawn and two hours before sunset. *Ae. albopictus* tends to bite during the day (ECDC website).

Culex pipiens/quinquefasciatus is zoo-anthropophilic, with a clear host preference for birds (poultry in particular). It is most aggressive at night, and its behaviour is mainly exophagic.

Anopheles mosquitoes bite at night. Vectors of human malaria are either strictly anthropophilic (e.g. *An. gambiae* and *An. coluzzii*) or else zoo-anthropophilic (e.g. *An. arabiensis* and *An. funestus*). They may be endophagic or exophagic, or both, and rest indoors or outdoors (endophilic/exophilic). Each species has its own peak(s) of night-time aggressiveness. However, following large-scale use of insecticide-treated repellent mosquito nets to protect people from night-time bites, some species of *Anopheles* have recently changed their peak of aggressiveness and bite either earlier in the evening or later in the morning (Moiroux et al., 2012; Sougoufara et al., 2014).

f) Seasonal abundance, dispersal capacity and adaptation to new environments

Presence of mosquitoes is entirely dependent on the presence of water for use as a breeding site. It also depends on climate conditions in which the mosquitoes can complete their life cycles. Thus in the humid tropics, mosquitoes may be present throughout the year. However, they are vulnerable to the heavy rains that wash out their breeding sites, which will reappear as areas of residual water. This is mainly the case for *Anopheles*. For urban and semi-urban mosquitoes such as *Ae. aegypti* and *Ae. albopictus*, which colonise artificial sites (flower pots, flower-pot saucers, badly maintained roof gutters, tyres, food tins), their presence depends chiefly on temperature, which must be over 20°C.

Dispersal in *Ae. aegypti* and *Ae. albopictus* takes place mainly by degrees because of their egg-laying behaviour, with eggs being distributed among different breeding sites with active dispersal from an entry point. Colonisation of new ranges by both species is the result of passive dispersal by human activity. While adults can be transported (by car, bus, aircraft, etc.), the main mode of dispersal is through eggs or larvae, particularly with trade in old tyres, which provide an ideal laying site for these two species. *Ae. albopictus* has thus been recorded as travelling over vast distances (Lounibos, 2002).

Dispersal in *Cx. pipiens/quinquefasciatus* can follow the same routes; however, mosquitoes of these species seem better able to travel downwind, with a migration of several kilometres in each generation (Jacquet et al., 2016; Lenormand et al., 1998).

The specificity of *Anopheles* breeding sites means that the risk of dispersal is limited at this stage. On the other hand, as in the other mosquito species, adults can be transported, with vehicles becoming resting places. This explains airport malaria. However, their biological dependence on specific environmental conditions, together with the existence of natural barriers (arid zones, mountain ranges), has prevented them from becoming established in new geographical areas, apart from *An. gambiae* (*arabiensis*) in Brazil in the 1930s (Parmakelis et al., 2008; Soper, 1943). Further examples of passive dissemination of

Anopheles through methods of transport and its role in malaria transmission have been reported (Giacomini and Brumpt, 1989).

Lastly, it is worth noting that, because of the proximity of their sites to their sources of food, *Aedes* travel less than *Anopheles*, which can live in sites much further away from human habitation. *Anopheles* females cover large distances in search of a blood meal and then return to lay their eggs at their breeding sites. In fact, as far as intrinsic dispersal is concerned, *Ae. albopictus* mosquitoes generally travel less than 100 m a day (Niebylski and Craig Jr, 1994), *Ae. aegypti* between 10 and 100 m on average in a lifetime (Winskill et al., 2015), and *An. gambiae* between 350 and 650 m in a day (Costantini et al., 1996).

Thus mosquitoes' varying dispersal capacities, combined with changes in climate, may result in greater or lesser mosquito population movements depending on their biological dependence on specific environmental conditions and their ability to adapt to new environments.

Table 1. Bio-ecological characteristics of mosquito species recognised to be major vectors of pathogens in French territories.

	Aedes aegypti	***Aedes albopictus***	***Culex pipiens* spp.**	***Anopheles* spp.**
Number of species	1 species	1 species	Species complex	Dozens of vector species out of hundreds of species
Likelihood of cross-breeding	Does not cross-breed with other species	Does not cross-breed with other species	*Cx. p. pipiens* and *Cx. quinquefasciatus* cross-breed	Cross-breeding possible in species complexes but rare
Pathogens carried	YFV, DENV, CHIKV, ZIKV, etc.	DENV, CHIKV, (YFV, ZIKV?), etc.	WNV, filarial worms, etc.	*Plasmodium*, filarial worms
Native range and geographical distribution	Native to Africa Common to tropical and subtropical regions	Native to Asia Common to tropical, subtropical and temperate regions	*Cx. p. pipiens*: temperate zones *Cx. quinquefasciatus*: native to North America, tropical and subtropical zones	Different species of vector *Anopheles* in various regions of the world, but generally very widespread
Mating	Close to hosts	Close to hosts	In swarms	In swarms
Egg-laying	One by one on humid substrates liable to flooding	One by one on humid substrates liable to flooding	In rafts on water surface	In large quantities on water surface
Breeding sites	Small artificial breeding sites	Small breeding sites, natural or artificial	Artificial sites varying in size	Mostly natural sites, varying in size
State of water	Clean water	Clean water	Polluted water, rich in organic substances	Clean water, limpid (light)
Habitat	Domestic Urban	Peri-domestic Urban or semi-urban invasive form	Domestic Urban and rural	Peri-domestic Mainly rural, some peri-urban cases
Resting places	Mostly endophilic	Exophilic with an endophilic tendency	Endophilic or exophilic	Endophilic or exophilic
Host preferences	Highly anthropophilic	Anthropophilic in urban areas but opportunistic	Zoo-anthropophilic (mainly ornithophilic)	Anthropophilic or zoo-anthropophilic
Sites of aggressiveness	Exophagic	Exophagic	Mainly exophagic	Endophagic and/or exophagic
Peaks of aggressiveness	Dusk and dawn	Daytime	Night-time	Different night-time peaks, adaptable
Dry season survival	Tolerates egg desiccation	Tolerates egg desiccation	No	Generally not but cases possible
Cold season survival	No	Winter diapause in eggs (adult survival in some populations)	Survival in adulthood	Survival possible for European *Anopheles* in adulthood or as larvae
Dispersal	Active: invasive by degrees Passive: means of transport, tyres, etc.	Active: invasive by degrees Passive: means of transport, tyres, etc.	Active: low Passive: means of transport, downwind, etc.	Limited despite significant travel between sites and food

2.3.2.4. Functional ecology

Before undertaking environmental risk assessment for vector control of a given mosquito population, it is necessary to have a proper understanding of the role of the target mosquitoes in the ecosystems that will be affected.

Of the over 3500 known species of mosquito, some play an important part in ecosystems. In the arctic tundra,[26] for example, mosquitoes can be very numerous and constitute a staple for certain birds during migration. They can also influence movements of caribou, whose migration routes tend to avoid swarms (Culler et al., 2015). In the same region, certain species of the genus *Aedes* have been able to establish species associations as orchid pollinators (Gorham, 1976).

However, for most species, including major vectors of disease, it is hard to determine theoretically mosquitoes' exact role in food webs in the absence of specific studies.

In general, we know that aquatic larvae filter microorganisms from the water and are prey to multiple predators, such as fish, dragonfly larvae and other aquatic insects (Culler and Lamp, 2009). In fact, mosquito larvae grow in a wide variety of watery environments, which can range from small temporary holes in the ground, leaves, and manmade items to permanent bodies of water. These environments, and the biotic community associated with each, vary greatly, meaning that mosquitoes' food-web relationships are context-dependent. For example, predation and competition between species change with duration and size of habitat, becoming more complex in large permanent bodies of water (Juliano, 2009).

Adult mosquitoes can contribute to plant pollination since they feed on the nectar of flowers, and they can be prey to spiders, amphibians, birds and bats (Medlock and Snow, 2008). These predators are mostly generalist and do not depend solely on mosquitoes for their subsistence, but species associations can occur. In East Africa, round Lake Victoria, there is a species of spider, *Evarcha culicivora*, that has developed a preference for blood-engorged *Anopheles* females in the course of its evolutionary history (Nelson and Jackson, 2006).

As for vector species, a case-by-case, region-by-region assessment, depending on the purpose of vector control, would be necessary to grasp the ecological impact of a strategy to eliminate a mosquito population.

Vector species adapted to the urban environment, such as *Ae. aegypti* and to a lesser extent *Ae. albopictus,* develop in temporary accumulations of small volumes of water, where interaction with other organisms such as competing species or aquatic predators may be rare. Similarly it might be expected that outside their native range invasive species such as *Ae. albopictus* and *Ae. aegypti* would have food-web relationships that would be less entrenched than those of native species owing to their more recent integration in the ecosystems invaded. The native species, on the other hand, would have older and more complex interspecific relationships, as in the case of those spiders having developed a preference for *Anopheles* (Nelson and Jackson, 2006).

2.3.3. Pathogen characteristics

Three types of pathogen may be distinguished – arboviruses, *Plasmodium* and filarial worms – in terms of mode of development in the vector and vertical transmissibility. Vertical transmission allows a pathogen to be transmitted directly to the offspring of an infected female mosquito. The vector mosquito then constitutes a natural reservoir for the pathogen, which may be epidemiologically significant during periods unconducive to its transmission to a vertebrate host.

[26] Mosquitoes in the arctic tundra have a very special ecology, which explains researchers' interest in them and the large number of papers devoted to them.

These pathogens share the following two features:

- They are ingested by a mosquito when an infected host with sufficient quantities of the pathogen in its blood is bitten;
- They are inoculated into a new host when the mosquito bites again looking for a blood meal.

They can be distinguished by their intermediate stages of development in the mosquito:

- The intra-mosquito phases of arboviruses and *Plasmodium* are similar in that the pathogens encounter the same physical barriers (gut epithelium and salivary glands) and go through amplification stages. In addition to these amplification stages, *Plasmodium* can be distinguished from arboviruses by a complex biological cycle, various transformations of the parasite and several successive stages of development, including sexual reproduction. In the case of arboviruses, the mosquito remains infected throughout its life and vertical (transovarial) transmission is possible but very rarely found. In the case of *Plasmodium*, the vector generally remains infected throughout its life, but there is no vertical transmission owing to the complexity and constraints of the parasite's biological development in the mosquito.
- The situation is different for filarial worms, which do not undergo an amplification phase in the mosquito but invade the thoracic muscles and go through a moulting phase before being reinoculated by active migration along the insect's proboscis. Filarial worms do not go through a prior stage of invading the salivary glands. The vector frequently becomes healthy again after infection by filarial worms. No vertical transmission is possible.

It is also worth noting that the various pathogens infecting mosquitoes differ in their pace of development. Thus DENV reaches mosquitoes' salivary glands (in the case of *Ae. aegypti* and *Ae. albopictus*) in 14 days, while CHIKV requires only 3 to 7 days (Vazeille et al., 2013). *Plasmodium* parasites pass through differentiation stages and need 7 to 14 days for the sporozoites to reach the salivary glands. However, the pace of development depends on both intrinsic, mosquito-related, factors and extrinsic, mainly temperature-dependent, factors. Thus in high temperatures *P. falciparum* sporozoites can reach mosquitoes' salivary glands in 7 days (Bourgouin and Boudin, personal observations). The infectivity and development stages of a virus such as DENV can also vary between two vector species, such as *Ae. aegypti* and *Ae. albopictus*, or two populations of the same vector species.

2.3.4. Other factors

- Genetic polymorphism of the different vector-system components

As mentioned above, it is important to take account of the genetic diversity of the populations of vector insects targeted by vector control. This diversity can be within species and within populations. The identification, particularly among *Anopheles*, of sibling or cryptic[27] species with different bio-ecological and adaptive traits may have an impact on the anticipated results of vector control techniques. Genetic polymorphism also affects pathogens and vertebrate hosts, including humans.

- Existence of reservoirs

The existence and distribution of pathogen reservoirs must be taken into account, particularly in low-transmission or re-emergence situations.

- Multiple factors influencing vector infection

Depending on the vector system, the risk of host-to-host transmission will hinge on the pathogen's access to the vector through the capillaries (the site of the bite) and on its concentration:

[27] Species that are morphologically indistinguishable.

In the case of arboviruses, transmission can occur only when the virus-carrying host is in the viraemic phase (presence of virus in the blood). This phase may be short-lived and vary in duration from one viral species to another. Viraemia levels (virus concentration in the blood) may also vary between hosts, which may be more or less effective in amplifying a given virus. For example, among the hosts of the West Nile virus, birds are effective amplifiers while humans and horses are dead ends for virus transmission (Gray and Webb, 2014). Depending on the susceptibility of vectors to a given virus, the viraemia level needed to infect them will vary. Lastly, it should be noted that the viraemic phase is not necessarily associated with symptoms in the host. Recent data have shown that asymptomatic individuals could transmit the dengue virus (Duong et al., 2015), which creates problems for epidemiological surveillance.

For malaria to be transmitted, the host must be carrying gametocytes, whose presence can be detected for several weeks after a malaria attack. With the aid of molecular biology technology, gametocyte carriage by asymptomatic carriers is now the subject of much research, as is the role of these asymptomatic carriers in perpetuating *Plasmodium* transmission, particularly in countries in the pre-elimination stage for malaria.

Filarial worms are also a case apart. Depending on the species and their vectors, some filarial worms are present in capillaries only at certain times of the day, which match the times when the vector mosquitoes bite.

- Influence of environment

The effectiveness of a vector system, and therefore of an infectious agent's transmission by a vector in a given environment, will depend in particular on their interaction and on the biotic conditions (host diversity, larval habitats, etc.) and the abiotic conditions (weather, climate, etc.) of this environment.

- Evolving systems

These vector systems are constantly evolving under the pressure of environmental and/or human changes. Given the complexity of vector systems, it is necessary to understand them properly in order to determine appropriate risk management measures, including vector control.

The great complexity and diversity of the vector systems at work thus highlight the need to adapt vector control strategies according to the pathogens and mosquito species concerned as well as the characteristics of the human population to be protected, taking account of the environment in which these three factors evolve, including the relevant habitat, climate and period of the year.

3. Use of genetically modified mosquitoes for vector control

This section endeavours to give an account of how GM mosquitoes can be used for vector control by showing the potential of different techniques for genetically engineering mosquitoes and specific examples of how these techniques may be applied. The specific features of these techniques in comparison with other vector control methods will be highlighted in Section 4.

3.1. Genetic engineering of mosquitoes and potential for vector control

Beyond its value in basic research for a better understanding of the biology of vector mosquitoes and their interaction with hosts and pathogens, genetic engineering of mosquitoes can be applied for vector control. As applied to mosquitoes, the development of transgenesis on the one hand and site-directed nuclease techniques on the other has now opened up two basic approaches to vector control strategy: reducing the density of vector mosquito populations and reducing the ability of these mosquitoes to transmit pathogens.

3.1.1. Mosquito transgenesis and potential for vector control

3.1.1.1. Transposon-mediated transgenesis

Applying transgenesis to mosquitoes, as in the case of other insects, was initially made possible by exploiting the natural properties of transposons. Transposons are mobile genetic elements that have a natural capacity to excise, transpose and reinsert themselves in the genome through the activity of a single enzyme, transposase (O'Brochta et al., 2014b). Insertion of a transgene of interest into a transposon thus enables it to be introduced into a genome (O'Brochta et al., 2014a). A transgenic individual can then be produced by inserting into the insect's germline a plasmid carrying a transposon including the transgene of interest. This can be done by embryo microinjection. To ensure stability of the transgene once inserted, binary systems have been developed by which the transposase can be supplied transiently from an auxiliary plasmid (Rubin and Spradling, 1982). Only the transgene of interest (which may be combined with reporter genes), inserted in place of the transposase, together with the other structural elements of the transposon, are then introduced into the target genome.

A number of improvements have been made to this basic system, such as the possibility of combining transgenes of interest with specific sex-, tissue- or stage-specific promoters (Hammond and Nolan, 2014). Another technical advance, adapted from pioneering work on *Drosophila* (Bischof et al., 2007), has been introduced for various mosquito species, to direct transposon insertion to a predetermined docking site[28] (Labbé et al., 2010; Meredith et al., 2011; Meredith et al., 2013; Nimmo et al., 2006; Pondeville et al., 2014; Volohonsky et al., 2015).

3.1.1.2. Successfully transformed mosquito species

Mosquito species are not all equally transformable. As far as vector species are concerned, *Ae. aegypti* was the first mosquito species to be genetically transformed (Jasinskiene et al., 1998) and is deemed the easiest to transform. More recently, transgenic mosquitoes of *Ae. albopictus* have been obtained, following the species' initial impact on public health (Labbé et al., 2010). *An. stephensi*, a malaria vector in India, was successfully transformed for the first time in 2000 (Catteruccia et al., 2000) and is also fairly easy to transform. *An. gambiae* is deemed the most difficult to transform: the first transgenic line was obtained in 2001 (Grossman et al., 2001). Some dozen laboratories worldwide are currently able to perform transgenesis of this species. Another species of *Anopheles* (*Anopheles albimanus*), a *Plasmodium* vector, has also been transformed (Perera et al., 2002); *Cx. pipiens* and *Cx. quinquefasciatus*, by contrast, seem difficult to transform, with only one team reporting genetic transformation of *Cx. quinquefasciatus* (Allen and Christensen, 2004; Allen et al., 2001). Limiting factors remain for some species whose embryos do not lend themselves to microinjection or which do not adapt well to laboratory handling and rearing conditions.

3.1.1.3. Possible applications in vector control

- *Can standard transgenesis[29] be used to reduce vector competence in a wild mosquito population?*

A number of transgenic mosquito strains resistant to pathogen transmission have been successfully developed in the laboratory (Isaacs et al., 2012; Jupatanakul et al., 2017; Yamamoto et al., 2016). However, any strategy seeking to interfere with vector competence in a vector mosquito population must include an effective mechanism for spreading the modification in the wild population. Without such a mechanism the frequency of the transgene in the population would simply be in proportion to the release of transgenic mosquitoes, and without selection pressure the transgene would inevitably

[28] The docking site itself is not, however, deliberately targeted within the genome; it is selected from various docking sites randomly created by insertion of a transposon into one of its target sequences occurring frequently in the genome.

[29] The term 'standard transgenesis' refers to transgenesis resulting in a genetic modification without gene drive. Gene drive is addressed in Section 3.2.2.3.

be diluted in the population or even disappear through genetic drift or counterselection if it conferred a selective disadvantage on the mosquitoes carrying it. Thus, without a mechanism to spread the modification in a population, standard transgenesis is not an option for reducing vector competence in a wild mosquito population.

- *Can standard transgenesis be used to reduce the density of a wild mosquito population?*

A transgene that confers a sterilising capacity on the mosquitoes carrying it can be used for vector control as part of a strategy to reduce the density of a wild mosquito population through repeated mass releases of sterilising mosquitoes. A number of conditions must be met: apart from the fact that the transgenic mosquitoes must be sexually compatible with the target population and competitive for mating with wild mosquitoes, genetic sterility must be conditional, preventing development of transgenic mosquito offspring in field conditions whilst allowing reproduction of mosquitoes in artificial rearing conditions. Only one example of this strategy has been developed to date: Oxitec's RIDL technique for *Ae. aegypti*, presented in Example 1 below (Section 3.2).

- *Can standard transgenesis play a part in other control techniques?*

As explained below, sexing, or separating individuals according to sex, is a technological barrier to a number of vector control techniques necessitating release of males only. Development of transgenic strains to facilitate sexing would be an important contribution to the success of a number of control methods. One example of development of a transgenic sexing strain has recently been reported for the New World screwworm (Concha et al., 2016). In mosquitoes, expression of the *M* (maleness) gene, recently discovered in *Aedes* and *Anopheles*, could be controlled by temperature-sensitive promoters in order to create such sexing strains (Adelman and Tu, 2016; Hall et al., 2015; Krzywinska et al., 2016).

3.1.2. Use of site-directed nucleases in mosquitoes and potential for vector control

Study of DNA repair mechanisms has suggested that, if it were possible to cause a sequence-specific double-stranded break in DNA, activation of endogenous repair mechanisms could be used to make targeted modifications in the genome. Two types of site-directed nucleases, or DNA cutting tools, have emerged since the 1990s. The first type, represented by zinc finger nucleases (ZFNs) and transcriptor activator-like effector nucleases (TALENs), is based on protein engineering, with the cleavage site determined by synthetic DNA-binding domains. The second, more recent, type is based on nucleic acid engineering, with the cleavage site determined by a single RNA molecule; it is represented by the CRISPR-Cas9 system and its variants.

Although this opinion focuses on the CRISPR-Cas9 system, the other types of site-directed nuclease can also be used for the same applications, including gene drive, although with a lengthier and more complex development phase. Other site-directed nucleases might also emerge in future.

3.1.2.1. CRISPR-Cas9 as a new genetic engineering tool

CRISPR-Cas9 is an enzymatic system discovered in prokaryotes, where it acts as an adaptive immunity mechanism. Insertion of short DNA sequences from infectious agents (e.g. bacteriophages) into prokaryotic genomes enables them to memorise past infections. In the event of a new infection, the repeat infectious agent is suppressed through nucleic acid recognition and cleavage by the Cas9 endonuclease (Jinek et al., 2012). Recently adapted as a tool of molecular biology, this easily programmable system can be used to make targeted DNA modifications by guiding Cas9 endonuclease cleavage at a specific site in the genome determined by an RNA molecule constructed to this end, which is known as 'guide RNA' or 'gRNA' for short (Jinek et al., 2012; Mali et al., 2013).

At this specific gRNA-determined site the CRISPR-Cas9 system can be used to generate point mutations, random or defined, and insert DNA fragments including transgenes. After targeted DNA cleavage by Cas9, the DNA can be repaired in either of two ways, depending on the DNA repair

mechanisms used by the cell: non-homologous end joining (NHEJ) may induce a random point mutation, whereas repair by homologous recombination with a sequence with close homology to the sequences flanking the break will result, depending on the homologous sequence available and used by the cell, in a defined mutation or DNA insertion (Komor et al., 2017). The homologous sequence may come from the homologous chromosome as well as from an exogenous DNA template (Taning et al., 2017). Experiments performed on mosquitoes show that the double-stranded DNA break is usually repaired by homologous recombination (Gantz and Bier, 2015; Gantz et al., 2015; Hammond et al., 2016),[30] but the variation reported suggests that the NHEJ rate will have to be determined for each new locus considered.

3.1.2.2. Potential of CRISPR-Cas9 system for vector control

Compared with transposon-mediated transgenesis, the CRISPR-Cas9 system makes it possible to:

- do without transposon-derived sequences to generate transgenic mosquitoes,
- target transgenes at a specific locus in the genome,
- generate targeted mutations without necessarily inserting or keeping transgenic elements in the genome (one possibility for a sexing strain),

but the major benefit of using the CRISPR-Cas9 system for vector control lies in the possibility of creating gene drive, enabling a genetic modification to spread within a natural population.

Any application of the CRISPR-Cas9 system not making use of this gene drive option will come up against the same limitations as standard transgenesis in terms of vector control, owing to lack of a mechanism for spreading the modification within a population.

3.1.2.3. Gene drive and its applications in vector control

Gene drive consists in increasing the odds that a genetic element will be inherited beyond the natural inheritance of 50% described in Mendel's laws,[31] thus resulting in the spread of this genetic element in the population even if associated with a certain genetic cost (Esvelt et al., 2014). Gene drive is a phenomenon that exists in nature. Genetic elements naturally endowed with this property are known as 'selfish'[32] or as having 'super-Mendelian' inheritance (Burt, 2003; Hastings, 1994). The point here is to exploit the natural phenomenon or redesign its mechanism in order to spread transgenes or inactivate existing genes in a natural population.

Since it was first conceptualised (Curtis, 1968), application of gene drive to vector control has been the subject of extensive research, particularly concerning use of these genetic elements naturally endowed with super-Mendelian inheritance (Hastings, 1994), with the specific example of homing endonucleases (Burt, 2003). The latter were put to use to create the first synthetic gene drive in a population of mosquitoes previously modified to contain the target for the endonuclease used (Windbichler et al., 2011). Adapting the specificity of these endonucleases to a given sequence present in a natural population is nevertheless a laborious and time-consuming task.

Owing to the ease with which it can be designed to target selected sequences, and the flexibility of its functional components, the CRISPR-Cas9 system offers new options for generating gene drive in a natural population. The procedures for using CRISPR-Cas9 for gene drive are very specific: unlike

[30] Depending on the locus tested, the NHEJ rate varies from 1.2% to 15% in males and from 1.2% to 21% in females (Hammond et al., 2016 ; Gantz et al., 2015). These two papers investigated four loci. The NHEJ rate will have to be determined for each new locus considered.

[31] According to Mendel's laws, in a diploid species a gene carried on a single chromosome (or to be more precise an autosome – a chromosome that is not a sex chromosome) is transmitted to half the offspring.

[32] The term 'selfish genetic elements' reflects the fact that these elements (genes, gene fragments, chromosomes and sets of chromosomes) are able to spread despite the cost that they may inflict on organisms. It has nothing to do with the use of the term by Dawkins in his 1976 book *The Selfish Gene*, which was meant to highlight the gene-centred view of evolution and which thus covered all genes. Nor is the term meant to be associated with any moral judgment of the genetic elements in question (Burt and Trivers, 2006).

standard use,[33] to develop a gene drive, the genes encoding Cas9 and its guide RNA, together with any transgenes of interest such as effector genes to be spread in the population, must be inserted together within the very same locus that is recognised by the guide RNA (the insertion disrupts the guide RNA target sequence) (Esvelt et al., 2014). In the presence of the transgenic chromosome, the initially unmodified homologous chromosome will be cut by Cas9 at the target locus, triggering repair by homologous recombination, thereby leading to the insertion of a copy of the Cas9-gRNA cassette. This will occur with each fertilisation following mating between a wild insect and a transgenic insect; the wild chromosomes will thus gradually be converted into transgenic chromosomes. If this mechanism operates effectively in the germline cells, prior to gamete formation, all the individual's offspring will inherit the transgene (Esvelt et al., 2014).

The paper providing proof of concept for CRISPR-Cas9 gene drive in *Drosophila* (Gantz and Bier, 2015) highlighted the invasive potential of this type of artificial construct. Based on this principle, several gene-drive-based vector control strategies may be considered, with two different objectives:

1. Gene drive to eliminate a vector population. Two strategies are currently under consideration:
    - Sex ratio manipulation in a population of target mosquitoes. One reported example of this strategy, currently under development, makes use not of CRISPR-Cas9 but a synthetic variant of a homing endonuclease (Galizi et al., 2014);
    - Spread of a genetic modification inactivating one of the mosquito's essential genes. This strategy is currently being tested through knock-out of a female-fertility gene (Hammond et al., 2016), described in Example 2 below (Section 3.3).
2. Gene drive to reduce the vector competence of a vector population, i.e. modifying the population to render it resistant to pathogen transmission. This may be done in two different ways:
    - Spreading of a genetic modification that will inactivate a gene that is essential to a mosquito's vector competence, for example a gene essential to pathogen development and/or transmission. The limiting factor here is to identify a gene of this type whose inactivation will not severely handicap the mosquito. Trials are under way to assess this principle with regard to inactivation of a *Plasmodium* sporozoite receptor in *An. gambiae* salivary glands (Eric Marois, pers. comm.);
    - Spreading of an effector transgene conferring resistance to pathogen transmission, which may be achieved in two different ways:
        - Interfering with mosquitoes' vector competence;
        - Tackling the pathogen directly. This strategy, currently being tested in *An. stephensi* for *Plasmodium* (Gantz et al., 2015), is described in Example 3 below (Section 3.4).

The specific examples of application of each of the genetic engineering techniques that are discussed in this opinion are presented in the sections below.

[33] To generate a genetic modification without developing a gene drive it is not necessary to insert the genes encoding the Cas9 endonuclease and its guide RNA into the genome: in practice, they can be expressed transiently or be supplied in the form of RNA; Cas9 can also be supplied in the form of purified protein (Komor et al., 2017). If they are inserted (possibly transiently prior to elimination by segregation), their location is of no importance for their successful use provided that they are expressed, and they do not have to be inserted together at the same locus.

3.2. Example 1: Oxitec's RIDL technique

Oxitec's RIDL technique is the only technique using GMOs that is currently developed to an operational level for vector control. It is an example of how standard transgenesis can be applied to mosquito vector control.

3.2.1. Principle

The principle of Oxitec's RIDL technique is not fundamentally new, being a variation of the sterile insect technique (SIT), which has been developed and used for several decades for insects other than mosquitoes.

The fundamental principle of SIT and the techniques derived from it consists in repeated mass releases of sterile or sterilising males that compete with wild males to mate with wild females, leading to a reduction in the target population or even elimination of this population if it is isolated.

Only males are released, since release of females would be likely (1) to increase pathogen transmission[34] and (2) to reduce the efficiency of the intervention by deterring some sterile males from mating with the wild females targeted.

Standard SIT makes use of males sterilised by irradiation. Successfully employed on other insects since the 1950s (Enkerlin et al., 2015; Vargas-Teran et al., 2005; Vreysen et al., 2000; Wyss, 2006), it is still being developed for mosquitoes (see Section 4.2.1 below).

Initial development of standard SIT for mosquitoes encountered specific problems relating to operational use of irradiation and/or the supposed negative effects associated with irradiation, which encouraged research into alternative methods of sterilisation (Phuc et al., 2007). RIDL (Release of Insects carrying a Dominant Lethal gene) has been designed to induce repressible dominant lethality through genetic modification.

The dominant lethality trait, also characteristic of radiation-induced random sterilising mutations in standard SIT mosquitoes, prevents development of hybrid offspring of the mosquitoes released, whether the latter are transgenic or irradiated, with wild mosquitoes. On the other hand, unlike the radiation-induced mutations in standard SIT mosquitoes, genetic modification of mosquitoes cannot be generated immediately prior to their release, which raises the problem of the development and rearing process for mosquitoes carrying the lethality trait. Here it is the repressibility of the transgenic lethality trait in the presence of an antidote that allows normal development of transgenic mosquitoes for both breeding- and release-oriented production.

3.2.2. Current state of research and production techniques

On this principle Oxitec has developed several *Ae. aegypti* mosquito strains, including OX513A.[35] Generated by standard transposon-mediated transgenesis, OX513A mosquitoes carry a synthetic transgene encoding the tTAV (tetracycline-repressible transcriptional activator variant) protein, a fusion of tetR (tetracycline repressor protein from *Escherichia coli*) and VP16 (transcriptional activator virion protein 16 of the herpes simplex virus), under the control of its own binding site *tet*O from *Escherichia coli*, and *Drosophila* regulatory sequences (Phuc et al., 2007). The tTAV protein is characterised by transcription factor activity that drives its own expression whilst disrupting expression of multiple mosquito genes to the extent of killing them ('transcriptional squelching' (Lin et al., 2007)). The system is controlled by a mechanism known as Tet-Off:[36] in the absence of

[34] It should be recalled that in mosquitoes only the females bite and can transmit pathogens.

[35] Another strain expressing a phenotype preventing production of reproductive females (flightless phenotype) has proven to have fitness costs that are too high for an effective population reduction strategy in operational conditions (Facchinelli et al., 2013). Other mosquito strains are currently under development (Oxitec, pers. com.).

[36] http://www.tetsystems.com/science-technology/ (accessed on 16 March 2017).

tetracycline, tTAV prevents development of transgenic mosquitoes (the tetR component of tTAV binds to *tet*O and induces lethal overexpression of tTAV through VP16); in the presence of tetracycline, tTAV is inactivated (as tetR has a greater affinity for tetracycline than for *tet*O), and the lethality associated with the *tTAV* gene is then repressed, allowing the strain to be reared in artificial conditions supplemented with tetracycline (Phuc et al., 2007).

In addition to the *tTAV* effector transgene, OX513A mosquitoes carry a fluorescent marker, DsRed2, from *Discosoma* coral, enabling the transgenic mosquitoes to be tracked (Phuc et al., 2007).

Furthermore, the OX513A line was selected from various transformants for its late-acting lethality: OX513A mosquitoes die at the larval or even pupal stage (Phuc et al., 2007). Late-acting lethality is useful for SIT strategies applied to insects whose population density depends on limited resources. This is the case for mosquitoes, whose population development is limited by the availability of egg-laying sites and nutrients for the larvae. These resources are thus factors of competition in the early stages of mosquito development. Lethality at the embryo stage, typical of standard SIT using irradiation, reduces the impact of these factors, which can lead to overcompensation. As a result, a substantial reduction in females' reproductive potential would not necessarily result in a significant decline in the size of the target population and might even establish a larger equilibrium population (Phuc et al., 2007). The advantage of late-acting lethality in terms of population reduction strategies' effectiveness has been clearly modelled: the number of sterile mosquitoes that would have to be released in proportion to the number of target wild mosquitoes in order to eliminate the population is lower with late-acting lethality than with embryonic lethality, and elimination is faster with effective release ratios in both cases (Phuc et al., 2007). According to additional modelling, this advantage would remain even if the OX513A larvae were up to 30% less competitive than wild larvae prior to their death (Phuc et al., 2007). This advantage of late-acting lethality in OX513A mosquitoes over embryonic lethality in standard SIT mosquitoes has yet to be demonstrated in the field.

The OX513A line has also been chosen for the high penetrance of its phenotype (i.e. the fact that a high proportion of transgenic mosquitoes effectively express the phenotype that the transgene is supposed to confer), although this penetrance is incomplete. Complete penetrance would entail 100% lethality for OX513A mosquitoes and their hemizygous offspring in the absence of tetracycline. However, 3 to 4% of transgenic mosquitoes survive in laboratory conditions without tetracycline (Phuc et al., 2007). This proportion drops to 2% in the environment, most likely due to less favourable conditions than in the laboratory (Dr Hadyn Parry, comment at HCB Oxitec hearing). The impact of incomplete lethality on the effectiveness of a population reduction strategy has also been modelled: while it is higher if the release ratio (number of mosquitoes released to number of target mosquitoes in the field) is low, its impact on strategy effectiveness is negligible for the ratios tested and for survivor levels under 5%, which is the case for the OX513A strain (Phuc et al., 2007).

The OX513A transformed line has been characterised in the laboratory. As expected with the Tet-Off control system, no difference in survival was found between OX513A mosquitoes and their non-GM equivalents in the presence of tetracycline, guaranteeing their production in rearing conditions supplemented with tetracycline (Phuc et al., 2007). Tested in laboratory cages after introgression of the transgene into a Malaysian genetic background, the OX513A males proved capable of inseminating as many females as non-GM males over the first few days, although they seemed to have a lower insemination capacity over the course of their lifetime and a higher cost of mating than near-isogenic non-GM males (Bargielowski et al., 2011). These findings were not deemed to preclude a RIDL strategy involving frequent field releases of excess OX513A males pending assessment of their performance in the field in competition with wild males (Bargielowski et al., 2011).

In addition to characterisation of transgenic mosquitoes in the laboratory and assessment of their competitiveness in the environment, production methods – specifically mentioned in the referral – and modes of deployment for OX513A mosquitoes are critical to the strategy's success in the field. More generally, these aspects have been considered largely responsible for the mosquito-specific

difficulties in implementing SIT strategies. A review of their development has been published by Carvalho et al. in connection with the deployment of OX513A mosquitoes in Brazil, including methods of larval production, blood feeding and sorting of males and females (Carvalho et al., 2014). Shared in various degrees by different mosquito-release strategies, the techniques of mosquito production and their associated challenges are compared in Section 4.3.3 in order to identify specific features relating to GM mosquitoes.

The specific features, risks, benefits and limitations associated with this technique are discussed at greater length later in this opinion (Section 4.3 and Section 6).

3.2.3. Outcomes of initial experiments worldwide

A step-by-step assessment system is established prior to any large-scale release of mosquitoes in the field. Mosquito strains are assessed first in the laboratory (Phase 1) and then in what are known as semi-field conditions, where mosquitoes are released in contained systems that imitate their natural environment and are placed in the field (Phase 2). These contained trials are used mainly to assess the sexual performance of transgenic strains and their competitiveness for mating with local strains. Phase 2 also includes site characterisation, pilot site selection and entomological and epidemiological data-gathering. Pilot trials in field conditions, on the scale of one or more villages in rural areas or one or more neighbourhoods in urban areas, can then take place to assess the efficacy of intervention in comparison with control sites (Phase 3). Phase 4 covers area-wide operational releases in the field in actual conditions of use including the relevant conventional vector control methods.

On this pattern, following a number of laboratory characterisation studies, experiments have been conducted with OX513A mosquitoes in the environment in Phases 2 and/or 3 in Malaysia (Lacroix et al., 2012; Lee et al., 2013), the Cayman Islands (Harris et al., 2012; Harris et al., 2011), Brazil (Carvalho et al., 2015) and Panama (Gorman et al., 2016). A Phase 4 experiment is under way in Brazil. Reported trials are described in Appendix 11 of the WG report. Their outcomes are set out below.

To date the experiments in Malaysia have been limited to characterisation of transgenic strains:

- Mating competitiveness of OX513A strains was assessed in semi-field conditions for different combinations of genetic backgrounds into which the transgene had been introgressed: between GM and non-GM males for non-GM females with the same genetic background (Malaysian), and between GM males with a Mexican genetic background and non-GM males with a Malaysian genetic background for non-GM females with a Malaysian genetic background. Both experiments showed that GM males were as likely as non-GM males to mate with non-GM females, demonstrating that the transgene had no negative consequences for the mating performance of the males tested and that use of different genetic backgrounds did not affect the outcome, at least in the combinations and conditions tested (Lee et al., 2013). This suggested that the RIDL approach might be applied without having to introgress the transgene into the local genetic background (Lee et al., 2013).
- Mosquitoes were released in the field to test the performance of OX513A mosquitoes in the environment compared with that of near-isogenic non-GM mosquitoes in order to identify any unintended effects associated with the transgene. Designed as the first stage of a precautionary step-by-step trial strategy, a small-scale release was made in an uninhabited forest area in December 2010. The conclusions from this trial, conducted in an atypical habitat for *Ae. aegypti* mosquitoes and where no offspring of the mosquitoes released were detected, are unclear: while the transgene had no negative effect on longevity and maximum dispersal distances of male mosquitoes in these conditions, the mean distance travelled by GM mosquitoes would seem to be significantly lower (Lacroix et al., 2012). The authors believe that this reduction would

not in principle make a RIDL strategy unfeasible. This must be tested, and these experiments must be repeated in more appropriate environments (Lacroix et al., 2012).

The other experiments reported tested the feasibility and efficacy of the RIDL approach for reducing a population of target mosquitoes in different environments. All these experiments were conducted with an OX513A strain with a Mexican genetic background.

The key parameters assessed in preparation for such experiments are:

- Proportion of transgenic males caught in adult traps, to estimate the **initial density of the wild population** in relation to the number of transgenic mosquitoes released;
- Proportion of transgenic larvae developed from eggs collected in ovitraps, to estimate the **mating fraction** of wild females with OX513A males, representing the **induced sterility rate**.

These factors are used to determine the **field competitiveness** of OX513A mosquitoes[37] together with the number of mosquitoes to be released to attain a given population reduction target depending on initial density.

The key parameter for progress and success of a RIDL strategy, **mosquito population density**, is measured:

- either indirectly, by an ovitrap index indicating the proportion of traps containing eggs of the target population,
- or more directly, by the number of target population adults caught in adult traps, which should be adjusted for trap efficiency, estimated by the recapture rate for sterile males.

The **reduction in mosquito population density** is measured from ratios of ovitrap indices and/or of numbers of adults recorded in the release zone at different stages in the experiment. The relative density reduction is inferred by comparing these figures for the release zone and the control zone.

The feasibility of the RIDL approach was tested for the first time in late 2009 on Grand Cayman, one of the three islands making up the British Overseas Territory of the Cayman Islands in the Caribbean (Harris et al., 2011). The characterisation parameters for the target population and for local performance of released mosquitoes, established on the basis of this first experiment, were then used to plan the first field trial for mosquito population reduction, conducted from May to October 2010 (Harris et al., 2012). Despite problems with mosquito production, resulting in a gradual restriction of the trial area from 55 ha to 16 ha, the potential of the strategy was validated, with a relative reduction in mosquito population density of approximately 80% according to an ovitrap index. Although 3.3 million GM mosquitoes were released somewhat irregularly over a 23-week period,[38] it has been estimated that a release of approximately 3,500 males per hectare per week, i.e. a ratio of 5:1, would have been enough to achieve this outcome. Furthermore, the authors emphasise that this outcome could be improved in the event of (1) less exchange with neighbouring areas (immigration of females fertilised by wild mosquitoes / emigration of GM males or of females fertilised by GM males) – which would be possible with a larger or more isolated area, (2) lower population survival in the form of eggs fertilised prior to release – possible with a longer operational programme – and (3) combination with other methods of control to reduce the initial mosquito density (Harris et al., 2012).

The experiment carried out in the state of Bahia in north-eastern Brazil from May 2011 to October 2012 also took place in several phases: a two-month preparatory phase to assess mosquitoes' performance during an initial series of releases and thus adjust the reduction strategy to local conditions, a one-year population reduction phase, and then a three-month maintenance phase

[37] Field competitiveness is calculated using the following formula: C = PW/(S(1-P)), where W is density of wild males, S is density of sterile males and P is proportion of sterile matings (proportion of fluorescent larvae in the case of OX513A mosquitoes) (Carvalho et al., 2015).

[38] A succession of release arrangements was used: (1) Across 55 ha for 6 weeks: 1,400 (95% CI: 990-1,800) adult males released per ha per week; (2) Across 32 ha for 6 weeks: 3,900 (95% CI: 2,600-5,300) adult males released per ha per week; (3) Across 16 ha for 11 weeks: 14,000 (95% CI: 13,700-14,500) adult males released per ha per week for the first 3 weeks, followed by 7,700 (95% CI: 6,900-8,500) adult males per ha per week, supplemented with 4,900 (95% CI: 3,800-6,000) adults per hectare per week eclosed from 5,600 (95% CI: 4,500-6,800) pupae released in the field.

(Carvalho et al., 2015). As in the Cayman Islands, the initial targets had to be scaled down in the course of the experiment owing to the difficulty of producing mosquitoes in the quantities needed to reduce the target population in the area concerned. The release area was therefore reduced halfway through, from 11 ha to 5.5 ha. Once tailored to local conditions, the RIDL strategy was ultimately validated, with a reduction in the *Ae. aegypti* population density of approximately 78% according to the ovitrap indices and 95% according to adult trapping data, for a total of approximately 15 million GM mosquitoes released over a year. The authors believe that mean releases in the region of 29,000 male mosquitoes per hectare per week would have been enough to achieve this outcome (Carvalho et al., 2015).

The experiment conducted in Arraijan in the suburbs of Panama City from April to October 2014 had the additional aim of assessing the effect of *Ae. aegypti* population reduction on a coexisting *Ae. albopictus* population (Gorman et al., 2016). A total of some 4.2 million *Ae. aegypti* OX513A male adult mosquitoes were released over a 6-month period across a 10 ha area, i.e. an average of approximately 15,700 adults per hectare per week. While *Ae. aegypti* egg-laying was reduced by 82% over 116 days and 93% over 200 days, no specific impact was found on *Ae. albopictus* population dynamics compared with the control sites in the course of the trial. However, the authors point out that it might be necessary to investigate the impact of longer-term reduction (Gorman et al., 2016). This experiment also enabled an assessment of the persistence of the transgene in the environment once releases had ceased: the proportion of eggs containing transgenic larvae in the ovitraps declined by 95% in 28 days. There were no detectable transgenic larvae 84 days after the last release (Gorman et al., 2016).

It should be noted that the field competitiveness of OX513A mosquitoes varies in these experiments (C=0.059 in the Cayman Islands, C=0.03 in Brazil and C=0.144 in Panama) (Carvalho et al., 2015; Gorman et al., 2016; Harris et al., 2012; Harris et al., 2011), which is not surprising in itself as it covers a number of parameters, including conditions of mosquito production, which may vary, and uncontrolled migration of mosquitoes to and from the release area. It must be distinguished from competitiveness measured in contained conditions, since, in the latter, OX513A mosquitoes have proven as competitive as the wild males tested, whatever their genetic background, in the experiments reported (Harris et al., 2011; Lee et al., 2013). No field competitiveness data have been published for mosquitoes used in standard SIT. It should nevertheless be noted that the data obtained with OX513A mosquitoes are within the range of values recorded for other insects.[39]

These experiments highlight the influence of local conditions on the resources needed to achieve a reduction in the mosquito population when employing a RIDL strategy. They also show the importance of initial characterisation of the target population and its environment, field competitiveness of OX513A mosquitoes, progress monitoring of reduction, and adaptability of releases according to outcomes and climatic variation, as well as the need for long-term stable conditions for mass mosquito production in order to scale up these results.

We should further note that the experiments reported have been assessed with strictly entomological criteria. Oxitec has nevertheless concluded that the mosquito releases in Brazil and Panama reduced the mosquito populations below the dengue transmission threshold (Carvalho et al., 2015; Gorman et al., 2016). To predict the experiments' impact on dengue transmission with entomological indicators, Oxitec has used the approach suggested by Focks et al., who determined transmission thresholds on the basis of number of pupae per person (as a proxy for the adult mosquito population), ambient temperature and herd immunity (Focks et al., 2000). The operational validity of this approach is controversial owing to the complexity of the relationships between

[39] C=0.3-0.5 for tsetse flies in Senegal (Bouyer et al., 2012); C=0.1 for the New World screwworm fly (Mayer et al., 1998; Vreysen, 2005), and C<0.01 for the Mediterranean fruit fly (Rendon et al., 2004; Shelly et al., 2007). It should nevertheless be pointed out that the Mediterranean fruit fly is known for its particularly low competitiveness owing to a highly complex mating ceremony.

entomological and epidemiological indicators[40] (Bowman et al., 2014; Fontenille et al., 2009; Wijayanti et al., 2016) (see WG report, Appendix 12).

A larger-scale experiment is under way in another region of Brazil, Piracicaba, in the state of São Paulo. Begun in a single neighbourhood in 2015, the experiment was broadened in 2016 to cover a 12 km^2 area with 60,000 residents (Servick, 2016). As a Phase 4 experiment, it could allow epidemiological data of interest to be gathered, enabling the efficacy of OX513A mosquito releases to be measured on the basis of impact on transmission of the diseases carried by these mosquitoes.

In conclusion, while each Oxitec experiment has indeed shown local effectiveness for reduction of field mosquito populations, the technique's efficacy regarding transmission of diseases such as dengue, Zika, chikungunya and yellow fever by these mosquitoes has not yet been established in the field. This is not specific to the RIDL technique. As far as members of the working group were aware, none of the emerging vector control techniques involving mosquito release had entailed epidemiological surveillance to date, unlike other control methods such as use of insecticide-treated mosquito nets or indoor residual spraying, whose epidemiological efficacy against malaria has been demonstrated (Lengeler, 2004; Pluess et al., 2010).

3.2.4. Marketing

RIDL mosquitoes are not yet on the market. Although in 2014 Oxitec obtained a licence from Brazil's National Biosafety Technical Committee (CTNBio) for unconditional release of OX513A mosquitoes in Brazil (a licence allowing the current large-scale experiment in the state of São Paulo), they cannot be marketed without formal permission from the Brazilian Health Regulatory Authority (ANVISA), whose appraisal is currently being delayed by preparation of a new regulatory framework.

3.3. Example 2: Gene drive for population elimination

3.3.1. Principle

Among possible gene drive techniques for population elimination (see Section 3.1.2.3), one method consists in spreading inactivation of a gene essential for female fertility.

This technique is based on release of sterilising males carrying a gene drive cassette that confers a female-sterility phenotype owing to its insertion within a gene essential for female fertility. The sterilising males initiate the spread of female sterility in the population from their first mating with wild females. Hemizygous individuals,[41] male and female, transmit the cassette to all their offspring owing to gene drive in the germ cells. The rapidly increasing frequency of the gene drive cassette results in a drop in population size owing to the sterility of the homozygous females.

The technique's success depends on two conditions in particular:

1- Genetic modification must induce sterility in homozygous females whilst preserving the fertility of hemizygous females, thus ensuring transmission of the modification to offspring;

2- Gene drive must take place only in the germ cells, prior to gamete production, thus allowing a hemizygous individual to transmit the modification to all its offspring whilst ensuring that the fertility of hemizygous females is not impaired somatically.

[40] Consider the prime example of Singapore, where significant dengue epidemics persist despite the considerable efforts made to reduce the *Aedes* population. A drop in entomological indices has not been reflected by a proportional decline in incidence of the disease, suggesting that virus transmission can continue at very low mosquito population densities that are hard to detect with entomological indices.

[41] Hemizygous individuals are initially hybrid offspring F_1, from mating between released males carrying the gene drive cassette (homozygotes) and wild females. In subsequent generations they are the hybrid offspring from mating between an individual carrying the cassette (homozygous male, hemizygous male or hemizygous female) and a wild individual. The homozygous males transmit the cassette normally to all their offspring. Only the homozygous females are sterile.

3.3.2. Current state of research

One application of this technique is currently being developed for the *An. gambiae* mosquito (Hammond et al., 2016), the main vector of the *Plasmodium* responsible for malaria in sub-Saharan Africa and Mayotte and one of the vectors of the filarial worms responsible for lymphatic filariasis in Mayotte.

Three female-fertility genes were tested, selected for the recessive female-sterility phenotype each of them conferred following disruption.

On the principle described above, a gene-drive cassette was inserted into each of these genes, containing: (1) the gene encoding the Cas9 endonuclease under the control of a reportedly germline-specific promoter, (2) a guide RNA designed to direct the endonuclease to cleave each of these wild-type genes, under the control of a ubiquitous promoter, and (3) a fluorescent marker (Hammond et al., 2016).

Gene drive tests on laboratory mosquito populations for each of the constructs confirmed the potential of this strategy whilst highlighting two technical problems that will have to be solved if the strategy is to be used successfully in the environment: (1) development of gene drive resistance and (2) a drastic reduction in hemizygous female fertility (Hammond et al., 2016).

Solutions are being developed for each of these problems: the gene-drive resistance phenotype, caused by DNA repair through non-homologous end joining in part of the population, could be circumvented by using several guide RNAs in the same cassette to target different sequences of the gene to be inactivated or by using several gene drive cassettes; the problem of reduced fertility in hemizygous females, caused by somatic expression of the Cas9 nuclease, seems already to have been solved by using a more germline-specific promoter (Austin Burt, pers. comm.).

The specific features, risks, benefits and limitations associated with gene-drive techniques for population elimination are discussed at greater length later in this opinion (Section 4.3 and Section 6).

3.4. Example 3: Gene drive for population modification

3.4.1. Principle

Among the various possible gene-drive techniques for rendering a population resistant to pathogen transmission (see Section 3.1.2.3), one method consists in propagating an effector transgene that will attack the pathogen directly.

This technique is based on release of males that, from their first mating with wild females, initiate the spread in the population of a gene drive cassette conferring on mosquitoes resistance to pathogen transmission through a 'resistance' transgene preventing the pathogen from developing in the mosquito. Hemizygous individuals[42] transmit the cassette to all their offspring owing to gene drive in the germ cells. The rise in frequency of individuals carrying the 'resistance' transgene in the mosquito population should lead to a reduction in pathogen transmission.

3.4.2. Current state of research

The limiting factor for this technique is identification of a resistance transgene that is effective against transmission of the target pathogen and not likely to facilitate transmission of other

[42] Hemizygous individuals are initially hybrid offspring F_1, from mating between released males carrying the gene drive cassette (homozygotes) and wild females. In subsequent generations they are the hybrid offspring from mating between an individual carrying the cassette (male or female, hemizygous or homozygous) and a wild individual. The homozygous individuals transmit the cassette normally to all their offspring.

pathogens. The risk of unforeseen effects on other microorganisms will be reduced if the resistance factor's mode of action is pathogen-specific.

The principle of this technique is currently being tested for *An. stephensi*, the principal vector of *Plasmodium falciparum* in Asia (Gantz et al., 2015). Here the resistance mechanism derives from a synthetic construct designed to produce two anti-*Plasmodium* antibodies, one targeting the *Plasmodium* ookinete protein Chitinase 1 and the other the circumsporozoite protein (Gantz et al., 2015). Unlike wild-type female mosquitoes, under the infection conditions expected in the field transgenic female mosquitoes expressing these antibodies do not produce *P. falciparum* sporozoites (the infectious form of the parasite) in their salivary glands and are therefore incapable of transmitting the parasite (Gantz et al., 2015).

To introduce the gene drive, mosquitoes were transformed with a gene drive cassette carrying: (1) the gene encoding the Cas9 endonuclease under the control of a reportedly germline-specific promoter, (2) a guide RNA designed to direct the endonuclease cleavage to a gene whose disruption phenotype will act as an additional drive marker (*kh*, white eyes), under the control of a ubiquitous promoter, (3) a fluorescent marker, and (4) effector transgenes encoding the anti-*Plasmodium* antibodies (Gantz et al., 2015).

In laboratory tests a high rate of gene conversion was recorded in generations following the first mating with wild-type females. As in the previous example of gene drive, solutions must nevertheless be found to the lack of tissue-specific Cas9 expression and the development of resistance through NHEJ (Gantz et al., 2015).

Other specific mechanisms of resistance to pathogen transmission may be considered, such as virus-specific interfering RNA, obtained by transgenic expression of a hairpin fragment from the genome of the virus to be eliminated (RNA interference (RNAi)). This type of approach could eventually be used against viral epidemics once it has been demonstrated that activation of the RNAi pathway by the transgene does in fact suppress transmission of the target virus. It may be asked whether the RNAi pathway would then be less available for attacking other viruses carried by the same species. It will therefore be important to study beforehand any changes in vector competence with regard to non-target viruses. Conversely, it should be possible to combine resistance to several viruses in a single transgene. Given its specificity, this mechanism would not in principle stop the emergence of an as yet unknown arbovirus.

The specific features, risks, benefits and limitations associated with gene-drive techniques for population modification are discussed at greater length later in this opinion (Section 4.3 and Section 6).

Thus use of GM mosquitoes is being considered through various techniques that could fit in with different vector control strategies. These techniques are being tested in specific applications, currently at different stages of development. The specific features, risks, benefits and limitations of the examples described in this section will be discussed further in the next sections of this opinion.

4. Specific features of vector control techniques using genetically modified mosquitoes

The referral asked for the specific features of vector control techniques using GM mosquitoes to be highlighted in comparison with methods already employed. In the spirit of the question we here propose to extend the comparison to other emerging techniques akin to techniques using GM mosquitoes. After a brief outline of existing vector control methods and these other emerging techniques, we shall highlight the specific features of techniques using GM mosquitoes in terms of possible objectives, efficacy and sustainability, technical constraints, and environmental and health risks.

4.1. Overview of existing vector control techniques

4.1.1. Use of biocides

The majority of the vector control techniques employed at present are based on use of insecticides (adulticides and larvicides), repellents and baits (combined with traps). Whether these products are chemical (synthetic or derived from plants) or biological, they are regulated as biocides in the European Union (EU, 2012).[43] As such, they are subject to the same marketing authorisation process, with the same assessment requirements regarding hazards, risks and efficacy. Biocides and their regulatory framework are described at length in the WG report (Section 3.2.1). We shall here focus on their uses and limitations.

1. Different uses and objectives of biocides in vector control

Adulticides

Adulticide treatments target blood-feeding adult females, solely responsible for pathogen transmission. Depending on the methods used, the purpose of adulticide treatments is to reduce vector density, vector longevity or host-vector contact:

- Indoor residual spraying of interior walls of homes is intended to reduce adult population density with the particular aim of reducing female longevity to shorten the activity period of older females, which are potentially the most dangerous for pathogen transmission. Indoor spraying is advisable if vectors are endophagic (prefer to feed indoors) or endophilic (prefer to rest indoors after blood meals). Persistence varies from 2 to 6 months depending on substrate and formulation;
- Space spraying generates a fog of very fine droplets intended to kill adult insects almost instantaneously upon contact and with very low persistence. Space spraying is recommended mainly for epidemics to lessen active adult mosquito densities quickly and bring down transmission;
- Insecticide-treated mosquito nets combine a physical barrier with chemical protection that repels or kills vectors even if the netting is damaged in parts. They are now treated industrially to ensure that they are effective over 20 washes and 3 years of use. Large-scale distribution has proven highly effective and is a mainstay of the Global Malaria Action Plan.[44]

[43] Biocides are used for chemical control and for some biological control applications. They are here given a section to themselves to emphasise the significance of regulations in their use for vector control. In the field of vector control, chemical control covers use of synthetic or plant-derived chemicals as repellents, attractants (combined with traps) or insecticides (Fontenille, 2009). Biological control is discussed in Section 4.1.2.

[44] The Global Malaria Action Plan (GMAP) is an initiative of the Roll Back Malaria Partnership (RBM), an international agency coordinating action against malaria (http://rollbackmalaria.org/about-rbm/aim-2016-2030/).

Use of adulticides is particularly subject to constraints and restrictions in the European Union. Of the four main families of adulticides used worldwide (pyrethroids and organochlorines, interfering with voltage-gated sodium channels, and organophosphates and carbamates, inhibiting acetylcholinesterase) (WHO, 2011), only fifteen or so of the molecules are still authorised in the European Union, all belonging to the pyrethroid family. Of the latter, the most widely used is deltamethrin. It is also the first choice of France's public vector-control operators owing to its environmental, technological and health profile. Natural pyrethrins, licensed for organic farming, are sometimes also used. Deltamethrin and natural pyrethrins are thus generally mentioned as the main authorised active ingredients, if not the only ones, to be used as adulticides by French operators, in prefectoral orders for both mosquito control and vector control more generally.[45]

Pyrethroids are highly toxic to aquatic organisms and cold-blooded animals. Adulticide treatments are therefore prohibited over or close to watering places, i.e. watercourses, ditches and permanent or transient bodies of water, including wetlands (Environment Code, Rural Code and Public Health Code). Use of pyrethroid adulticides is therefore limited to natural areas excluding wetlands and to urban and peri-urban areas, subject to strict compliance with a certain number of regulatory and environmental restrictions and constraints.

Larvicides

Larvicide treatments are intended to reduce vector population density. They are applied to species with an aquatic stage in their development cycle and whose breeding sites are relatively stable, identifiable and accessible to operators.

Larvicides can also be used indirectly through auto-dissemination: placed in ovitraps or resting traps in the form of powder, they are effectively disseminated by the adult females that land there. On a small scale this strategy has proven very effective with pyriproxyfen (a juvenile hormone analogue) for controlling *Ae. aegypti* in Peru (Devine et al., 2009) and subsequently *Ae. albopictus* in Spain (Caputo et al., 2012). The contaminated females deposit the pyriproxyfen in their breeding sites, thus drastically reducing the rate of adult mosquito emergence from the larvae concerned. The difference in attractiveness between dissemination stations and natural sites requires further study in the field to optimise the number of traps per hectare and their efficacy before large-scale use can be contemplated. Moreover, given the large number of traps per hectare that have to be set, this technology is not considered cost-effective at present.

The following can be used as larvicides:

- Synthetic growth regulators, such as juvenile hormone analogues, which disrupt larval development and prevent metamorphosis into pupae and adults, or ecdysone analogues, which inhibit chitin synthesis during moulting;
- Biological biocides, such as the spinosyns A and D produced by the actinomycete bacteria *Saccharopolyspora spinosa*, which are effective for larval mosquito control but are non-selective (Hertlein et al., 2010), or the protein crystals formed by *Bacillus thuringiensis israelensis (Bti)* and *Lysinibacillus sphaericus* (*Lsph*, formerly known as *Bacillus sphaericus* (*Bs*)), whose larval toxicity is very specific owing to their mode of action through receptors after ingestion (Ben-Dov, 2014; Regis et al., 2001).

To date, three growth regulators (diflubenzuron, pyriproxyfen and S-methoprene) and the entomopathogenic bacteria *Bti* and *Lsph* have been authorised as larvicides in the European Union. However, the vast majority of natural environments subject to mosquito control operations have

[45] Deltamethrin and other pyrethroid active ingredients still on the market are also employed in a variety of ways by private service providers in disinfection and insect/rodent control for every type of public health use (including mosquitoes but also all domestic pests, cockroaches, bugs, termites, etc.). Some of these substances are also sold off the shelf in garden centres and hypermarkets in appropriate forms for domestic use (Flit gun, coils, plugs, diffusers, etc.).

varying types of protection status. At EU level, the Habitats Directive (Natura 2000) requires impact studies. Locally, each Member State can apply special rules for certified protected areas. This is the case, in France, for the nature reserves run by France's Coastal Protection Agency and for the Camargue Regional Nature Park, for example. With these new environmental protection requirements, use of *Bti* is obviously preferred in natural environments, since growth regulators are usually outlawed because of their lack of selectivity regarding non-target aquatic invertebrates, and *Lsph* is very little used or else used in association with *Bti*. In urban environments all larvicides still on the market are used, but here again most public operators tend to base their strategies mainly just on *Bti.*

Repellents

Used for personal protection against vectors, repellents are intended to limit host-vector contact ((Duvallet et al., 2011), 'Good clinical practice recommendations on personal protection against vectors' produced by the Society of Travel Medicine and the French Society of Parasitology). There are repellents for dermal application and to treat clothing, other fabrics or mosquito nets. Active ingredients authorised by the EU include DEET (N,N-diethyl-m-toluamide) and IR3535 (3-[N-Butyl-N-acetyl]-aminopropionic acid, ethyl ester). The ingredients KBR3023 (1-(1-methylpropoxycarbonyl)-2-(2-hydroxyethyl)piperidine/Icaridin) and PMDRBO (mixture of cis- and trans-p-menthane-3,8 diol, or 2-hydroxy-α,α,4-trimethylcyclohexanemethanol) are currently being assessed at the EU level. It should also be noted that permethrin and deltamethrin, combined with trans-tetramethrin, are authorised for treatment of clothing, fabrics and mosquito nets. Permethrin and cypermethrin are used for pre-treated mosquito nets.[46]

Baits

Baits are used in association with trapping systems. They are here discussed under physical control (Section 4.1.3).

2. Limitations of biocide use

Since vector control methods are based to a large extent on use of insecticides, the main obstacles facing control programmes are the development of insecticide resistance and the reduction in the number of molecules available for public health treatments. The environmental and health impact of some biocides is a further limitation on their use: regulatory assessment has severely restricted the number of biocides authorised and established stringent rules for their use. The health impact of biocides will be discussed at greater length in comparison with that of techniques using GM mosquitoes and other vector control techniques (see Section 4.3.4).

Development of insecticide resistance

Resistance in a target species may be defined as a heritable change in the sensitivity to an insecticide (Nauen, 2007). In essence, it is an adaptation to a new environment through selection pressure from one or more insecticides as part of a natural selection process. Resistant individuals carry one or more genetic mutations (known as resistance alleles) encoding proteins that interact with the insecticide. Thus the mutated proteins prevent the insecticide from reaching its target, for example by degrading it or by modifying the target, allowing the insects carrying these mutations to survive what would normally be lethal doses of insecticide (Labbé et al., 2011). Insecticides do not cause onset of these mutations directly but select for the individuals carrying them, since they are then able to survive and reproduce in the presence of these insecticides. Consequently, resistance allele frequency increases in populations exposed to an insecticide over generations. Some species may be resistant to a very wide range of compounds, both chemical and biological.

[46] http://social-sante.gouv.fr/IMG/pdf/tableau_repulsif_recos_mars_2016.pdf.

The growth in vector populations with multiple resistance mechanisms is a real threat to vector control programmes. Interactions between several resistance mechanisms are not necessarily additive for the phenotype (Raymond et al., 1989) but may generate substantial resistance multiplicatively, as found in *Cx. quinquefasciatus* for mutation of the *kdr* gene (sodium channel protein) when associated with cytochrome P450 mutations, with permethrin resistance levels up to 10,000 times higher than in susceptible larvae (Hardstone et al., 2009). A significant decline in the efficacy of insecticide-treated mosquito nets and indoor residual spraying has been recorded in the south of Benin for *An. gambiae* adults showing metabolic resistance associated with mutation of the pyrethroid target site (N'Guessan et al., 2007). In Martinique, resistance in *Ae. aegypti*, a dengue and chikungunya vector, has reached levels such that pyrethroid (deltamethrin) and pyrethrin space sprays have become ineffective for reducing vector densities (Marcombe et al., 2011).

Resistance management strategies and problems of implementation

Resistance management strategies have been developed to maintain the efficacy of vector control. They consist in using several molecules with different modes of action to reduce the selection pressure from a single molecule and the selective advantage of resistant individuals if resistance is associated with a fitness cost. These strategies are based on sequential use of insecticides over a certain period (rotations) or area (mosaics), or on combining a number of molecules in a mixture. Given that public operators in France have a preference for a single adulticide (deltamethrin) and a single larvicide (*Bti*), these resistance management strategies are hard to implement on French territory.

Another difficulty stems from the fact that the same product can be used both as a biocide for vector control and as a plant protection product for farming purposes under different regulations.[47] It is therefore necessary to take into account agricultural use of biocides when managing resistance development in mosquitoes. Ideally, use of the same product for two different purposes should be coordinated at the landscape level.

Paucity of available insecticides and limited development

While innovation in plant protection for farming is thriving on the whole, no new molecules have been developed specifically for public health over the past twenty to thirty years. This situation is attributable to a number of causes, including economic and regulatory factors, such as higher regulatory standards for assessing and limiting unintended effects in terms of toxicology and ecotoxicology, the variety of regulations between countries (an economic obstacle) and the prospect of a meagre return on investment given the limited size of the market concerned.

The situation is particularly critical with regard to available insecticides in France, which has a number of overseas territories in areas where some diseases are endemic (malaria, dengue, chikungunya, Zika, etc.). Research into alternative adulticides with modes of action that differ from that of pyrethroids is urgently needed to restore the efficacy of vector control in the territories where *Ae. aegypti* is resistant and to prevent the risk of resistance to this family of insecticides in *Ae. albopictus* elsewhere. Approached by the Environment, Health and Labour Ministries in 2009, 2012 and 2015, and more recently by the Ministry of Agriculture, the French Agency for Food, Environmental and Occupational Health and Safety has delivered three opinions to this end (Anses, 2011, 2013, 2016).

This being so, it is necessary to develop vector control methods that are alternative and/or complementary to biocide use; some are already available and being used, including biological control and physical/environmental control, which are described below.

[47] Under Article 2 of Regulation (EU) No 528/2012, some biocidal products may be used for purposes that fall within the scope of other regulations. This is the case for use of biocides for plant protection, which comes under the regulation on plant protection products (Regulation (EC) No 1107/2009 concerning the placing of plant protection products on the market).

4.1.2. Biological control

In the field of vector control, the principle of biological control is to use a 'natural enemy' of an arthropod to reduce its population and thus reduce the risk of pathogen transmission. Among the natural enemies of mosquitoes, predators and pathogens may be distinguished (Fontenille et al., 2009).

Predators

- Mosquito larvae-eating fish

Use of fish that eat mosquito larvae is effective for mosquitoes living on permanent bodies of water and limited in number so that they can be monitored. Larvae-eating fish of the *Aphanius dispar* species have thus been used for malaria control in Djibouti and *Gambusia affinis* for *Anopheles sacharovi* in Greece, *An. stephensi* in India, and several North African and Middle Eastern *Anopheles* species (Chandra et al., 2013; Collins and Paskewitz, 1995).

As for France, mosquitofish were introduced in Corsica in 1924 and on the continent between 1927 and 1931 in order to control malaria (Walton et al., 2012). Introduction of exotic larvae-eating fish such as mosquitofish does, however, risk creating an ecological imbalance because these predators are usually not exclusive in their choice of prey (Chandra et al., 2008). However, for mosquito control in urban areas and to control the nuisance of *Aedes stegomyia* mosquitoes, the advice commonly given to the technical departments responsible for maintenance of municipal parks containing ornamental ponds and to residents with ponds or ornamental pools in their gardens is to stock them with fish (koi carp, goldfish, etc.) as an effective means of control.

- Copepods

Biological control of *Aedes* also entails use of copepods of the genus *Mesocyclops* (Howard, 2013). These 1 to 2 mm predatory crustaceans are able to kill 20 to 40 young *Aedes* larvae a day. Releasing them into domestic water storage containers can reduce larval density by 95-100% over several months. This method has been applied as part of integrated control programmes in South-East Asia (Vietnam and Thailand) (Kay and Nam, 2005; Kittayapong et al., 2006; Nam et al., 2012). It has also been tested in Florida (Baldacchino et al., 2015). More recently, semi-field trials with *Macrocyclops albidus* have shown promising results for *Ae. albopictus* control in Italy (Veronesi et al., 2015). Copepods also seem to attract female mosquitoes, which look for copepod-inoculated laying sites; permanently filled water containers would thus seem to act as ovitraps (Marten and Reid, 2007). While copepods would appear to be a useful tool for integrated vector management in urban areas, it must be borne in mind that they cannot survive if the containers dry out (Marten and Reid, 2007). Use of copepods in metropolitan or overseas France would require prior research, including identification of an autochthonous species that preys on the target mosquitoes and does not carry local pathogens, in order to avoid introducing an invasive species and minimise impact on the local ecological balance.

- *Toxorhynchites*

There are also mosquito larvae that prey on other larvae. Mosquitoes of the genus *Toxorhynchites*, for example, have highly voracious larvae that grow in small sites such as tree holes and leaf sheaths (that cannot be reached by other methods and contain important vectors); some species can be reared and produced in large numbers. These mosquitoes do not feed on blood. This control method is not used in Europe as these are tropical and sub-tropical species. Experimental releases of *Toxorhynchites* were carried out in French Polynesia in the 1970s; the species adapted well, but the reduction in *Ae. polynesiensis* and *aegypti* populations was not enough to bring down filariasis and dengue transmission respectively (Rivière et al., 1979).

Pathogens

Entomopathogenic bacteria, fungi and viruses have been considered for vector control.

- Entomopathogenic bacteria

Bti and *Lsph* bacteria are effective larvicides, characterised by their specificity of action. Since they are approved as biocides, their use and modes of action are described in the section on use of biocides (Section 4.1.1).

- Entomopathogenic fungi

Infection of adult mosquitoes with *Beauveria bassiana* and *Metarhizium anisopliae* spores leads to their death in a few days, potentially reducing 80-fold the capacity of *Anopheles* to transmit *Plasmodium* sp. (Blanford et al., 2005). The infected mosquitoes also become more susceptible to neurotoxic insecticides, making it possible to use them in synergy with chemical control methods (Farenhorst et al., 2009). The main limitation on large-scale use is their high sensitivity to abiotic factors such as temperature, humidity and ultraviolet radiation, which affect their infectivity.

- Entomopathogenic viruses

Insect-specific viruses – densoviruses and baculoviruses – have also been mentioned, but research is still at the experimental stage. Densoviruses are very specific entomopathogenic viruses: a number of toxicity studies for other animal species have shown that the infectivity and virulence of densoviruses are limited to the insect genus or family from which they have been isolated. For example, AeDNV (a species isolated from a laboratory colony of *Ae. aegypti*) is able to infect mosquitoes of the genera *Aedes* and *Culex* but not those of *Anopheles* (Carlson et al., 2006). After vertical transmission from the female to the breeding site, the virus is spread locally by the larvae. If the virus density is low, the larvae produce infected adults, whereas at higher virus densities a large proportion of the larvae die. Breeding sites remain infected even after lengthy desiccation (over a year) (Carlson et al., 2006). A preparation of AeDNV consisting of infected *Ae. aegypti* larvae in a phosphate-buffered saline solution mixed with glycerol and known as 'Viroden' (Buchatskiĭ et al., 1986) has been successfully tested as a microbial pesticide in wide areas of Ukraine, Russia and Tajikistan (Carlson et al., 2006).

N.B. In the European Union, use of entomopathogens for vector control is governed by the Biocides Regulation. At the moment only Bti *and* Lsph *bacteria are approved as biocides; they have been described in Section 4.1.1.*

4.1.3. Physical and environmental control

In the field of vector control, physical and environmental control is intended mainly to eliminate breeding sites, modify adult habitats, promote use of attractant traps and provide physical protection preventing host-vector contact.

Physical or mechanical control

Physical or mechanical control covers vector trapping methods (to reduce abundance), host-vector contact prevention methods and, by extension, methods of avoiding contact with the host (Fontenille et al., 2009).

Many types of trap are available for *Aedes*, targeting either gravid females (ovitraps) or females seeking hosts (BG-Sentinel® traps (Biogents AG, Regensburg, Germany) or Mosquito Magnet® traps (http://www.mosquitomagnet.com/)) (Baldacchino et al., 2015). These traps are widely used not just for vector control but also for passive surveillance, detection of species dependent on artificial micro-habitats and study of vector population dynamics (Dowling et al., 2013).

Mass trapping with attractants has been suggested as a means of reducing adult mosquito populations (Kline, 2007). A number of experiments to control *Ae. aegypti* using lethal ovitraps have seen varying degrees of success (Baldacchino et al., 2015). Development of oviposition stimulants might lead to much better control of populations with this type of trap. Studies in Brazil and Italy, for example, have shown that BG-Sentinel® traps, used on their own or with a CO_2 source or a BG-Lure®,

are effective in trapping *Ae. aegypti* and *Ae. albopictus* (Baldacchino et al., 2015), although they have not demonstrated efficacy in reducing populations on the scale required for vector control purposes.

In the end, adult-mosquito trapping strategies, whether used on a large-scale for community control purposes or domestically for personal protection, are not really new but have been updated, with new trapping systems being developed, some of which are at the assessment stage. Their feasibility and efficacy must be verified on a case-by-case basis (species, biotopes, rural environment, urban environment, size of area to be protected, species' attractability, etc.) without ruling out complementary control methods such as water management, irrigation control, *Bti* treatment, etc. However, there is no epidemiological proof of concept for the efficacy of mosquito trapping as a vector control tool at present.

Physical control measures for preventing host-vector contact consist in putting mosquito screens on doors and windows to limit entry of endophagic mosquitoes and employing mosquito nets as a means of personal protection. In use for centuries, the latter are gradually being replaced by insecticide-treated fabrics (see Section 4.1.1 on use of biocides).

Environmental control

Environmental control covers all measures designed to make the environment unconducive to development of vector populations (Fontenille et al., 2009). Environmental management measures may be temporary or permanent and seek to eliminate breeding sites or disturb adult resting places.

Temporary changes include use of irrigation systems allowing occasional draining of breeding sites to prevent larvae from completing their development, removal of water plants (weed-cutting) to reduce refuge areas for larvae of sun-intolerant species, and day-to-day management on an individual, community or public basis. For mosquito species using artificial accumulations of water (flower-pot saucers, tins, water storage containers, etc.) such as *Ae. albopictus* and *Ae. aegypti*, public awareness-raising is essential to limit the availability of these larval habitats, either by mechanical removal or by sealing off any such accumulations of water. Public health measures are also necessary, with refuse management being extremely important.

Permanent changes include drainage of wetlands, renovation of ditches and concrete channels to prevent water from stagnating, and habitat improvement. The latter covers a range of recommendations for adapting or changing the design and layout of street furniture, of engineering structures, of sewage and storm drainage systems, and of buildings and structures of every kind in order to avoid creating sustainable breeding sites able to accommodate and facilitate proliferation of mosquitoes in a human habitat, particularly in urban and peri-urban areas. Ideally, this approach should be preventive; at present it is mainly remedial.

To round off this overview of existing vector control techniques, attention is drawn to the following comment by WHO: despite ongoing and intensive control efforts, including improvements to and extensive use of insecticide-treated mosquito nets, diseases transmitted by mosquitoes, such as dengue and malaria, continue to pose an enormous global health burden (WHO, 2014). WHO experts have stated that existing tools cannot be relied on to eradicate malaria. Limitations of current control methods include: inability to reach mosquito larval breeding sites and adult resting sites; development of resistance to insecticides; compliance and infrastructure issues; concern about the impact on the environment and/or toxicity to humans; and, importantly, cost. Thus, for both operational and economic reasons, there is a recognised need for new, sustainable, and cost-effective vector control tools (WHO, 2014).

The HCB Scientific Committee notes that while it has considered innovative vector control techniques it has not neglected appropriate use of conventional control techniques in suitable combinations, nor has it discounted the socio-economic aspects (addressed by the Economic, Ethical and Social Committee).

4.2. Other emerging techniques based on mosquito release

Alongside emerging vector control techniques based on use of genetically modified mosquitoes, related techniques also based on mosquito release are being developed.

4.2.1. Standard sterile insect technique (SIT)

Principle

The principle of SIT is not new and is explained in Section 3.2.1, as used in the RIDL technique. It aims to reduce the density of or even eliminate a target mosquito population through repeated mass releases of sterile male mosquitoes. In standard SIT the males are sterilised shortly before release, mainly by ionising radiation. Sterilisation may also be chemically induced.

General background and specific case of mosquitoes

Conceptualised in the 1950s (Knipling, 1955), standard SIT was first used in 1955 (Bushland et al., 1955). It has since notched up a number of successes, such as elimination of New World screwworm (*Cochliomyia hominivorax*) in North and Central America between 1957 and 2006 (Wyss, 2006) and in Libya in 1992 (Vargas-Teran et al., 2005), and elimination of tsetse flies (*Glossina* sp.) on the Zanzibar island of Unguja (Vreysen et al., 2000). At present it is mainly used for crop pests, particularly fruit flies (Enkerlin et al., 2015).

Standard SIT is nevertheless one of the emerging vector control techniques for mosquitoes, since its application to the latter is still under development. Benedict and Robinson described in 2003 the numerous attempts to use the technique for different mosquito species since the early 1960s (Benedict and Robinson, 2003). Whatever the species, failures were attributed mainly to three factors: (1) insufficient mosquito production, due in part to the lack of sexing strains (to separate males and females), (2) loss of fitness in sterile males, and (3) immigration of wild mosquitoes into release areas (Benedict and Robinson, 2003).

Current state of research

Apart from the aspects specific to radiation itself, the difficulties associated with techniques of producing and using SIT mosquitoes are common to all methods entailing mosquito release and are more significant if the techniques require mass rearing. Recent improvement in rearing methods for a number of mosquito species from the genera *Anopheles* and *Aedes*, developed by the FAO/IAEA Insect Pest Control Laboratory (IPCL) in association with a number of researchers worldwide have thus benefited all these emerging techniques (Bourtzis et al., 2016; Lees et al., 2015). The greatest challenges remain development of a reliable sexing technique and aerial release systems for mosquitoes (see Section 4.3.3).

In the specific case of standard SIT, protocols for mosquito irradiation at doses sufficient to induce complete sterility without significantly reducing the males' performance are now well established for a number of important species. Tests in large laboratory and field cages have shown that the sterilised males of several species are as competitive as untreated males with regard to wild females (Madakacherry et al., 2014; Oliva et al., 2012; Oliva et al., 2013; Yamada et al., 2014), although it is necessary to confirm this competitiveness by field releases. In a promising sign for field competitiveness, irradiated *An. arabiensis* males released in Sudan proved capable of participating in mosquito swarms and even initiating them (Ageep et al., 2014). Some protocols may deliberately set a 95% sterility target to prioritise male competitiveness, but this principle is not recommended by the IAEA, which advocates complete sterility for the males released (see Section 4.3.4).

Review of SIT experiments in the field

Although no standard SIT project has yet reached the stage of operational release to reduce or eliminate a mosquito population area-wide, numerous advances over the past decade have made it

possible to validate the technique and initiate a number of pilot projects. The best-documented recent demonstration of the potential of standard SIT for controlling mosquitoes was provided by pilot trials with *Ae*. *albopictus* in Italy between 2005 and 2009 (Bellini et al., 2013).[48] A number of field trials are in the pipeline as part of technical cooperation projects with the IAEA for different mosquito species in a wide range of environments in the Indian Ocean, the Americas, Africa and Europe (WG report, Section 3.2.1 and Appendix 11). Particular mention may here be made of the SIT experiment in Germany (WG report, Appendix 11) to eliminate the first invasive *Ae. albopictus* mosquitoes as part of an integrated control programme to forestall a more substantial invasion (Becker et al., 2017). As for French territories, no trials are planned in metropolitan France despite effective establishment of *Ae. albopictus* in thirty of its departments; preliminary experiments have been carried out in semi-field conditions in Réunion with a view to improving control of *Ae. albopictus* in the various islands of the south-western Indian Ocean, since *Ae. albopictus* was responsible for a major chikungunya epidemic there between 2005 and 2007 (Damiens et al., 2016; Oliva et al., 2012; Oliva et al., 2013).

Future development prospects: SIT combined with auto-dissemination of larvicides

It has recently been suggested that sterile males could be used to contaminate specifically wild females with juvenile hormone analogues such as pyriproxyfen (Bouyer and Lefrançois, 2014) in order to scale up the larvicide auto-dissemination method described above (see Section 4.1.3). This method could also be used to disseminate other biological control agents such as bacteria, viruses and fungi, as has been successfully tested for fruit flies (Flores et al., 2013). A recently initiated CIRAD and IRD research project (ERC Revolinc) plans to test this technique for *Ae. albopictus* with densoviruses (Bouyer et al., 2016). According to preliminary modelling, this could reduce 10- to 100-fold the quantity of sterile males needed to eliminate a target population (Pleydell and Bouyer, 2016).

Thus although the principle of the standard sterile insect technique (SIT) is not new and has already been successfully applied to other pests and vector insects, its use for reduction or elimination of mosquito populations is still under development. Numerous field trials are in preparation worldwide, in technical cooperation with the IAEA, to target different species of *Aedes* and *Anopheles*. As for French territories, no trials are planned in metropolitan France, but a trial with *Ae. albopictus* is in preparation in Réunion.

4.2.2. *Wolbachia*-mediated techniques

Wolbachia

Wolbachia pipientis (Rickettsiaceae) are intracellular bacteria that are very frequent in arthropods; according to recent estimates, 40% of arthropod species are infected (Zug and Hammerstein, 2012).

Wolbachia infect both males and females but are transmitted exclusively from mother to offspring. Endowed with various properties that have the effect of manipulating reproduction of their hosts (e.g. male-killing, feminisation, parthenogenesis induction[49] and cytoplasmic incompatibility), *Wolbachia* promote production of infected females and their own spread in host populations. In this respect, *Wolbachia* are individuals with super-Mendelian inheritance, which could be used to spread genetic traits in a population through gene drive[50] (see Section 3.1.2.3).

[48] Sterile male pupae, separated by sieving and released on five urban sites of between 16 and 45 ha, induced between 18 and 68% sterility in native mosquito populations according to egg-hatching measurements. The results of these trials suggest that it would be necessary to induce a minimum female sterility level of 81% to achieve elimination of the local population (Bellini et al., 2013).

[49] As far as the working group experts are aware, parthenogenesis (reproduction from an unfertilised female gamete), male-killing and feminisation have not been reported for mosquitoes.

[50] Since genetic transformation of *Wolbachia* has hitherto proven impossible, they have not been considered for spreading transgenes to date.

Cytoplasmic incompatibility (CI) and pathogen interference (PI)

Wolbachia usually induce cytoplasmic incompatibility (CI) in mosquitoes.[51] CI is characterised by conditional mortality of embryos derived from mating of an infected male with a female that is either uninfected or infected with an incompatible *Wolbachia* strain. Infected males can therefore have offspring only with females infected with a compatible *Wolbachia* strain. Unlike males, infected females are able to produce viable offspring whatever the status (infected or not) of the males with which they mate, unless these males are specifically infected with an incompatible *Wolbachia* strain (this is known as bidirectional CI). CI thus encourages reproduction of infected females. Since *Wolbachia* are exclusively transmitted by females, CI promotes the spread of their infection in the mosquito population (Caragata et al., 2016).

Wolbachia bacteria can also interfere with transmission of certain viruses or other parasites by some mosquitoes. In other words, they interfere with the vector competence of some mosquitoes for certain pathogens. This phenotype is known as pathogen interference (PI) (Moreira et al., 2009).

Wolbachia's capacity to generate CI and PI phenotypes in their hosts makes them a potential tool of choice for vector control. Two approaches are possible: the incompatible insect technique and the spread of pathogen interference. They are explained below.

4.2.2.1. Incompatible insect technique (IIT)

Principle

The incompatible insect technique (IIT) is a variant of SIT stemming solely from *Wolbachia* cytoplasmic incompatibility (IC). It enables reduction or elimination of a target mosquito population through repeated mass releases of *Wolbachia*-infected male mosquitoes. Unlike standard SIT, these mosquitoes are not sterile but are sterilising because of their incompatibility with local females: *Wolbachia*-infected males cannot have viable offspring with local females that are either uninfected or infected with an incompatible *Wolbachia* strain.

Benefit of combining IIT with SIT: IIT-SIT strategy

In the case of standard SIT and of RIDL (another technique derived from SIT) it has been pointed out that it is important to release only males in order to prevent increased pathogen transmission on the one hand and to maximise the efficiency of interventions on the other (see Section 3.2.1). In the case of IIT this is essential if the technique is to work: accidental release of *Wolbachia*-infected females would result in modification of the local *Wolbachia*-free population rather than its reduction because of the possibility of fertile mating between released females and wild males and the spread of *Wolbachia* in the population that this would entail. Given the limited reliability of current sexing methods, two solutions have been suggested:

1- Use of bidirectional cytoplasmic incompatibility (the released mosquitoes being infected with a *Wolbachia* strain incompatible with the strain infecting the wild population) to ensure that any females released will have no offspring with wild males. However, it is not possible to prevent the released females from mating with the released males. Nor can this option be used for a wild population that is *Wolbachia*-free;

2- As a more effective alternative, it is possible to combine IIT with low-dose irradiation SIT to sterilise females without aiming to sterilise males, whose sterilisation requires higher doses of irradiation. This rules out the risk of accidentally releasing fertile females, while male sterility is ensured through *Wolbachia*-induced CI, possibly backed up by irradiation (Arunachalam and Curtis, 1985; Brelsfoard et al., 2009; Calvitti et al., 2015).

[51] There is an exception to this rule: the *Wolbachia* strain found in some *An. gambiae* mosquitoes does not seem to induce CI with uninfected *An. gambiae* (Shaw et al., 2016).

In comparison with use of SIT on its own, the IIT-SIT combination has the further advantage that the females accidentally released will possess any PI effect associated with *Wolbachia*, which should lessen their ability to transmit the pathogen. Since PI is not automatic, it must be checked prior to release. And it should also be checked that irradiation has not affected the PI and CI phenotypes associated with *Wolbachia*, although this would be inherently impracticable, since each irradiated individual is unique. Random irradiation mutations might affect *Wolbachia*'s CI capacity (and therefore operation of IIT) and its PI capacity (and therefore the vector competence of accidentally released females). However, because the females are sterile, any undesirable mutation in *Wolbachia* would have no chance of spreading in the environment, since the *Wolbachia* bacteria would not be transmitted (if it is assumed that female sterility is 100%). In the absence of a 100%-effective sexing method, the IAEA recommends a combined IIT-SIT approach rather than SIT on its own.

4.2.2.2. Spread of pathogen interference (PI)

Principle

The technique for spreading pathogen interference consists in using *Wolbachia* bacteria to modify a mosquito population to make it less able to transmit a given pathogen. In this it is similar to a gene drive technique that spreads a modification interfering with mosquitoes' vector competence (see Section 3.1.2.3). This technique is based on both the CI and PI properties of *Wolbachia*, with CI conferring on *Wolbachia* its capacity to spread in the population and PI in principle providing the reduced pathogen transmission phenotype.

The technique is based on release of male and female mosquitoes[52] infected with a *Wolbachia* strain. The infected females initiate spread of *Wolbachia* in the population from their first mating with wild males. Releases continue until the frequency of *Wolbachia* infection reaches the threshold at which the infection can invade the target population. Owing to its PI properties, the rise in frequency of *Wolbachia* in a mosquito population should lead to a reduction in pathogen transmission.

For this technique to be successful, the *Wolbachia* strain used must meet a number of conditions:

1- To allow invasion and continuing infection of a target mosquito population, it must be characterised by a high and stable unidirectional CI[53] with local mosquitoes, high maternal transmission and a low fitness cost for target host mosquitoes;

2- To ensure long-term resistance to pathogen transmission, it must induce stable PI over time, not significantly affected by environmental conditions.

These conditions are contingent on the nature and stability of CI, PI and maternal transmission and the possible fitness cost of *Wolbachia* for the mosquito. While the conditions relating to CI seem relatively easy to anticipate, the others are harder to foresee and guarantee over time (see below).

It should be noted that, unlike all the other techniques based on mosquito release, this one is based on release of females, with the potential risk of transmission of associated pathogens (see Section 4.3.4).

[52] It should be noted that a release of *Wolbachia*-carrying females only would also result in the spread of *Wolbachia* in the field population, which would then have a genetic background closer to the original population than if males were also released. However, since sorting is not necessary for the success of a population modification strategy, it is not used for the sake of avoiding a substantial technical constraint. It may further be noted that release of males accelerates the population modification process, since their mating with wild *Wolbachia*-free females prevents the latter from having offspring.

[53] Unidirectional CI is induced when the mosquitoes released carry *Wolbachia* while the wild mosquitoes are not infected with any *Wolbachia*, in contrast to bidirectional CI, which is induced when wild mosquitoes are infected with a *Wolbachia* strain that is incompatible with the strain infecting the released mosquitoes.

4.2.2.3. Current state of research and review of experiments

Wolbachia *in mosquitoes*

Not all mosquito species are naturally infected with *Wolbachia*. Of the vector species discussed in this opinion, *Cx. pipiens* and *Ae. albopictus* are so infected (Caragata et al., 2016). Mosquitoes of the *Cx. pipiens* complex are infected with various strains belonging to the *w*Pip *Wolbachia* clade (Atyame et al., 2014). These infections have reached fixation (i.e. all individuals are infected worldwide) (Duron et al., 2005). Similarly, *Ae. albopictus* is bi-infected with *w*AlbA and *w*AlbB virtually everywhere across the globe (Tortosa et al., 2010). In these natural associations, *Wolbachia* are often low-density and with a low fitness cost, that is, they do not unduly impair the insects' ability to reproduce while inducing cytoplasmic incompatibility. However, they result in very little pathogen interference (Zug and Hammerstein, 2015).

Mosquito transinfections and effect on CI and PI

Consequently, pilot vector-control programmes in Australia and South-East Asia often make use of transinfection, which is artificial infection of a new non-natural host by injecting *Wolbachia* into adult females or, more effectively, into the egg cytoplasm (Hughes and Rasgon, 2014). It is also possible to infect a non-natural host with *Wolbachia* by introgression if there is no barrier to mating because of a species' reproductive isolation.[54] If the recipient host is already naturally infected, it can either be cleared of its *Wolbachia* by antibiotic treatment prior to transinfection or be superinfected through artificial transinfection with an additional *Wolbachia* strain.

These transinfections may be stable and heritable in some cases but are often difficult to establish and may require culturing of *Wolbachia* on cells of the new host over generations (Hughes and Rasgon, 2014), entailing possible genetic drift in the bacteria. Some transinfections of vector mosquitoes have proven impossible, despite numerous attempts, particularly for *An. gambiae*, which transmits the malaria pathogen (Hughes and Rasgon, 2014). The influence of the microbiota, and particularly the role of *Asaia*, has been demonstrated for establishment of *Wolbachia* in a new host (Hughes et al., 2014; Rossi et al., 2015).

In all the cases studied, transinfection causes CI, and the effects of *Wolbachia* are usually greater in these new 'non-natural' associations, which are often correlated with high infection densities. Possible changes in density and CI are discussed in Section 4.3.4. The molecular mechanism of CI is currently being clarified (Beckmann et al., 2017; LePage et al., 2017).

PI properties, on the other hand, together with the fitness cost of *Wolbachia* for the host mosquito, vary according to the *Wolbachia* strain transinfected, the host, the pathogens concerned and the environmental conditions (Caragata et al., 2016). In some fairly rare cases, mosquito/*Wolbachia* interaction leads to increased viral or parasitic intensity. This is the case for *w*AlbB in *Cx. tarsalis*, which increases susceptibility to West Nile virus (Dodson et al., 2014). The molecular mechanism of PI is still not properly understood and appears to be multiple in nature: transinfections are associated with extensive transcriptional changes in the host with increases in immune gene expression, variation in mitochondrial activity, changes in gene methylation, and increased levels of reactive oxygen species that could be linked to iron metabolism – all changes more or less connected with the PI phenotype (Brownlie et al., 2009; Caragata et al., 2016; Sicard et al., 2014). *Wolbachia* and viruses are known to be in competition for host lipids and other resources. In addition, *Wolbachia* produce small RNAs and alter the small RNA profile of their hosts, which might also contribute to PI (Hussain et al., 2011; Woolfit et al., 2015).

[54] Introgression is not considered to be transinfection (Hughes and Rasgon, 2014), but it may result in the same properties: *Aedes polynesiensis*, a vector of *W. Bancrofti* filarial worms and occasionally of DENV in French Polynesia, has been infected by introgression from an interfertile species, *Aedes riversi*. The *Wolbachia* strain from *Ae. riversi* has induced CI in *Ae. polynesiensis*, which could be used for a population reduction strategy (Brelsfoard et al., 2008).

Other Wolbachia-*associated effects that might be used for vector control*

Wolbachia and impact on longevity: some virulent strains of *Wolbachia* such as *w*MelPop, a mutant strain from a laboratory *Drosophila* (and its derivatives), have been suggested in order to shorten the lives of vector mosquitoes, making them unable to transmit parasites (Sinkins and O'Neill, 2000). Since the *w*MelPop line also expresses complete CI, it has been suggested that this could facilitate invasion of natural populations and persistence over time (McMeniman et al., 2009). However, because infection with *w*MelPop-CLA has extremely detrimental effects on its hosts, this goal is considered elusive, particularly in dry conditions (Yeap et al., 2011). Field trials in Australia and Vietnam have shown that the associated fitness cost would not allow the strain to become sustainably established in natural populations (Nguyen et al., 2015).

Review of initial IIT and IIT-SIT experiments

The first landmark experiment with a CI phenotype dates back to 1967, when *Wolbachia*'s role in the phenotype had not yet been clarified: natural bidirectional CI between two populations of *Cx. quinquefasciatus* (then known as *Culex pipiens fatigans*) of the *Cx. pipiens* complex allowed the local population of mosquitoes transmitting lymphatic filariasis in an isolated village in Burma to be eliminated after nine weeks of mass releases of incompatible males (5,000 a day) (Laven, 1967). This trial was the first success of a SIT variant applied to mosquitoes (Benedict and Robinson, 2003).

More recently, with the identification of *Wolbachia*'s role in CI, specific strains of *Wolbachia* have been introduced into mosquitoes deliberately, either by introgression (Atyame et al., 2011; Brelsfoard et al., 2008) or by transinfection using microinjection (Calvitti et al., 2010), to reduce or eliminate vector mosquito populations by mass release of infected males.

Small-scale releases were carried out to test IIT in the field (1) from late 2009 on *Aedes polynesiensis* in French Polynesia (O'Connor et al., 2012) and (2) from 2014 on *Ae. albopictus* (Mains et al., 2016) and *Ae. aegypti* (J. Gilles, pers. comm.) in the United States. Although not resulting in complete elimination, these pilot trials brought about population reductions that were all the greater the more isolated the conditions (e.g. the remote reef islets of French Polynesia) and provided experimental evidence that *Wolbachia* bacteria did not become established from the males released. More recently an experiment in French Polynesia, not yet reported in a scientific paper, seems to have achieved a 95% reduction in the *Ae. polynesiensis* population of a 75 ha reef islet in the Tetiaroa atoll over 7 months (see WG report, Appendix 11).[55]

Other IIT applications are currently being developed, either at earlier stages of experimentation in laboratory or semi-field conditions or in field trials. Thus an IIT vector control strategy is being developed in Réunion for application in a number of islands in the south-western Indian Ocean for *Cx. p. quinquefasciatus* mosquitoes, which carry filarial worms and the virus responsible for Rift Valley fever. Following introgression into local populations of the *w*Pip(Is) strain from a population of *Cx. p. pipiens* from Turkey, the infected males were tested in the laboratory and in semi-field conditions with very promising results in terms of CI and competitiveness (Atyame et al., 2015; Atyame et al., 2011).

IIT and IIT-SIT vector control strategies are being developed for *Ae. albopictus* in Guangzhou, China. Since *Ae. albopictus* mosquitoes are naturally infected with two *Wolbachia* strains (*w*AlbA and *w*AlbB) (Bourtzis et al., 2014), a triple-infected line (*w*AlbA, *w*AlbB and *w*Pip) has been developed. This line is fully incompatible with the naturally double-infected strain and is also refractory to the dengue virus (Bourtzis et al., 2016). Data from semi-field conditions suggest that this triple infection generates only a minor fitness cost for *Ae. albopictus*, making it possible to use in an IIT-SIT approach (Zhang et al., 2015b).

[55] http://www.tahiti-infos.com/L-experience-des-moustiques-steriles-a-Tetiaroa-est-concluante_a146430.html; http://www.ilm.pf/node/2508.

The IIT-SIT approach, combining low-dose irradiation with *Wolbachia* transinfection, has been validated in the laboratory: irradiation doses of 40 Gy completely sterilised *Ae. polynesiensis* females with no obvious effect on the survival and competitiveness of the males (Brelsfoard et al., 2009); however, the same doses of irradiation had more mixed effects on *Cx. pipiens* males depending on the populations tested (Arunachalam and Curtis, 1985). More recent studies have examined the impact of the IIT-SIT combination with different irradiation doses on the robustness and competitiveness of triple-infected *Ae. albopictus* mosquitoes in semi-field conditions and on the lesser risk associated with accidental release of females (Zhang et al., 2016; Zhang et al., 2015a; Zhang et al., 2015b). Since the results with irradiation doses as low as 28 Gy were promising, pilot field trials to test this approach were initiated on selected sites in Guangzhou (Bourtzis et al., 2016). The IIT-SIT combination is also being assessed in the field in Thailand for *Ae. aegypti* mosquitoes doubly infected with *w*AlbA and *w*AlbB. Another IIT-SIT experiment is planned in Singapore for *Ae. aegypti* mosquitoes infected with *w*AlbB in comparison with use of IIT on its own[56] (see WG report, Appendix 11).

Thus experiments using *Wolbachia* for population reduction or elimination are growing in number – particularly in Asia and the United States but also in the Pacific with French Polynesia – on populations of *Ae. aegypti*, *Ae. albopictus* and *Ae. polynesiensis* inundated with males carrying various *Wolbachia* strains and strain combinations. These trials are on the way to producing extensive data that will make it easier to gauge the utility of the incompatible insect technique (IIT), whether or not in combination with low-dose irradiation (IIT-SIT), and determine the best conditions for using it.

Review of initial experiments with the technique for spreading PI

Techniques for spreading *Wolbachia* in mosquito populations to make them resistant to pathogen transmission owing to *Wolbachia* pathogen interference have been tested in a number of countries, including Brazil, Colombia, Indonesia, Australia and Vietnam under the Eliminate Dengue Program.[57]

It has been demonstrated in the laboratory that different strains of *Wolbachia* can not only protect against infection from arboviruses such as DENV, CHIKV (Moreira et al., 2009; Walker et al., 2011) and more recently ZIKV (Aliota et al., 2016; Dutra et al., 2016) but also against parasites such as *Plasmodium spp.* (Bian et al., 2013; Caragata et al., 2016; Moreira et al., 2009). It has also been shown that the *w*Mel *Wolbachia* strain from *Drosophila melanogaster*, which interferes with development of the dengue virus in *Ae. aegypti*, has a relatively low fitness cost and a capacity to spread in semi-field conditions (Walker et al., 2011). This being so, the population modification strategy was tested by spreading *w*Mel in two target populations of *Ae. aegypti* near Cairns in Queensland, Australia (Hoffmann et al., 2011).

Through the CI mechanism, after releases of male and female mosquitoes transinfected with *w*Mel, the bacteria effectively invaded *Ae. aegypti* populations in the target areas and successfully established themselves in a few months (Hoffmann et al., 2011). Studies suggest that the effects of *Wolbachia* on the host remain stable more than two years after final releases: field-collected *Ae. aegypti* mosquitoes infected with *w*Mel displayed limited rates of dengue virus infection, replication and dissemination after laboratory exposure to three dengue serotypes (DENV-1, DENV-2 and DENV-3) (Frentiu et al., 2014). In addition, *w*Mel retained its ability to induce complete CI with uninfected mosquitoes, although associated with a fecundity cost (Hoffmann et al., 2014). *Wolbachia* infections have remained at high levels in both populations, between 80 and 100%, with uninfected individuals being attributed to immigration. This is still true five years after the final releases (presentation by

[56] http://www.nea.gov.sg/public-health/environmental-public-health-research/wolbachia-technology/project-wolbachia-singapore.

[57] The Eliminate Dengue Program is a not-for-profit global research initiative led by Monash University in Australia. The initiative has been broadened to cover a number of governments, research institutes and philanthropic groups around the world, including The Foundation for the National Institutes of Health (Grand Challenges in Global Health Initiative of the Bill & Melinda Gates Foundation) (http://www.eliminatedengue.com/program).

S. O'Neill, Zika Summit, April 2016, Paris). However, taking these data as a whole, Hoffmann and colleagues concluded that there would be no rapid invasion of surrounding areas by *Ae. aegypti* mosquitoes infected with *w*Mel because the selective disadvantage would be too high (Hoffmann et al., 2014). Combined with a species dispersal rate that is too low, this fitness cost prevents the modified population of *Ae. aegypti* mosquitoes from reaching the critical frequency (30% (Hoffmann et al., 2011)) for fixing the transinfected strain in neighbouring populations. The need to reach a critical threshold for initial relative density entails mass rearing, even if less extensive than for SIT (see Section 4.3.3).

In preparation for larger-scale trials in Brazil, the *w*Mel strain transinfected into *Ae. aegypti* mosquitoes from Australia has been introgressed into *Ae. aegypti* mosquitoes in the Rio de Janeiro area by backcrossing. Laboratory assays show strong CI, a high maternal transmission rate and little impact on fecundity of local mosquitoes (Dutra et al., 2015). Modelling was used to simulate the spread of *Wolbachia* in five suburbs of Rio de Janeiro according to their socio-demographic characteristics and the latter's influence on the size of the existing mosquito population in order to optimise future mosquito release strategies for each site (Dutra et al., 2015).

Lastly, the feasibility of a strategy exploiting a different *Wolbachia* strain, *w*MelPop, which shortens the life of infected *Ae. aegypti* and reduces dengue virus replication, has been tested in Australia and Vietnam, where dengue is endemic. The results have been mixed, but in every instance *w*MelPop infection fails to become established in the population owing to its high fitness cost (Nguyen et al., 2015). This strain cannot therefore be used as part of a sustainable population modification strategy (Nguyen et al., 2015).

Thus a number of experiments for spreading pathogen interference (PI) using *Wolbachia* have been initiated as part of the Eliminate Dengue Program, three of which have been reported in published papers. All these experiments seek to reduce the vector competence of natural *Ae. aegypti* populations for different arboviruses by transinfecting them with various strains of *Wolbachia*. These trials have shown a varying capacity on the part of *Wolbachia* strains to invade and become established in wild *Ae. aegypti* populations depending on their fitness cost for the hosts. The most promising results have been obtained with *w*Mel, with infection becoming sustainably established in a local mosquito population near Cairns in Australia. While laboratory assays have shown that field-collected transinfected mosquitoes maintain reduced vector competence for dengue more than two years after the final releases, epidemiological findings are needed to prove the technique's efficacy against transmission of the disease. Experiments are in progress in Brazil, Colombia, Indonesia, Australia and Vietnam. An experiment is planned in New Caledonia.

N.B. It should be noted that *Wolbachia* are not the only bacteria that could be used for vector control: it has been shown in the laboratory that ingestion of *Chromobacterium Csp_P.* extracellular bacteria by *An. gambiae* and *Ae. aegypti* mosquitoes reduces their vector competence for *P. falciparum* and the dengue virus respectively (Ramirez et al., 2014). Here the idea is to use transinfection that is extracellular, rather than intracellular as in the case of *Wolbachia*, and to design control strategies that do not depend on the CI characteristics specific to *Wolbachia*.

4.2.3. Paratransgenesis techniques

Another form of vector control using bacteria that are intracellular or part of the mosquito's microbiota is being considered. Based on use of genetically modified bacteria, this technique is known as paratransgenesis (Favia, 2014; Lampe and Bongio, 2014; Weiss and Aksoy, 2014). Paratransgenesis consists in genetically modifying not the vector insect itself but a symbiotic microorganism harboured in its tissues.

This microorganism could be made to express one or more molecules capable of disabling or killing the pathogen in the mosquito. In the case of mosquitoes the following challenges have to be overcome:

- Identifying a symbiotic microorganism stably associated with the target vector species and that is transmitted vertically, culturable *in vitro* and genetically transformable. *Asaia* bacteria are the microorganisms that have been the most studied in this respect for *Anopheles*, and they could also be used for *Aedes*. A *Wickerhamomyces* yeast is another possibility. *Wolbachia* bacteria, which are intracellular and not culturable *in vitro*, pose technical problems that are too complex for them to be considered for paratransgenesis at present;
- Identifying one or more proteins or peptides capable of disabling or killing the pathogen in the mosquito and which can be expressed under the control of an appropriate promoter and secreted in sufficient quantities by the selected microorganism whilst remaining functional and without affecting that microorganism's viability. Single-chain antibodies (nanobodies), antimicrobial peptides and components of the venom of certain arthropods having shown anti-parasitic activity *in vitro*, as well as interfering RNAs, are all being considered. Using antimicrobial peptides from the insect's own immune system for this purpose brings with it the risk that pathogens resistant to these peptides may be selected, which could make the vector's natural population hypersusceptible to these pathogens;
- Reintroducing the genetically engineered organism into the mosquito, checking the efficacy of transmission prevention and finding a means of infecting wild populations with the genetically modified bacteria. In the latter case, advantage could be taken of the fact that the microorganisms under consideration are viable in sugar solutions such as nectar, which allow preparation of sugary baits containing the microorganism, and vertical transmission would then take over.

Research is being actively conducted into paratransgenesis as a tool to control *Plasmodium* and the *Trypanosoma* carried by the tsetse fly (*Glossina*) in Africa and by bugs (*Triatoma*) in America. Compared with a genetically modified insect, introduction of genetically modified bacteria in the field is likely to be harder to control. There is a high likelihood of non-target insects acquiring the genetically modified symbiont, which would then persist in these insects and the environment. The fact that bacteria such as *Asaia* are not host-specific (Favia, 2014) might nevertheless be considered an advantage in terms of efficacy, since it enables several malaria vectors to be targeted at once, whereas standard transgenesis makes it necessary to proceed species by species. However, the general issue of risks associated with the wider dispersal of the genetically modified microorganism would then have to be addressed. Since it is not as close to field application as other emerging techniques, paratransgenesis will not be discussed further in this opinion.

4.3. Comparison of GM mosquito techniques with other vector control techniques

The vector control techniques using GM mosquitoes (GM mosquito techniques) considered in this comparative assessment are Oxitec's RIDL technique – the only technique using transgenic mosquitoes that has been developed to an operational level at present – and the two examples of CRISPR-Cas9 gene drive reported to date, one for the purpose of population elimination and the other for population modification, although they are still at an early stage of development.

To keep the appraisal relatively concise whilst at the same time relevant and informative for the purpose of identifying the specific features of GM mosquito techniques, the most representative techniques possible have been selected from the various types of vector control methods described in Sections 4.1 and 4.2, either because they are actually used in France (as in the case of pyrethroids and *Bti* for biocide control), because they have been publicly discussed, and/or because projects have been reported in scientific papers (as in the case of mosquito-eating fish as predators and of densoviruses as pathogens for biological control). Emerging techniques involving mosquito release, which are central to the referral along with GM mosquitoes, have all been considered, apart from paratransgenesis, which seems further from being operational.

The summary table below lists the various techniques' modes of action and shows, for reference, their regulatory framework (discussed under risk assessment criteria in Section 5), their stage of development[58] and the scale of their use in Europe (Table 2).[59]

The specific features of GM mosquito techniques have been examined with a view to clarifying the benefits and limitations of their use for vector control in France in terms of possible objectives, efficacy and sustainability, technical constraints and environmental and health risks.

[58] The different stages of development were described in relation to the trials for the RIDL technique (Section 3.2.3) and are defined in the glossary.

[59] Site-directed mutagenesis is mentioned as a non-transgenic GM alternative that could be rapidly developed with the recent advent of new molecular tools. It is, however, limited by the absence of a mechanism for spreading the modification in the population. There are currently no projects in this field, and it will therefore not be discussed further in this opinion.

Table 2. Different vector control techniques: mode of action, possible objective(s), regulation, stage of development and scale of use in Europe.

Vector control technique	Mode of action	Objective (effect on population)	EU regulation[3]	Stage of development	Scale of use in Europe
1. Physical and environmental control					
1.1. Elimination of breeding sites by environmental management	Physical	Reduction	Unregulated	na	Case by case
1.2. Physical protection	Physical	Possible reduction	Unregulated	na	Urban (*Aedes* spp.)
2. Biocide control					
2.1. Adulticides: pyrethroids	Neurotoxicity	Reduction	Biocides	Phase 4	Urban
2.2. Larvicides: *Bti*	Cytotoxicity on gut epithelium	Reduction	Biocides	Phase 4	Rural and urban
3. Biological control					
3.1. Predators: mosquito larvae-eating fish	Predation	Reduction	Unregulated	Phase 4	Little used
3.2. Pathogens: densoviruses (entomopathogenic viruses)	Pathogenicity	Reduction	Biocides	Phase 3	Not yet used
4. SIT					
4. Standard sterile insect technique (SIT) by irradiation	Males sterilised by irradiation	Reduction / Elimination[1]	Unregulated	Phase 3	Not yet used
5. *Wolbachia*-mediated techniques					
5.1. Incompatible insect technique (IIT)	Sterilising males through *Wolbachia*-mediated cytoplasmic incompatibility	Reduction / Elimination[1]	To be determined[4]	Phase 3	Not yet used
5.2. Technique for spreading pathogen interference (PI)	Reduced vector competence associated with *Wolbachia*	Modification	To be determined[4]	Phase 3-4	Not yet used
6. Genetic modification of mosquitoes					
6.1. Standard transgenesis: RIDL technique	Sterilising males, carrying a repressible lethality trait introduced by genetic modification	Reduction / Elimination[1]	GMOs (transgenic)	Phase 3-4	Not yet used
6.2. Site-directed mutagenesis	Mutation of a gene needed for pathogen transmission	Partial modification	Legal status under discussion	Never tested	Not yet used
7. Genetic modification with gene drive					
7.1. Gene drive for population elimination	Using gene drive to spread a genetic modification inactivating an essential female-fertility gene in the target mosquito population	Elimination / Eradication[2]	GMOs (transgenic)	Phase 1	Not yet used
7.2. Gene drive for modification of a population to make it resistant to transmission of a given pathogen	Using gene drive to spread two possible modifications in the target mosquito population: 1. A transgene conferring resistance to transmission of a pathogen 2. A genetic modification inactivating a mosquito gene essential for vector competence	Modification	GMOs (transgenic)	1. Phase 1 2. In the planning stage	Not yet used

[1] Population elimination conceivable in the case of an isolated population and/or large-scale area-wide treatment (example of New World screwworm in Libya).

[2] Possible to eradicate the species in theory.

[3] In the European Union the applicable regulations are for biocides (Regulation (EU) No 528/2012) and GMOs (Directive 2001/18/EC).

[4] The regulatory status of insects transinfected with *Wolbachia* in the European Union has not yet been raised, since no application for release has been submitted to date.

4.3.1. Possible objectives

Apart from conventional techniques to reduce host-vector contact (e.g. mosquito nets, repellents) or vector lifespan (e.g. indoor spraying with biocides), there are two broad categories of vector control techniques, distinguished according to their effects on the target insect population (see Table 3):

1. Techniques to reduce the number of vector insects in a given area without affecting the vector competence of the remaining insects (known as 'population reduction techniques')

   Depending on their potential, these techniques can form part of vector control strategies aimed at varying degrees of target population reduction, the primary objective being to bring the vector population below the threshold required for transmission of a given pathogen. On this basis, three different vector control strategies are possible in practice, depending on the degree of reduction sought:

   - **Strategies for reduction** of a population density below the threshold required for transmission of a pathogen,
   - **Strategies for** (local) **elimination** of an isolated vector population,
   - **Strategies for** (global) **eradication** of a vector species.

   This broad category covers most existing vector control techniques (the others relate to host-vector contact or vector lifespan), and, of the emerging techniques and techniques under development described in this opinion, all those entailing release of sterile or sterilising males, whether using transgenic mosquitoes (RIDL and gene drive for elimination) or not (SIT, IIT), with a range of possible objectives depending on the technique:

   - Conventional vector control techniques concern population reduction; they cannot be used to eliminate a population unless associated with other techniques, since their efficacy is less with lower mosquito densities (see Section 4.3.2);
   - The emerging IIT, SIT and RIDL techniques can be used for both a population reduction strategy and an elimination strategy, since their efficacy increases with low mosquito densities (see Section 4.3.2). For high densities, however, their use must be preceded by a conventional vector control technique for better overall efficiency (see Section 4.3.2); the risks associated with these techniques will therefore depend on the context in which they are used and especially the strategy of which they form part, since the risks associated with population reduction are not the same as those associated with elimination (see Section 4.3.4);
   - Only gene drive techniques have the theoretical potential to eradicate a species, although they may be confined to eliminating a local population if employed in isolated conditions. In the, likely, event of inactivation through resistance development (see Section 4.3.2), a gene drive technique will be unable to transcend the reduction stage.

2. Techniques to reduce the inherent ability of a population's individuals to transmit a pathogen without necessarily affecting the density of that population (known as 'population modification techniques')

   These techniques form part of what are known as population modification strategies for vector control. As in the case of reduction, population modification can be carried out on various scales depending on how the modification is spread.

   In this category we find emerging techniques that spread a new trait in the target population, as in the case of the *Wolbachia*-mediated PI technique and gene drive for population modification. No existing techniques are able to affect mosquitoes' vector competence.

Table 3. Classification of vector control techniques according to their effect on a population and the possible objective of control (GM mosquito techniques in bold).

Mode of action	**Population reduction techniques** Operating on vector population density				**Population modification techniques** Operating on the inherent ability of a population's individuals to transmit a pathogen
Control strategy / possible objective	- Population reduction strategy - Population elimination strategy - Species eradication strategy	Reduction	Elimination	Eradication	- Population modification strategy
Technique	- Physical and environmental control[1]	x			- Spreading of new trait in a target population that interferes with mosquitoes' ability to transmit a pathogen, by means of: o *Wolbachia*-mediated spread of PI[3] o **GD for population modification**[4]
	- Biocide control[1]	x			
	- Biological control[1]	x			
	- Techniques based on release of sterile or sterilising males:				
	o Standard SIT	x	x		
	o **RIDL**	x	x		
	o IIT	x	x		
	o **GD for elimination or eradication**	(x)[2]	x	x	

[1] These 'non-genetic' methods may also aim at reducing host-vector contact and shortening vector lifespan to reduce the intensity of pathogen transmission. These techniques cannot eliminate a vector population unless they are combined with other techniques.

[2] If gene drive is inactivated, an elimination or eradication strategy may be automatically blocked at the reduction stage.

[3] In current trials, infection with *Wolbachia* has not extended beyond the release area owing to the selective disadvantage conferred on the mosquito by the bacterial strain used, meaning that *Wolbachia* is maintained in the local population but does not spread beyond.

[4] If gene drive is inactivated, a general modification strategy may be automatically blocked at the local population modification stage. Modification of an isolated population could be another objective of gene drive, provided that the risk of gene drive spreading beyond the target area was accepted.

Thus vector control techniques using GM mosquitoes can be employed for population reduction in the same way as a number of existing techniques and other emerging techniques. They differ from existing techniques in their ability to eliminate a local population and modify a mosquito population. This can also be achieved by other emerging vector control techniques. Of these techniques, only gene drive could theoretically eradicate a mosquito species.

4.3.2. Efficacy and sustainability

A technique's efficacy and the duration of its effects should ideally be measured by entomological and epidemiological surveillance indicators while it is being applied (WG report, Section 4.2 and Appendix 12). Efficacy and duration may also be characterised beforehand by other criteria, including:

- Time needed for a technique to achieve its objective;
- Duration of effects (does a technique's effect persist or is it necessary to maintain application?);
- Spread of effects (is a technique's effect limited in area or can it spread without further intervention?);
- Flexibility or adaptability of a technique's use in the light of monitoring data, and even the possibility of stopping application of the technique and reversing its effects if the monitoring data show adverse effects associated with it;
- Actual efficacy versus theoretical efficacy (could there be a gap between the efficacy recorded when the technique is first applied and the efficacy that might be expected of it in principle?);

- Potential loss of efficacy over time, whether through development of resistance in target populations or through drift in the technique or loss of functionality other than through target resistance;
- Competitiveness of males (is the efficacy of techniques involving release of males affected by different levels of competitiveness in the males released?);
- Efficacy depending on target population density (is efficacy of techniques differentially affected by target population density?);
- External factors that may influence or interfere with a technique's success.

Assessment of these criteria for the various vector control techniques under consideration has brought out distinctive features or similarities of the different types of GM mosquito techniques by comparison with other vector control techniques, as set out below. The extent to which such an assessment is theoretical depends on the data available for each technique.

1. Time needed for a technique to achieve its objective

A distinction can here be drawn between control techniques whose initial effects, recorded within a few hours to a few days of a technique's deployment, directly reflect its objective and techniques whose objective of reducing or even eliminating a population is achieved several months after initial application. The first group includes existing techniques such as biocide control (adults and larvae die within 24 hours of exposure to pyrethroids or *Bti* respectively) and biological control, whose speed of action depends on the number of predators or pathogens released (larvae die within a few hours of exposure to mosquito-eating fish and within a few days of exposure to densoviruses). The second group covers emerging techniques involving release of mosquitoes, including GM mosquitoes (control of a wild mosquito population is achieved within a few months, the period varying according to a number of parameters such as target area, initial mosquito density, number of mosquitoes released, etc.). The techniques in the first group will be the techniques to be used in emergency situations.

Techniques based on release of GM mosquitoes, like any other techniques based on mosquito releases, cannot therefore be viewed as a quick answer to an emergency situation. They must form part of a management strategy using various combinations of techniques on a long-term basis.

2. Duration of effects (does a technique's effect persist or is it necessary to maintain application?)

Local characteristics, such as variations in climate or immigration of vector mosquitoes into the treated area, may affect the duration of a control technique's effects. Apart from this common environment-related aspect, vector control techniques can be distinguished by the duration of their effects or action:

- The effects of some techniques are non-persistent after a one-off application; maintenance is necessary to ensure their efficacy over time. This is so for temporary physical and environmental control measures (such as elimination of breeding sites) and use of chemical biocides (such as pyrethroids) or biological biocides that are unable to reproduce in operational conditions (such as *Bti*);
- Conversely, some techniques have a persistent effect. This is the case for permanent changes resulting from physical and environmental control (e.g. drainage of wetlands);
- Some show intermediate durability: the need to maintain biological control varies depending on the invasive capacity of the predators or pathogens used. The effect of densoviruses may last several years.

In this context, techniques based on sterile or sterilising males, whether GM or not, are distinguishable by varying duration of effects depending on the situation in which they are used:

- As part of a reduction strategy, SIT, RIDL and IIT will have limited efficacy over time and will require maintenance, whereas as part of an elimination strategy the effect of these techniques will last until wild vectors immigrate into the treated area again. The duration of the elimination will then depend on the extent to which the treated area is isolated;
- In the case of local elimination through gene drive, the effect will again last only until the wild vectors re-immigrate, since the gene drive cassette disappears with the mosquito population. Only in the event of (global) eradication, nowadays unlikely, with no population recovery, would gene drive techniques have a lasting effect and be sustainable. Thus the technique of elimination through gene drive does not necessarily offer a permanent solution for populations of pathogen vectors.

Persistence of the effects of population modification techniques offering resistance to pathogen transmission depends on persistence of the modification in the population and on the stability of its phenotype. Persistence of the modification depends on the balance between its fitness cost and its transmission mechanism (in the case of *w*MelPop, the fitness cost is such that the bacteria cannot persist in the population (Nguyen et al., 2015)); the stability of its phenotype depends on the underlying molecular mechanism and possible changes over time and on the likelihood of resistance developing in the pathogen population:

- The published example of gene-drive-mediated resistance to *Plasmodium* transmission suggests stability over time once the modification has been established in the population. The actual establishment of the modification, however, may only be partial owing to development of resistance to gene drive (see point 5 below on resistance development, Gantz et al., 2015);
- By contrast, the *Wolbachia*-mediated PI phenotype may undergo variation depending on environmental conditions (Zug and Hammerstein, 2015). It may also be subject to functional drift (*Wolbachia* density reduction, reduction of *Wolbachia* maternal transmission, *Wolbachia* loss) or development of resistance in the pathogen population (see point 5 below).

3. <u>Spread of effects</u> (is a technique's effect limited in area or can it spread without further intervention?)

Vector control techniques can also be distinguished by spread of their effects beyond the treated area or the mosquito release area.

Of the existing techniques, only biological control methods are able to extend beyond the initial treatment area depending on the invasiveness and dispersal potential of the predators and pathogens used.

Of the techniques using GM mosquitoes, only some gene drive techniques have the theoretical ability to spread unlimitedly in a population. In practice, this spread will probably be limited by resistance development associated with mutations or polymorphisms at the guide RNA recognition site in the population. In some cases this limitation in the spread of gene drive may be considered an advantage. Otherwise, appropriate technical design strategies could reduce the likelihood of such developments (see point 5 below on resistance development).

Wolbachia's ability to spread PI depends on the fitness cost of *Wolbachia* to the mosquitoes. In fact, the *w*Mel *Wolbachia* strain, currently being tested by the Eliminate Dengue Program in Australia, is unable to spread beyond the release area owing to a selective disadvantage that is too high for the host mosquito, even though it persists within the release area (Hoffmann et al., 2014).

Other techniques based on mosquito release – mainly release of sterile or sterilising males, whether GM or not (SIT, RIDL, IIT) – are unable to spread their sterilising effect beyond the males released (the possibility of survivors among RIDL mosquito offspring through incomplete penetrance of the

trait, noted again in point 4 below, would not entail spread of a sterilising phenotype throughout a population).[60]

Self-limiting to self-sustaining continuum of techniques

These characteristics of duration and spread of a technique's effects are often amalgamated in the concepts of self-limiting and self-sustaining, which are commonly used in this field of research but are difficult to grasp because they combine two different dimensions (space and time) and therefore two different continuums. Two groups of vector control techniques can thus be identified on the basis of the ability of their effects to persist and spread, with some techniques being positioned on a two-dimensional continuum between these two extremes of self-limiting and self-sustaining:

1) **Self-limiting** techniques, whose effects are limited in space and time unless maintained

In the case of self-limiting vector control techniques involving release of modified insects, the modification will disappear from the population unless it is reintroduced by regular release.

Self-limiting techniques include most population reduction techniques, encompassing (1) conventional vector control methods (other than permanent physical changes, such as drainage of wetlands, and biological control techniques such as mosquito-eating fish and densoviruses, which have some degree of persistence in the field (Carlson et al., 2006; Suchman et al., 2009)), as well as (2) emerging techniques involving release of sterile or sterilising males, whether GM or not, for an objective of population reduction (SIT, IIT, RIDL).

2) **Self-sustaining** techniques, whose effects spread across space and last over time without requiring maintenance

To be more precise, in the case of vector control techniques involving release of modified insects, the modification's persistence over time and spread across space in the target population depend on the balance between the dynamics of the modification's spread and any selective disadvantage conferred on modified individuals.

Self-sustaining techniques include population modification techniques, depending on the modification's ability to spread, and all gene drive techniques, whether for modification, elimination or eradication.

The technique of *Wolbachia*-mediated spread of PI in a population is an example of a self-sustaining technique for which the bacteria's fitness cost to the mosquito may be an obstacle to its spread (Hoffmann et al., 2014). In the population modification technique using gene drive, the gene drive cassette may also fail to invade a population if the rate of conversion of wild-type chromosomes by gene drive is too low in comparison with any selective disadvantage associated with the modification. In most cases of homing-based gene drives, however, the theoretical spread of the gene drive cassette in mathematical models is very swift, even for substantial fitness costs (Unckless et al., 2015).

Use of standard transgenesis techniques (i.e. without gene drive) or site-directed mutagenesis for population modification would require heavy maintenance to offset natural counterselection or lack of selection of these transgenes and mutations in the absence of a system for spreading them; these techniques are therefore not considered to be self-sustaining (these cases are theoretical; nothing has been published on this subject).

The gene drive technique for elimination, on the other hand, may be considered self-sustaining, since in this case the gene drive cassette should spread spontaneously in the population and induce sterility in homozygous females, which should ultimately result in elimination of the population.

[60] The spread of a technique's effects must be distinguished from the persistence or invasiveness of the elements released, discussed below in point 4 on actual versus theoretical efficacy in terms of residual fertility and in Section 4.4.3.4 in terms of risk.

Advantages and drawbacks of self-sustaining as opposed to self-limiting techniques and consequences in terms of flexibility and adaptability

Self-sustaining techniques have the advantage of not requiring maintenance or large-scale facilities but the drawback of being very inflexible and even, in theory, uncontrollable or irreversible if the spread of *Wolbachia* or the relevant gene drive cassette were to encounter no obstacles. It should be noted that existing examples of population modification using *Wolbachia*-mediated PI do not allow spread because of the fitness cost of the *Wolbachia* strain used, together with the low dispersal capacity of the transinfected mosquito species. In the case of *Ae. aegypti* transinfected with *w*Mel in Australia, population modification would seem to take place and persist in the release area if the mosquitoes released exceed the initial relative density of 30%, but it does not spread beyond (Hoffmann et al., 2011). This spatial limitation may be regarded as a drawback in that it limits spontaneous progression of PI in the mosquito population but also as an advantage in that it enables the area of populations transinfected with *Wolbachia* to be controlled.

Conversely, self-limiting techniques will call for demanding maintenance in the long term (see rearing constraints, Section 4.3.3). It should be noted that SIT, IIT and RIDL will be more expensive as part of a population reduction strategy than as part of a population elimination strategy. These self-limiting techniques have the advantage of being controllable and adjustable in the light of monitoring data.

As far as monitoring is concerned, it should be noted that OX513A RIDL mosquitoes carry a fluorescent marker linked to the repressible lethality transgene, allowing effective monitoring of transgenic mosquitoes in the environment and offering the possibility of adapting how the technique is applied. SIT mosquitoes can be covered with fluorescent powder when they leave the rearing facilities, also enabling them to be monitored and their releases to be adjusted accordingly. However, it would not be possible to detect any offspring in the environment in the event of residual fertility. *Wolbachia*-carrying mosquitoes can be detected by using molecular markers for *Wolbachia* bacteria. Lastly, the gene-drive mosquitoes referred to in the literature carry a fluorescent marker which should also make it possible to monitor them, although without any direct possibility of modifying strategy implementation.

4. Actual efficacy versus theoretical efficacy

The actual efficacy of the different vector control techniques may depart from their anticipated theoretical efficacy – based on the principle of the technique – for reasons other than a potential loss of efficacy in the field over time (developed in point 5).

Thus the possible existence of a residual fertility rate must be taken into consideration for techniques based on release of sterile or sterilising males:

- As far as the RIDL technique is concerned, we have seen that there could be roughly 2% of survivors in OX513A mosquito offspring in the environment. This is due to incomplete trait penetrance, since the transgene is intact but does not produce the expected phenotype. Its occurrence is considered sufficiently low in each generation not to have any significant impact on the technique's effectiveness (Phuc et al., 2007);
- For SIT as well one cannot rule out the possibility that non-sterile irradiated males may be released. A 95% sterility target might even be deliberately set in order to improve the competitiveness of the males released, but this is not recommended by the IAEA because of the potential risks associated with it (see Section 4.3.4);
- For IIT a residual fertility rate for released males carrying *Wolbachia* with uninfected field females might be found in the event of incomplete cytoplasmic incompatibility. It has recently been shown in the laboratory that different temperature regimes can reduce the cytoplasmic incompatibility induced by some *Wolbachia* (Ross et al., 2017).

As for the experimental gene drive system for elimination proposed by Hammond et al. (2016), efficacy in spreading the gene drive cassette has been found to be reduced, owing to partial sterility of females hemizygous for the cassette, optimum spread being based on the principle of sterility of homozygous females only (Hammond et al., 2016). This technical problem is on the way to being solved by increasing the tissue specificity of *Cas9* expression in germline cells (A. Burt, pers. comm.).

Regarding the technique of *Wolbachia*-mediated spread of PI, the maternal transmission of some *Wolbachia* characterised in the laboratory might be less in the field depending on environmental conditions such as temperature (Ross et al., 2017). Since some *Wolbachia* are more sensitive than others to these environmental fluctuations, these new data should make it possible to select the *Wolbachia* strains for field use accordingly (Ross et al., 2017). Environmental conditions might also affect the efficacy of interference by some *Wolbachia* strains with pathogen transmission by mosquitoes (Zug and Hammerstein, 2015).

The problem of the gap between actual efficacy and theoretical efficacy is therefore not specific to GM mosquitoes. It exists for all emerging techniques based on mosquito release, with a varying impact on the efficacy of techniques in the field. It may be associated with (1) a residual fertility rate for techniques based on release of sterile or sterilising males, (2) compromised spread of a gene-drive cassette or compromised transmission of *Wolbachia* in population modification strategies, or (3) impairment of PI efficacy in some *Wolbachia* strains, depending on environmental conditions.

The consequences of these gaps between actual and theoretical efficacy for the different techniques in terms of risk will be addressed in Section 4.3.4.

5. Potential loss of efficacy over time

The sustainability and/or efficacy of these techniques may be limited by (a) **resistance development in target populations**, or by (b) **drift in the technique or loss of functionality**. This will depend on the technique's mode of action:

a. Loss of efficacy through **resistance development** may occur if the vector control technique operates through a genetic target – in the mosquitoes or pathogens concerned – for which there are viable alternative allelic states that could circumvent the technique's mechanism.

   i. In the case of population reduction techniques, any individuals carrying these alternative allelic states will become the majority of the population as these vector control techniques are applied over generations:

      - **Biocides:** Such selection of resistant mosquitoes is common with the use of many synthetic biocides such as pyrethroids. A resistance management plan should be implemented to maintain the efficacy of these biocides over time (see Section 4.1.1). It might also be possible for mosquitoes to develop resistance to the pathogenicity mechanism of densoviruses, even though, unlike synthetic biocides, these densoviruses could evolve in turn. On the other hand, development of mosquito resistance to *Bti* is very unlikely given that it contains six larvicide toxins targeting different receptors, some of them acting in synergy (Ben-Dov, 2014).[61] In the case of *Bti*, therefore, it is not the absence of targets but the multiplicity of different targets that considerably reduces the likelihood of an individual being resistant; such an individual would have to carry resistance alleles for each of the six toxins, since none of these alleles would constitute a selective advantage in itself;

[61] To be more precise, *Bti* contains 4 major Cry toxins and at least 2 minor Cyt toxins that act in synergy with the Cry toxins and whose presence reduces the risk of resistance development in targets (Ben-Dov, 2014).

- **Gene drive techniques** also operate through genetic targets: (1) the guide RNA recognition sequence (required for targeting cleavage by the Cas9 endonuclease) and (2) its flanking sequences (required for homology-directed repair). Thus, in a field population, individuals having a sequence at the guide RNA target site that is different from the predefined guide RNA recognition sequence to the extent that the Cas9 endonuclease does not cleave the site, or having sequences flanking the guide RNA target site that are polymorphic to the extent that homologous recombination – and therefore insertion of the gene drive cassette – cannot take place, will be refractory to gene drive. This is also the case for individuals carrying mutations at the guide RNA target site owing to non-homologous end joining following a previous cleavage by Cas9. In the case of gene drive for the objectives of elimination or eradication, the presence of refractory individuals because of genetic polymorphism would allow the population to recover after partial reduction. For individuals mutated following non-homologous end joining, there are two possible outcomes, depending on the effects of the induced mutations on the fertility gene in which the guide RNA recognition sequence is located: individuals carrying mutations that inactivate the fertility gene are bound to disappear as a result of natural selection, while individuals carrying mutations that do not affect the function of the gene will contribute to population recovery. It should be noted that, if it is considered undesirable, this possible interruption in gene drive can be minimised in future by simultaneous use of several separate gene drive cassettes and/or of several guide RNAs in the same cassette;

- **Techniques that do not operate through genetic targets are not conducive to development of resistance to the technique's mode of action**. This is the case for physical and environmental control measures[62] and use of predators. It is also the case for emerging techniques entailing release of SIT, IIT or RIDL OX513A male mosquitoes: the first are sterilised by non-specific irradiation; the second carry *Wolbachia* bacteria inducing cytoplasmic incompatibility making it impossible to produce offspring with any female mosquitoes that are uninfected or infected with an incompatible *Wolbachia* strain; the third transmit a conditional lethal gene to their offspring, which works by non-specific repression of transcription (transcriptional squelching) (Lin et al., 2007) induced by overexpression of the *tTAV* transgene, itself made possible by the absence of tetracycline in the environment, regardless of the target-mosquito genes. It is therefore unlikely that SIT, IIT or RIDL OX513A mosquitoes would induce development of resistance in their targets unless a fraction of the female population targeted possessed the behavioural trait of avoiding the irradiated mosquitoes, the mosquitoes carrying *Wolbachia*, or the OX513A genetic strain used in the RIDL technique respectively, and this fraction would be selected over generations as the technique was being applied. This hypothesis of so-called **behavioural resistance** would be unlikely for SIT mosquitoes given their extreme heterogeneity, since irradiation generates random mutations differing from one individual to another.[63] As for *Wolbachia*-carrying mosquitoes, there are phenotypes, particularly fitness phenotypes, associated with *Wolbachia*, but to date none that are recognised and/or avoided by *Wolbachia*-free mosquitoes

[62] Cases of behavioural resistance have nevertheless been found in *Anopheles*, in which some of the population biting earlier or later than the majority get round use of mosquito nets by inhabitants. It has not yet been shown whether this is behavioural plasticity or selection of behavioural resistance alleles.

[63] It should nevertheless be noted that behavioural resistance of this kind has been found for sterile fruit flies (McInnis et al., 1996). However, this finding is not easily transposable to mosquitoes, since the mating of fruit flies involves a complex courtship ritual. Besides, the genotypes and rearing conditions of the fruit flies used have been so adapted that this behavioural resistance is no longer found (K. Bourtzis, pers. comm.).

or mosquitoes lacking *Wolbachia* bacteria of a specific strain, as far as the experts of the working group are aware. In the case of RIDL mosquitoes, it would be conceivable that the only reared strain, OX513A, currently used by Oxitec for all its sites, might be sufficiently different from field mosquitoes for it to be avoided by some of the mosquito population;

ii. For population modification techniques, two types of resistance are possible: (1) resistance to modification itself, and (2) resistance to the desired phenotype in the modified population. Individuals resistant to modification will come under selection pressure of varying degrees, depending on the associated fitness cost (or selective disadvantage). No cost would result in simple coexistence of the population refractory to modification and the modified population. The possible development of these two types of resistance are presented for *Wolbachia*-mediated spread of PI and gene-drive-mediated population modification techniques below:

- As regards the **technique for spreading PI via *Wolbachia***, population modification (i.e. spread of *Wolbachia*) results from the phenomenon of cytoplasmic incompatibility also responsible for IIT (population modification here resulting from the fact that females carrying *Wolbachia* are also released). Following the line of argument set out above for IIT, resistance to population modification through avoidance of *Wolbachia*-carrying males would be unlikely. However, circumvention of the desired PI effect following population modification is theoretically possible. This particular resistance phenomenon might occur if a *Wolbachia*-carrying mosquito's vector competence was not affected for certain pathogen strains already existing in the field (as might be expected with pathogen interference (PI)). These PI-resistant strains would then be gradually selected in the field. Since PI's mode of action has not yet been unravelled, it is not clear whether it operates through a genetic target in the pathogen and whether strain polymorphism with regard to PI could be easily selected;

- As regards the **gene drive technique for population modification**, the possibilities of resistance outlined above owing to pre-existing polymorphism or *de novo* mutation in the targets also apply here and could lead to selection of individuals resistant to modification. As for the desired phenotype after modification of a mosquito population into a population with reduced capacity for pathogen transmission, the resistance to pathogen transmission conferred by the transgene could be less effective than anticipated for certain field pathogen strains. A broad spectrum of isolates should be tested to ensure the technique's efficacy. If we take as an example the production of antibodies targeting *Plasmodium* (Gantz et al., 2015), this phenotype could theoretically be overcome by *Plasmodium* strains that would not be recognised by these bodies. Since the targeted molecule in *Plasmodium* shows very little natural variability, the likelihood of *Plasmodium* strains resistant to this vector control technique being selected is very low to virtually nil.

Thus although the risk of loss of efficacy through development of resistance to a technique's mode of action is common in biocides and constitutes one of their major limitations, it is unlikely in RIDL GM mosquitoes, as in other emerging vector control techniques based on release of sterile or sterilising SIT and IIT mosquitoes, since their respective modes of action do not involve genetic targets. This might give application of these emerging techniques a significant advantage in the long run. It should nevertheless be noted that behavioural resistance to RIDL mosquitoes and possibly to *Wolbachia*-carrying mosquitoes could develop, although this has not been recorded to date, which might reduce the durability and sustainability of these techniques. Unlike IIT, SIT and RIDL techniques, gene drive techniques operate through genetic targets and are therefore

conducive to resistance development, whether on account of pre-existing polymorphisms in the population or mutations induced by non-homologous end joining in the sequence recognised by the guide RNA. However, technical developments might minimise resistance development in order to eliminate a population without risk of recovery or achieve complete population modification. Moreover, gene drive for the objective of population modification, as in the technique of *Wolbachia*-mediated spread of PI, could theoretically encounter development of resistance to the desired phenotype after population modification, even if this possibility seems very unlikely in the case of the reported example of antibodies targeting *Plasmodium*.

b. Some vector control techniques might also lose their efficacy through **drift in the technique or loss of functionality:**

- Quality control for synthetic **biocides** suggests that intrinsic drift is impossible, but for some biological biocides, such as densoviruses, a decline in pathogenicity might occur if the virus adapts to the host mosquito (this point relates to both development of resistance in mosquitoes, discussed in the previous point, and functional drift of the technique, since it entails interaction and co-evolution of two organisms);
- As far as the **RIDL** technique is concerned, the system could lose its functionality in the event of genetic drift in *tTAV* during rearing in the presence of tetracycline. It may be remembered that in the absence of tetracycline, tTAV prevents development of transgenic mosquitoes (the tetR component of tTAV binds to *tet*O and induces lethal overexpression of tTAV through VP16) while in the presence of tetracycline, the lethality associated with the *tTAV* gene is suppressed (as the tetR component of tTAV has a greater affinity for tetracycline than for *tet*O); however, the presence of tetracycline in rearing facilities will not prevent functional genetic drift in *tTAV*. If there is loss of function, the hemizygous offspring inheriting the mutated *tTAV* gene would remain viable in the absence of tetracycline. Oxitec tests the functionality of *tTAV* every six generations. No instance of loss of function has been detected to date (Oxitec, pers. comm.). In the event of survival of field mosquitoes carrying an inactivated tTAV, they would nevertheless be just as susceptible as wild mosquitoes to a further RIDL intervention using mosquitoes carrying functional tTAV (full system functionality could be restored through purification of the OX513A strain through elimination of non-functional alleles);
- Functional drift in **IIT** might be found in the event of a significant accidental release of females. IIT would be less effective in achieving population reduction, since the *Wolbachia*-carrying females would be compatible with the field males.[64] It should be noted that the risk of potential loss of efficacy owing to release of females would be reduced by using a mosquito strain that was bidirectionally incompatible with field mosquitoes and would be nil in combination with SIT (see Section 4.2.2.1);
- With the technique of *Wolbachia*-mediated spread of **PI**, the field population acquires *Wolbachia* bacteria, which are supposed to reduce the vector competence of female mosquitoes. Theoretically this technique could drift off course if there is a decline in density or even loss of *Wolbachia*. As far as the experts in the working group are aware, no loss of *Wolbachia* in transinfected populations has been recorded to date, but it will have to be tested in offspring of modified populations at different ages and in varying conditions with different biotic and abiotic parameters

[64] There would be partial population replacement. The IIT strategy would be partly tranformed into a PI strategy, potentially with no confirmation of the PI (pathogen interference) property. It should be stressed that this property is not common.

such as heat, infection, insecticide resistance, etc. Temperature-dependent variations in densities and maternal transmission of *Wolbachia* have been recorded in experimental conditions (Ross et al., 2017). Evolution in *Wolbachia* density and virulence has been reported in natural populations (Echaubard et al., 2010; Weeks et al., 2007). Another theoretical possibility of functional drift in the PI technique would be an evolution in the interaction between *Wolbachia* and its host possibly leading to gradual reduction in the protection conferred on mosquitoes by *Wolbachia*;

- Last but not least, **gene drive** techniques might be naturally inactivated in the event of chromatin methylation, inactivation by piwi-interacting RNAs (piRNAs)[65] or mutation of the gene drive cassette. In reality, inactivation by chromatin methylation seems unlikely in mosquitoes, since little methylation has been detected in *Aedes* and none in *Anopheles*. It seems, however, that both natural and artificial transposons, to which a gene drive cassette is comparable as an exogenous element, could be inactivated by the piRNA pathway (E. Marois, unpublished data, and (Vodovar et al., 2012)). Further epigenetic inactivation pathways might be discovered in future.

Thus there is a possibility of functional drift for the RIDL technique, even if it is controllable and quickly rectifiable. Gene drive techniques may also lose functionality over time, which is more problematic for their continuing efficacy in the field. This risk is not specific to GM mosquitoes. Techniques using *Wolbachia* may also drift off course from other causes. It should be noted that SIT differs from other emerging techniques in that it has no identified risk of functional drift.

In both cases it is important to stress that **monitoring** can be used to detect development of resistance in the target population and loss of functionality in the vector control methods employed, and this enables the control technique to be adapted accordingly: **application of self-limiting techniques can then be stopped and corrected (e.g. change of insecticide, cessation of mosquito releases); for self-sustaining techniques, it will be a question not of stopping their use, which, by definition, will be impossible, but of superimposing another method of vector control (a different *Wolbachia* strain, a different gene drive, other techniques, using different modes of action).**

N.B. These possibilities of resistance development in target populations or of drift in the technique or loss of functionality have here been considered in terms of loss of efficacy of the relevant vector control technique. The potential impact in terms of environmental and health risks will be addressed in Section 4.3.4.

6. Competitiveness of males

This issue concerns all techniques involving release of males, including techniques using GM mosquitoes (RIDL and gene drive).

The efficacy and cost-effectiveness of techniques involving release of males can vary depending on the competitiveness of the released males in relation to the wild females, i.e. the released males' probability of mating with wild females as compared with that of wild males.

It is first necessary to stress the importance of the impact of mass-rearing conditions on the competitiveness of the males released, whatever the vector control techniques used. Whether we are talking about SIT, IIT, IIT-SIT combined or RIDL, these techniques require industrial rearing, with production processes that may prove harmful if the insect's biology is not respected. It should also be noted that the various data available in the literature are not always directly comparable owing to

[65] The piwi-interacting RNA pathway is a natural mechanism for silencing transposons through RNA interference. A transgene in *Anopheles* has been shown to be inactivated by this pathway (S. Blandin, E. Marois, pers. comm., paper in preparation).

different methods of measuring competitiveness (ovitraps versus adult traps), different trial conditions (semi-field trials in greenhouses, which provide a quality index for the insects, as opposed to field trials, which take into account a number of additional factors), different species of mosquitoes studied, and the different vector control techniques used. It is therefore difficult to distinguish the effect of mass rearing from the effect of irradiation, *Wolbachia* or RIDL on males' competitiveness and ultimately to compare the performance of these techniques with each other in this respect.

We may nevertheless note the following points:

- Because of the random effects of irradiation, we might expect the competitiveness of irradiated males released with SIT to be systematically affected, but this is not always the case, according to data from cage experiments on mosquitoes, to be confirmed in the field, and field data for other insects (see Section 4.2.1);
- Some strains of *Wolbachia* may affect the competitiveness of infected males. This was the case for *w*MelPop in *Ae. aegypti*, a technique that has in fact been abandoned because of the harmful effects that it entailed (see Section 4.2.2). Other strains show a very small impact on the field competitiveness of the infected males released with regard to uninfected females. This is the case for the strain originating from *Ae. riversi* after introgression into *Ae. polynesiensis* (C=0.68) (O'Connor et al., 2012);
- Because of the specificity of the transgene inserted, no impact on the competitiveness of transgenic males was anticipated for the RIDL technique. It has nevertheless been found that the males of Oxitec's OX513A line were only 3 to 15% as likely as wild males to mate with wild females (i.e. C=0.03 to C=0.15). This difference would seem to be attributable not to the transgenic technique as such but to adaptation of the line to mass rearing conditions. It should be pointed out that OX513A mosquitoes have proven to be as competitive as non-GM mosquitoes in contained conditions (Harris et al., 2011; Lee et al., 2013) and that this level of field competitiveness is not exceptional in comparison with data obtained with other SIT insects (C=0.3 to 0.5 for tsetse flies in Senegal (Bouyer et al., 2012); C=0.1 for the New World screwworm (Mayer et al., 1998; Vreysen, 2005) and C<0.01 for the Mediterranean fruit fly (Rendon et al., 2004; Shelly et al., 2007), although the latter is known for its particularly low levels of competitiveness owing to a very complex courtship ceremony) (see Section 3.2.3);
- Initial examples of gene-drive males are unlikely to be less competitive than wild males in theory, but their competitiveness will have to be tested case by case for each new line. The issue of the competitiveness of the males released is, however, less important for gene drive owing to the rapid spread of the transgene in the genetic background of the target population.

The impact of males' low competitiveness on the efficacy of such techniques could be offset by increasing the number of males released, which would have the effect of lessening the technique's cost-effectiveness and might pose a problem with regard to economic viability.

This issue is not specific to techniques using GM mosquitoes; it concerns all techniques based on release of male mosquitoes.

7. <u>Efficacy depending on target population density</u>

The efficacy of vector control techniques can vary significantly depending on the density of the target population:

a. Conventional vector control methods, such as biocides, are supposed to be effective irrespective of target insect density. However, this theoretical efficacy comes up against a number of limitations, meaning that, in practice, efficacy diminishes in step with target population density, which itself diminishes during a vector control campaign aimed at reducing this density. Two practical constraints reduce this efficacy:

- The systematic aggregated (non-continuous) distribution of insect populations and the impossibility of reaching all infested sites, which diminishes the efficacy of some methods such as aerial aerosol spraying (Adam et al., 2013) or trapping systems using toxic baits, which cannot be placed on all of these sites (Bouyer et al., 2013);
- Use of predators, parasitoids and parasites, which become less effective as the density of prey declines (Krivan, 2007), since non-specific predators focus on the most abundant species of prey.

These methods thus allow a rapid initial reduction in the population density of the mosquitoes concerned (desirable in health emergencies, for example) but have little chance of eliminating them.

b. Conversely, reduction techniques such as SIT, RIDL and IIT will be all the more effective if the density of field mosquitoes is low with a constant number of mosquitoes being released, since the ratio of sterile males to wild males will increase as the population is reduced by the control campaign. These techniques involving mosquito release cannot be used efficiently in seasons when mosquito populations are exploding unless preceded by techniques effective at high densities, such as use of biocides. Releases are then optimised with reference to the density of field mosquitoes and adjusted according to the effectiveness indicators for the technique over time, as an insufficient ratio of sterile males could lead to an increase in the next generation of mosquito adults by improving larval survival because of a reduction in larval densities in breeding sites (through sterilisation of a proportion of the eggs), thus having the opposite effect from that expected. This effect, called density-dependent overcompensation, is usual in mosquitoes (White et al., 2010). It may be noted that this effect should not be found in a RIDL strategy owing to late-acting lethality in the larvae (Phuc et al., 2007).

In other words, the efficacy of conventional methods of vector control is independent of density beyond a certain density threshold of the target mosquito population; below this threshold it declines with density until it is nullified before it can lead to elimination. Conversely, reduction techniques such as SIT, RIDL and IIT can only be effective below a certain density threshold of the target mosquito population (depending on the ratio of released males to wild males and on the competitiveness of the males released in comparison with wild males); beneath this threshold, they are all the more effective when the density is lower, thus leading to elimination of the population (Feldmann and Hendrichs, 2001).

Moreover, some methods of environmental control such as reduction of breeding sites may require a significant effort if the target population is not limited by the number of these sites. Thus if only 50% of larval habitats are used by *Ae. albopictus* in a given area, reducing the overall number of larval habitats by 75% will reduce the occupied breeding sites by only 50%, and therefore the adult population by about the same figure, depending on the quality of the sites.

These different context-dependent efficacy profiles for the various vector control techniques mean that compatible, complementary techniques ought to be combined in an integrated vector control approach. If GM mosquito techniques are to be used successfully in the environment, they will thus have to be used in combination with other vector control techniques depending on the target population density.

8. External factors that may influence or interfere with a technique's success

External factors may influence or interfere with the success of certain vector control techniques. For example, climatic and meteorological factors interfering with the target mosquitoes' biology and life cycles must clearly be taken into consideration in all control techniques. These factors may also

interfere directly with some techniques, such as application of biocides or release of mosquitoes, whether GM or not.

Local farming practices may affect the success and sustainability of biocide control. In fact, some farming practices use the same molecules as biocides for plant protection purposes. As uses of biocides and plant protection products are regulated independently of each other (see Section 4.1.1), the risk that mosquitoes may develop resistance to these molecules becomes extremely difficult to manage over the area concerned. This problem is heightened by the differences in the products' scale of use for farming and for mosquito control and by the fact that mosquito control operators more often than not are unaware of the plant protection practices in the area. These multiple regulations for the same molecules are a basic problem for local vector-control managers/stakeholders with regard to controlling mosquitoes' resistance.

4.3.3. Technical constraints

The technical constraints considered in this section highlight the issue of mosquito rearing facilities for vector control techniques involving release of mosquitoes, including GM mosquitoes.

Existing vector control techniques are certainly not without technical constraints either, but these constraints may be of an entirely different nature. Thus, for information and comparison purposes, biocides require industrial production and special transport to their application sites. They must be handled and applied according to special procedures with various spreading equipment and techniques whilst complying with disposal requirements for pesticide waste and packaging. All of these procedures are adapted to the different toxicity characteristics of these biocides for health and the environment.

Specific technical constraints associated with mosquito rearing and release include:

- Need for rearing on various scales and consequences in terms of infrastructure,
- Setting-up and operation of mosquito rearing,
- Dependence on biological resources such as blood, and related issues (source, quality),
- Separation of individuals according to sex (need, feasibility),
- Mosquito release system.

1. <u>Need for rearing on various scales and consequences in terms of infrastructure</u>

Mosquito rearing is needed for techniques involving release of mosquitoes on varying scales:

- Mass rearing is necessary for all techniques requiring repeated mass releases for reduction purposes (SIT, IIT, RIDL), entailing substantial infrastructure and therefore significant investment;
- Rearing requirements are much lower for gene drive techniques, whether for population modification or reduction, owing to the intrinsic invasiveness of the transgene that it is wished to spread. A single mosquito could in theory be enough to initiate spread of the transgene through CRISPR/Cas9-mediated gene drive, even if, in practice, larger releases are planned in order to accelerate the spread of the modification (Austin Burt, pers. comm.). These techniques could in theory be used by an existing laboratory without the need for infrastructure investment;
- The requirements of the technique of *Wolbachia*-mediated spread of PI are in between, since it needs little or no maintenance, depending on the scale of the target region, once the strain has been established in the field. In practice, such establishment can occur only if the releases enable the initial relative density threshold to be exceeded, which depends on the

fitness cost associated with *Wolbachia*. This threshold will determine the size of the rearing facilities required;

- It should be noted that mosquito rearing is also necessary for production of densoviruses, which are then dispersed through dead mosquito larvae.

2. <u>Setting-up and operation of mosquito rearing</u>

The quality of mosquito rearing is key to the efficacy of vector control. Mosquito rearing is more or less complex to set up and operate depending on the species and the vector control technique requirements:

- Standard operating procedures must be adjusted according to the species. Rearing protocols are in the process of being optimised for *Ae. aegypti* and *albopictus* and developed for *Anopheles* (Bourtzis et al., 2016);
- Mosquito rearing can be set up more or less rapidly depending on the conditions required by the different techniques. Ideally, the lines of mosquitoes released should be as close as possible to the wild mosquitoes in the target area, especially when the goal is population modification, in which the genome of the reared mosquitoes will hybridise with that of the wild mosquitoes and contribute to the population of the target area:
    - Irradiated mosquitoes (SIT) are the fastest to produce from a local strain.[66] After a minimum period of acclimatisation to rearing, few generations of reproduction in the rearing facility are needed owing to the mosquitoes' high fertility rate, which minimises the genetic drift that might occur because of differential adjustment of genotypes to rearing conditions;
    - Mosquitoes transinfected with *Wolbachia* bacteria (IIT and the technique for spreading PI) are more difficult to produce and often require many generations to allow the infection to become established, a process that is probably associated with varying degrees of genetic co-adaptation by the mosquitoes and the bacteria. Starting with a local mosquito strain is therefore harder, and it takes longer to establish the technique. One alternative to direct transinfection of a local strain consists in crossing it with a strain in which the infection is established and to restore the local genetic background by backcrossing (six generations will restore over 98% of the local genotype). The desired *Wolbachia* bacteria can thus be introgressed into a local strain within a few months. This was what was done, through nine generations of backcrossing, to obtain the *w*Mel_Br *Ae. aegypti* lines for release in Brazil from Australian lines infected with *w*Mel (Dutra et al., 2015);
    - Transgenic mosquitoes raise the problem even more acutely, since a local mosquito strain is not necessarily the best suited to laboratory conditions and genetic transformation. Consequently the OX513A line of *Ae. aegypti* was initially developed from the Rockefeller strain. Colonised in the 1930s, this strain is well suited to laboratory conditions and conducive to transformation but has lost traits related to field performance. In preparation for field releases, the insertion was therefore introgressed into a more recently colonised strain. This strain, of a Mexican genetic background, was generated using more than 20 homozygous female founder parents to ensure a certain level of genetic diversity (Lee et al., 2013). The resulting OX513A line has been maintained in the laboratory and in rearing facilities for over 100 generations. It has been used for all reported field trials, in conditions as various as

[66] This statement would have to be qualified for *Anopheles*, since not all species of *Anopheles* necessarily adapt well to rearing, even in the *An. gambiae* complex.

Malaysia, the Cayman Islands, Brazil and Panama. The result is (1) good adaptation to optimised rearing conditions, (2) evaluation of the same strain over several years, enabling its characterisation to be consolidated and its stability confirmed, but (3) a possible decline in competitiveness with local wild males (estimated to be between 3 and 15% according to trials conducted), which is offset only by release of extra mosquitoes. Oxitec does not seem to be considering introgression of the RIDL genetic cassette into strains derived from local populations. In theory, gene drive mosquitoes have the same disadvantages associated with selection of mosquito strains well-suited to laboratory rearing and genetic transformation, but the strain's genetic background is less important, since the transgene cassette will spread in the wild genetic background as the gene drive progresses. The gene drive cassette can in any case be introgressed into a local strain in the laboratory prior to release.[67]

3. Dependence on biological resources such as blood, and related issues

Rearing mosquitoes means having to provide blood so as to allow egg maturation in fertilised females. This raises questions concerning the quantity and quality of the blood and related questions concerning its source and sterilisation.

- Source: The blood may come from dedicated rearing facilities as in the case of Oxitec, from human blood banks as in the Eliminate Dengue Program for releases in Australia, or from abattoirs as is often the case in SIT programmes. The volumes of blood needed are not that great and should not be considered a barrier: a female requires only a few microlitres of blood to lay 50 to 60 eggs (in the case of *Ae. albopictus*) or 60 to 80 eggs (in the case of *Ae. aegypti*). The release of one million males should not require more than one litre of blood. Blood distribution methods can be optimised to reduce these volumes even further, for example by using membranes.
- Quality: It is imperative to check the quality of the blood supplied to the rearing facility. The blood must be sterilised in order, on the one hand, to eliminate the bacteria it contains and which could be harmful to rearing and, on the other, to destroy pathogens that could potentially be transmitted by released females. The absence of pathogens in the blood must be ensured for techniques requiring release of females or in which females may accidentally be released. The mosquitoes released are the offspring of reared females fed with the blood supplied. In other words, the females released have not been fed this blood directly, but their mothers were. Thus, if the supplied blood were contaminated and not sterilised, the females released might well spread a pathogen in their hosts in the environment if they inherited it from their mothers through vertical transmission.

Blood can be sterilised using various procedures:

- Sterilisation of blood by irradiation goes hand in hand with use of SIT, since the irradiator used to irradiate the mosquitoes can also be used to sterilise the blood, at doses of 1000 to 2000 Gy;
- The method of sterilisation used in mosquito rearing facilities for *Wolbachia* techniques should be verified, especially for population modification strategies, which entail releases of both males and females. In the Eliminate Dengue Program in Australia the blood comes from human blood banks and has therefore been tested for absence of pathogens;
- Oxitec has stated that it uses a private company that supplies it with blood that is certified sterile, taken from animals reared for this purpose. Quality control for blood used in RIDL mosquito rearing facilities will have to be examined.

[67] This statement would also have to be qualified for *Anopheles*.

N.B. Alternatives for artificial meals are under development. Their efficacy for egg production has not yet been optimised. The ease of obtaining animal blood has provided little incentive for such developments.

4. Separation of individuals according to sex

Sexing, or separating individuals according to sex, is necessary for techniques requiring release of males only. This covers all techniques involving mass release of sterile or sterilising males (SIT, IIT, RIDL). Exclusive release of males is also preferable for gene drive mosquitoes. Thus:

- For SIT and RIDL, sexing and exclusive selection of males makes it possible, firstly, to maximise the competitiveness of the males released for mating with wild females and, secondly, to avoid releasing biting females that may be carrying pathogens, even if they are unable to produce offspring;
- For IIT, sexing is not only important for the reasons outlined above for SIT and RIDL, but it is above all essential in order to avoid spread of *Wolbachia* in the population from released females, since the males released can sterilise only *Wolbachia*-free females. Use of bidirectional CI or low-dose irradiation (IIT-SIT) can, however, preserve the functionality of the technique by preventing the females released from having offspring with wild males (Section 4.2.2.1);
- For gene drive mosquitoes, release of females is avoided in order not to increase the population of biting females that may be carrying pathogens. It should be noted that gene drive techniques do not in theory require mass release of mosquitoes;
- The technique of *Wolbachia*-mediated spread of PI is the only technique based on release of female mosquitoes. Since males can also be released, thus accelerating population modification (since mating between released males carrying *Wolbachia* and wild females prevents the latter from having offspring), the technique does not require sexing.

The risks associated with release of females are examined in Section 4.3.4.

The feasibility of sexing differs according to species:

- Sexual dimorphism exists in *Ae. aegypti* and *Ae. albopictus*, the male pupae being smaller than the female, which can be used to sort the pupae by sieving. However, the sexing systems available are still cumbersome and cost-ineffective and are not 100% effectual (for *Ae. albopictus*, Bellini et al. report a recovery rate of only 30% for males, with a residual presence of 1.2% for females (Bellini et al., 2013)); they will have to be improved for large-scale releases. After mechanical sorting of pupae, Oxitec states that it carries out quality control processes with the target of limiting release of *Ae. aegypti* females to 0.2% at most (FDA, 2016). Releases in the Cayman Islands, Brazil and Panama consisted of 99.93%, over 99.97% and 99.99% of males respectively (Carvalho et al., 2015; Gorman et al., 2016; Harris et al., 2012);
- This dimorphism is less pronounced in *Anopheles*, and their pupae, which are less rigid, are less able to withstand the separator used for *Aedes*; a significant technical development will therefore be necessary to separate male from female *Anopheles* effectively, even for small-scale trials. As for transgenic mosquitoes, fluorescent marker systems to differentiate males and females are being tested to select males by flow cytometry (COPAS: complex object parametric analyser and sorter).

Faced with these needs, the IAEA has undertaken to fund a five-year project (2013-2018) to develop a sexing system that is effectual and cost-effective for large-scale release of male mosquitoes (Gilles et al., 2014). Other initiatives are in progress. Oxitec is testing various types of innovations to improve their in-house sexing system (Hadyn Parry, evidence to HCB's Oxitec hearing). The recent

identification of a gene controlling production of male mosquitoes in *An. gambiae* opens up the possibility of producing transgenic mosquitoes conditionally supplying male-only offspring (Krzywinska et al., 2016).

5. Mosquito release system

There are no effective mosquito release systems to date owing to low natural dispersal of adult mosquitoes and their lack of robustness (loss of legs, dehydration when released from aircraft), unlike release systems developed for other insects such as tsetse flies, for example (Mubarqui et al., 2014). This matter requires improvement. A system of releasing mosquitoes by drone might be studied subject to suitable aviation regulations on French territories.

Thus there are serious technical constraints relating to infrastructure and logistics that are associated with rearing and release of mosquitoes, whether GM or not. These constraints vary depending on the species of mosquito concerned: a species' biology has a considerable impact on (1) rearing conditions, the quality of which is crucial for ensuring the competitiveness of the mosquitoes released, and (2) sexing techniques, which is a major technological barrier associated with mosquito release. They also vary according to the number of mosquitoes to be released, determined by the techniques and strategies of which they form part. Indeed, (1) SIT, RIDL and IIT when used for reduction require functional mass rearing facilities in the long term to ensure repeated mass releases (this must be qualified if the same techniques are used for elimination); (2) the *Wolbachia*-mediated technique for spreading PI requires medium-size rearing facilities for an intermediate period, with size and period varying according to the fitness costs associated with the *Wolbachia* strain used; (3) gene drive techniques necessitate smaller rearing facilities. It should be pointed out that the ease of setting up mosquito rearing using local mosquitoes also varies according to technique: it will be simpler and faster for irradiated mosquitoes than for mosquitoes infected with *Wolbachia* and for GM mosquitoes. Setting up rearing for the latter can be simplified and accelerated by introgression, into local strains, of *Wolbachia* or the transgenic insert respectively from lines previously transinfected or transformed. Although this seems to be what is done in the Eliminate Dengue Program, it does not seem to be the strategy adopted by Oxitec, which has used a single *Ae. aegypti* line for the various releases that have been reported to date, including in regions remote from the source of the line's genetic background. Lastly, a vital factor in terms of risk prevention, common to all techniques involving mosquito release is quality control of the blood used to feed reared females so as to avoid all risk of introducing pathogens into the environment if females are released.

4.3.4. Environmental and health risks

Nota bene:

(1) The general information below on the potential risks of the different vector control techniques is provided for guidance. To carry out a formal assessment of the risks associated with a given mosquito release technique it would be necessary to have factual data from a specific application for authorisation of release. In particular, the criteria for use of GM mosquitoes (see Section 5) will have to be determined and assessed on a case-by-case basis.

(2) Although this information is intended to identify the various risks associated with the different vector control techniques, the review below will also underline the paramount importance of other variables critical to assessing these risks, such as the species of mosquito targeted (invasive, autochthonous, endemic, etc.), the environment concerned (urban, rural, etc.), the geographical situation (degree of isolation), the possible objective of control (reduction, elimination, eradication, modification, etc.), and so on.

To identify any specific risks of techniques using GM mosquitoes in comparison with other techniques, a cross-analysis of the risks associated with the various vector control techniques has been carried out under the following headings:

- Specificity of a vector control technique's mode of action and consequences in terms of direct impact on non-target organisms and on health;
- Risks associated with a vector control strategy's objective (population reduction, elimination or modification) rather than with the technique itself;
- Risks in the event of a vector control technique's loss of efficacy through resistance development or functional drift;
- Specific risks associated with mosquito release, arising from the possible persistence or invasiveness of the mosquitoes released (or elements associated with these mosquitoes) on the one hand and with possible release of females on the other.

Additional issues specific to different vector control techniques are then discussed:

- Techniques using transgenic mosquitoes: possible new products;
- RIDL technique: use of tetracycline;
- Gene drive techniques: collateral mutagenesis by the CRISPR-Cas9 system and its consequences, the specific risk of transfer of a gene drive cassette and its consequences, the specific question of eradication of a species, the risk of insertion of an 'undesirable' gene into the gene drive cassette, unpredictable risks and the means of pre-empting them, and the precautions to be taken for research;
- Use of *Wolbachia* bacteria: the risk of increasing mosquitoes' vector competence for transmission of local pathogens and the potential for horizontal gene transfer to, from or via *Wolbachia* or transfer of *Wolbachia* between mosquito species.

1. *Specificity of a vector control technique's mode of action and consequences in terms of direct impact on non-target organisms and on health*

Ideally a vector control technique should affect only the vector mosquitoes that it targets. The various techniques studied here have different degrees of specificity of action. A lack of specificity could lead to a direct impact on organisms other than the mosquitoes targeted, including humans.

Techniques that operate through mating between released mosquitoes and field mosquitoes (including techniques using GM mosquitoes) have a specific sphere of direct action that does not go beyond the species concerned and any other interfertile species.[68] It should here again be pointed out that both *Ae. aegypti* and *Ae. albopictus* are monophyletic groups, inasmuch as they each consist of a single species. They present a strong reproductive barrier with all other species of mosquito,[69] thus guaranteeing the specificity of action of techniques based on release of *Ae. aegypti* or *Ae. albopictus*. This is not the case for *Anopheles*, where possible interfertility within a species complex such as *An. gambiae*[70] must be taken into consideration in risk assessment. Of techniques relying on mosquito release, gene drive techniques have an even narrower specificity of action within the species, since only individuals having homology with the guide RNA target sequence and the flanking sequences can be modified and in turn transmit the gene drive. The risk that a technique

[68] Strictly speaking, interfertile mating is possible only within a species and interfertile 'species' should in fact be taken to be subspecies.

[69] It should be noted that satyrism has been observed between *Ae. albopictus* and *Ae. aegypti*, with mating between *Ae. albopictus* males and *Ae. aegypti* females resulting not in hybrid offspring but in sterilisation of the females.

[70] In the *An. gambiae s.l.* species complex, genetic exchanges have been observed in the environment between *An. gambiae s.s.* and *An. coluzzii*. Interfertility has also been reported between *An. gambiae s.s.* and *An. arabiensis*, although very limited in scope. These three species are vectors of *Plasmodium*.

may lose efficacy owing to presence of polymorphic individuals and the possible solutions to counter this possibility have been discussed above (see Section 4.3.2).

The situation is more varied for biocide control, whose specificity of action will depend on the characteristics of the biocides used:

- Pyrethroids, such as deltamethrin, are not selective. Although moderately toxic to mammals and slightly toxic to birds and earthworms, they are highly toxic to aquatic organisms, even though, after exposure, an ecosystem may be expected to recover within a few months. They are also very toxic to bees and pollinators (Afsset, 2007a). In terms of public health, deltamethrin presents essentially acute toxicity (Afsset, 2007a). More recently, an adverse effect on brain development has also been suggested (Viel et al., 2015). These characteristics have resulted in significant restrictions and precautions for use (see Section 4.1.1), enabling the associated risks to be minimised in practice;
- *Bti* activity seems restricted mainly to certain families of Diptera of the suborder Nematocera, including Culicidae (mosquitoes) and Simuliidae (black flies responsible for river blindness in Africa) (Boisvert and Lacoursière, 2004; Boisvert and Boisvert, 2000), for which it is employed. Other families of nematoceran Diptera, such as Chironomidae and Tipulidae, which may have indirect effects on the food web according to some authors (see section on indirect impacts), and some insect families of other orders, such as Heteroptera and Trichoptera, may also be susceptible to *Bti* but at much higher doses than those effective for Culicidae and some 5 to 1000 times greater than operational doses (Boisvert et Boisvert, 2000). Thus, despite their sensitivity to *Bti*, a number of studies have failed to find disruption to chironomids after *Bti* treatments (Duchet et al., 2015; Lagadic et al., 2016; Lundstrom et al., 2010; Vaughan et al., 2008). In terms of public health, *Bti* is characterised by absence of acute toxicity, moderate toxicity to aquatic organisms and no direct toxicity to birds, mammals and bees (Afsset, 2007b). More knowledge is nevertheless needed concerning impact on terrestrial arthropods and aquatic biodiversity as well as toxicity to humans, in particular chronic toxicity (Afsset, 2007b);
- Densoviruses are specific for a family or subfamily; the densoviruses used for mosquitoes are specific either for the family Culicidae, namely all species of mosquito, or for one of the mosquito subfamilies (Culicinae or Anophelinae)[71] (Carlson et al., 2006).

It should here be recalled that biocide control is regulated differently from GMOs, on the basis of Regulation (EU) No 528/2012, which takes due account of the biocide's spectrum of toxicity and ecotoxicity and provides for corresponding restrictions on use.

As for **biological control**, it should be noted that mosquito-eating fish such as guppies and mosquitofish can feed not only on all Culicidae larvae but also on a broad range of zooplankton, insects and crustaceans and even fish juveniles (including their own species) in the environments into which they are introduced; ultimately Culicidae form only a small proportion of their diet and one that is not chosen in preference to the rest (El-Sabaawi et al., 2016; Fraval, 2002; Gkenas et al., 2012). Therefore, to avoid the risk of creating predictable ecological imbalances, their use should in principle be limited to isolated urban habitats colonised mainly by mosquitoes.

Lastly, **physical and environmental control** techniques have a wide range of direct environmental consequences depending on the measures taken: to give two examples at opposite extremes, emptying water out of flower-pot saucers to get rid of breeding sites around houses will have a very specific effect on urban *Aedes* mosquitoes, whereas, in contrast, draining a wetland can seriously disrupt an entire local ecosystem.

[71] The *Aedes* densonucleosis virus (AeDNV) is able to infect several species of the genera *Aedes*, *Culex* and *Culiseta* of the subfamily Culicinae; however, it is unable to infect *Anopheles maculipennis* of the subfamily Anophelinae (Carlson et al., 2006).

It should be emphasised that, **while the specificity of action of a vector control technique can reduce its environmental impact, it will not necessarily increase efficacy of disease control**: specificity that is too narrow may adversely affect this efficacy, and therefore efficacy of control for a given pathogen, since some pathogens may be transmitted by other mosquito species. For example, several viruses transmitted by *Ae. aegypti* are also transmitted by *Ae. albopictus* (see Section 2.3.2). In regions where both species coexist, control by release of *Ae. aegypti* or *Ae. albopictus* mosquitoes can target only one of these species. Conversely, control of *An. gambiae s.s.* (a vector of *Plasmodium*) by release of mosquitoes could affect other interfertile (sub)species of the *An. gambiae* complex that are also *Plasmodium* vectors, such as *An. coluzzii.*[72] This lesser specificity of action could thus be advantageous for malaria control. With regard to environmental risks, a vector control technique's specificity for a mosquito species or species subset has the advantage of preserving other mosquito populations within the target ecosystem (see below).

Thus, by comparison with existing techniques, techniques using GM mosquitoes, like all other techniques involving mosquito release, are distinguished by unprecedented specificity of action for vector control, confined to the species of mosquito released. The specificity of action of gene drive techniques can be further focused within a species according to the target sequence defined by the guide RNA. This specificity of techniques based on mosquito release is an advantage for reducing the impact of vector control on health and the environment. It does, however, entail as many individual interventions as there are non-interfertile vector mosquito species to be targeted.

2. Risks associated with a vector control strategy's objective rather than with the technique itself

Depending on its objective, a vector control technique may lead to **reduction** (in varying degrees) or **modification** of the target population. Reduction, elimination or modification of the target mosquito population will have typical environmental effects whatever the technique used.

a. Population reduction techniques, whether or not involving mosquito release, should all entail prior assessment of:

- *the ecological role of the target populations, particularly for local elimination or eradication of a given species (as a theoretical objective for gene drive, see point 7), in order to assess the impact of a target mosquito population reduction, depending on its extent and duration,*
- *the specificity of the reduction technique and the risk that populations of non-target organisms might be unintentionally affected,*
- *the potential for unintended replacement of the eliminated population – given that an ecological niche has been vacated – by, for example, a population of mosquitoes with a vector competence of greater concern than that of the eliminated population*,
- *the health consequences of reducing or eliminating a vector population.*

i. Reduction or elimination of a mosquito species population may represent an environmental risk arising from the role it plays in the ecosystem, particularly through its position in the food web.

The particular ecological function of the vector mosquito species considered in this report does not seem to have been documented in any scientific papers. General information about the functional ecology of mosquitoes and the expected characteristics of the relevant vector species in the light of their habitats and/or invasiveness potential (see Section 2.3.2.4)

[72] It should be noted again that such interfertility is nevertheless very limited. More generally, since inter(sub)species mating is rare, it will have little effect on the other species of the complex, except in the case of gene drive, where the occasional hybrids, provided that the target sequences are sufficiently similar to be recognised by the guide RNA and allow homologous recombination, might be enough to initiate invasion of another (sub)species.

suggest that the impact on ecosystems of reducing the density or eliminating a population of an invasive urban mosquito species such as *Ae. aegypti* or *Ae. albopictus* will be less than that of an autochthonous species in a natural environment, such as *An. gambiae.* However, any functions acquired by invasive mosquitoes will have to be considered in an appropriate assessment.

The impact of mosquito population depletion on the species that are dependent on them must be taken into account. It should be noted that, owing to their life cycle, mosquitoes live in an aquatic habitat at the larval stage and a terrestrial one as adults. The effect of reducing or eliminating a mosquito population will hinge mainly on its position in the food web:

- At the adult stage: Among mosquito predators, some are generalists and do not depend exclusively on mosquitoes for subsistence (reduction or elimination of mosquito populations would in principle have a fairly limited effect on these predators), but species associations could develop. Only one example of preferential association has been recorded: that of a spider living around Lake Victoria in East Africa that has developed a marked food preference for blood-carrying *Anopheles* females (Nelson and Jackson, 2006). Any disappearance of *Anopheles* in the region might affect not only the diet of these spiders but also their reproduction, since recognition between males and females is influenced by chemical signals associated with the blood diet (Cross et al., 2009). What impact would different vector control techniques have on this spider? Existing techniques cannot eliminate a mosquito population entirely; they can only reduce, more or less specifically and durably, the density of all the local mosquito populations. As for vector control techniques based on mosquito release, they only target a single species (and any interfertile species); they cannot therefore eliminate all *Anopheles* and deprive these spiders of their preferred diet. Since the set of species present is specific to a particular region, it must be considered on a case-by-case basis prior to any reduction or elimination of a given population of vector mosquitoes. There are hundreds of sympatric mosquito species in the tropics.[73] In the case of this spider, the impact of the various techniques considered would probably be limited. More generally, a case-by-case and region-by-region assessment will be necessary to evaluate the environmental impact of a reduction or elimination strategy for a given mosquito population.
- At the larval stage: Mosquito larvae are both prey and predator. As predators they have an important regulatory role for protozoan communities in larval habitats. Reducing their density or eliminating them could therefore affect the species composition of these protozoans, with a significant cascade effect on the biomass and species composition of bacteria in these environments (Cochran-Stafira and von Ende, 1998; Pace et al., 1999). As prey, mosquito larvae belong to the diet of numerous organisms (insects, fish, etc.). Local elimination or reduction of the population of a mosquito species can therefore affect other organisms in the environment through its impact on the food web.

Population reduction techniques (such as biocides but also, more specifically, SIT, IIT and the RIDL technique) will have a more or less transient impact on microbial communities and mosquito predators depending on the percentage reduction. This is always true of the natural habitats where *Anopheles* mosquitoes are to be found but not of the non-permanent sites in mainly urban environments where *Ae. aegypti* and *Ae. albopictus* live. Microbial communities and mosquito predators in natural habitats would be particularly affected by mosquito elimination, since the target *Anopheles* species are endemic there; it should, however, be noted that techniques specific for a species will spare other mosquito species in

[73] The VectorMap website http://vectormap.si.edu/MosquitoCountryList.htm lists mosquito species by country.

that environment. Conversely, elimination of *Aedes* will have a smaller impact on the surrounding microbial communities and on *Aedes* predators, since the target *Aedes* species are invasive in urban environments.

ii. Reduction or elimination of a vector mosquito population carries with it the possibility that a new species, itself a vector of disease, may replace the population thus reduced or eliminated.

This has not been found in the case of conventional vector control techniques for population reduction (use of biocides, for example). Since these techniques are non-specific, replacement by other mosquito species is unlikely. Population reduction techniques based on mosquito release are more specific. The RIDL trial in Panama was an opportunity to test the hypothesis of replacement of *Ae. aegypti* by *Ae. albopictus*. In this particular trial, no increase in the *Ae. albopictus* population was found after reduction of *Ae. aegypti*, but longer-term studies are needed (Gorman et al., 2016). Note should also be taken of a doctoral thesis recording the invasiveness of *Ae. albopictus* in the presence of the resident species *Ae. aegypti* in Réunion without this being obviously connected with an *Ae. aegypti* control strategy (Bagny, 2009). It has, however, been suggested that the decrease of *Ae. aegypti* in Réunion might have been triggered by interactions with *Ae. albopictus* and that vector control campaigns in the 1950s (thus non-specific) might have accelerated the process (Bagny et al., 2009). In this case it is reasonable to assume that use of a vector control technique specific for *Ae. aegypti* would facilitate its replacement by *Ae. albopictus* and, in a situation where there may be competition between mosquito larvae, would likely increase the speed of *Ae. albopictus* invasion. The balance of power between *Ae. aegypti* and *Ae. albopictus* inclines to *Ae. albopictus* in the above-mentioned studies in Réunion, but it cannot be assumed that this will always be the case for other populations of these species in other contexts. Thus wherever *Ae. albopictus* and *Ae. aegypti* cohabit, it would always seem safer to recommend the concomitant use of control techniques each targeting one of these two species.

iii. Lastly, with regard to human health, after elimination of a vector mosquito population in a given region, the generations of a human population that have not been exposed to the pathogen will be entirely naive in terms of immunity if this pathogen begins to circulate again following immigration of new mosquitoes able to transmit it. In other words, a population's loss of immunity due to initial reduction in incidence of the disease could then facilitate return of epidemic outbreaks if the disease were to reappear. Risk management strategies and close monitoring are needed to detect and manage any such consequences of vector control. Loss of populational immunity must be weighed against the benefit derived from disappearance of the disease for a time.

Thus techniques with the objective of population reduction are nothing new. The novel features associated with releases of sterile or sterilising mosquitoes, including GM mosquitoes, are their specificity and ability to reduce a mosquito population to the point of elimination, with more lasting effects than reduction alone. It should be noted that the specificity of these techniques entails fewer risks to non-target organisms and to health. However, the fact that they have the potential to eliminate a population **makes it necessary to explore the target species' role in the ecosystem in greater detail. The impact of reducing or eliminating a mosquito species population must be assessed case by case and region by region in terms of its position in the food web in its larval and adult states. It is to be expected that reduction or elimination of a population of an invasive urban mosquito species such as *Ae. aegypti* or *Ae. albopictus* will have a more limited impact on ecosystems than that of an autochthonous species in a natural environment, such as *An. gambiae*, even though invasive mosquitoes might acquire a function that would have to be considered. Lastly, the specificity and durable nature of elimination makes it necessary to take into account potential replacement of the target**

population by a population of another vector species as well as the issue of a human population's loss of immunity. In regions where *Ae. albopictus* and *Ae. aegypti* cohabit, concomitant use of control techniques each targeting one of these two species would help to reduce the risk of one replacing the other.

b. Population modification techniques should entail:

- *Prior assessment of the characteristics of the modified population in comparison with the native population, particularly in terms of ecological role, fitness, vector competence and nuisance. Since the specific objective of this vector control technique is reduction of pathogen transmission by the mosquito population, this characteristic must be monitored in the environment over generations;*
- *Consideration – because of the persistence and invasiveness of the modifications introduced into the native population – of the evolution and long-term effects of the factors responsible for these modifications (e.g. the transgenes involved in gene drive for population modification and the* Wolbachia *bacteria for the* Wolbachia*-mediated technique to spread PI) and potential vertical transfer to any interfertile (sub)species or horizontal transfer to other organisms or microorganisms.*

Further to mosquito population-modification experiments using the *Wolbachia*-mediated technique for spreading PI to interfere with dengue transmission, no changes in nuisance or the ecological role of the mosquito population have been reported[74] (address by S. O'Neill, Zika Summit, April 2016, Paris). In Cairns (Australia) five years after final releases of *Wolbachia*-carrying mosquitoes, it has been found (1) that the *Wolbachia* bacteria are well-integrated and stabilised in the local population, (2) that they are linked to an approximately 15% decline in fitness, and (3) that the population's phenotype of reduced vector competence for the dengue virus has persisted to date (address by S. O'Neill, Zika Summit, April 2016, Paris). However, it is still too soon to determine the potential medium- and long-term effects of this strategy.

The possibility of population modification is a new development introduced by techniques involving release of mosquitoes, both transgenic (for gene drive) and non-transgenic (with *Wolbachia*). In principle, population modification has less impact on the ecosystem than population reduction owing to the stability of target mosquito population densities. This statement must be qualified according to the impact of the population's new characteristics. The effect of the modification should in theory be limited to interference with pathogen transmission by the mosquito, but it must be assessed more comprehensively over time.

3. Risks in the event of a vector control technique's loss of efficacy

We have seen in Section 4.3.2 that the efficacy of the various techniques could be restricted or compromised in varying degrees by development of resistance in target populations or by drift in the technique or loss of functionality. Beyond the need to adapt vector control techniques if loss of efficacy is detected, the environmental and health consequences of these various types of evolution can be considered and any specific features of techniques using GM mosquitoes identified:

***a.* Risks associated with potential for resistance development in the target population**

We have seen that development of resistance to the mode of action of the RIDL technique, as in the case of SIT and IIT, is unlikely, contrary to certain biocides, such as pyrethroids and densoviruses, and contrary to gene drive techniques and potentially to *Wolbachia*-mediated spread of PI (depending on the molecular mechanism involved in PI), for which resistance development must be taken into account because of their mode of action through genetic

[74] It should be noted that the object of population modification is to reduce pathogen transmission rather than mosquito nuisance.

targets. In addition, potential for development of behavioural resistance may be considered for all the various techniques based on mosquito release. What are the associated risks?

Resistance to a technique's mode of action

- Development of **insecticide** resistance in mosquitoes, as found for **pyrethroids**, is a critical issue for vector control, since an insecticide gradually loses its efficacy against whole populations of vector mosquitoes. However, mosquitoes' resistance to an insecticide has no direct consequences in terms of environmental impact. Detection of such resistance will lead to the insecticide no longer being used if the recommended management measures fail. There might nevertheless be some indirect environmental consequences if this resistance initially led to the use of higher doses of the insecticide concerned (up to, or even over, the registered dose[75]) or if it required use of other molecules, or other control methods, which could perhaps be more harmful to the environment. There might also be a greater health risk if the mosquitoes selected as resistant to an insecticide had increased vector competence for a given pathogen (Alout et al., 2013).
- Similarly, development of **densovirus** resistance in mosquitoes would have no direct consequences for the environment. The control technique would be halted and a different one put in place (for example, use of a different densovirus strain or a cocktail of strains).
- As for **gene drive** techniques, we have seen that they could lose their efficacy of propagation if resistance developed through pre-existing genetic polymorphism or mutations induced by non-homologous end joining in the sequence recognised by the guide RNA (see Section 4.3.2). Two cases must be distinguished in terms of risk assessment. In the case of gene drive techniques for elimination or eradication, any recovery of the population through polymorphic or mutated individuals with no loss of functionality of the fertility gene (see Section 4.3.2) would not in principle be harmful to the environment. In the case of gene drive techniques for population modification, polymorphic or mutated individuals would coexist with the modified population. The gene drive would not be as effective as expected, but this would still not constitute an environmental risk. However, in order to anticipate any undesirable consequences, it would be useful to study the consequences of resistance development to natural gene drives (genetic elements endowed with super-Mendelian inheritance, see 3.1.2.3), even though the molecular mechanisms underlying the latter are different. A possible impact on the dynamics of genetic drift in the target mosquito population, which could result in speciation events, genome rearrangement or a change in the reproductive mechanism, might thus be considered, as found in natural systems (Lindholm et al., 2016).

  Lastly, concerning the potential for development of resistance to the desired phenotype after population modification by gene drive owing to selection of pathogen strains insensitive to the pathogen-transmission resistance mechanism (a very low-probability event if the mechanism is highly specific, as in the case of the antibodies targeting *Plasmodium*, see Section 4.3.2), the gene drive would no longer serve its vector control objectives, but this evolution would not in principle present any particular risks for the environment. There might be a related health risk if some pathogens selected for their insensitivity to the resistance mechanism spread by gene drive turned out to be more virulent for human beings. A reversal drive would then be needed to eliminate the mosquitoes carrying this gene drive cassette (see below, point 7).

[75] Registered doses are calculated taking into account a safety factor that varies according to the product. Use of doses higher than the registered dose is allowed up to 20% in excess.

- Similarly, if any pathogen strains insensitive to *Wolbachia*-mediated **PI** were selected, the PI technique would no longer serve its purpose. Without remedial action, even if releases were halted the *Wolbachia* bacteria would continue to spread in the mosquito population (unless its selective disadvantage was too high for the hosts). There would in principle be no particular environmental risks, but there might be a greater health risk if the pathogen strains selected as insensitive to PI were more virulent for humans or animals. Pathogens would thus have to be assessed regularly for virulence. Other vector control methods would therefore have to be developed to suppress this new mosquito population or the *Wolbachia* bacteria it hosted.

Thus development of resistance to a technique's mode of action must be monitored for risk in case of selection of mosquitoes with greater vector competence from mosquitoes selected as biocide-resistant and selection of pathogens that are more virulent for humans and animals from any pathogens selected as insensitive to *Wolbachia*-mediated PI or to the resistance mechanism spread by gene drive.

Behavioural resistance

- Any development by field mosquitoes of behavioural resistance to irradiated mosquitoes, *Wolbachia*-carrying mosquitoes or genetically modified OX513A mosquitoes in connection with **SIT, IIT or RIDL** would also be without any consequences for the environment: mosquito releases would be stopped once such resistance was detected, the mosquitoes already released would have no viable offspring (owing to their intrinsic sterility/sterilising trait on the one hand and the field mosquitoes' behavioural resistance on the other) and their genetic characteristics (individual genetic background, mutations, *Wolbachia* bacteria or transgene cassette) would not remain in the environment once they had decomposed after death.
- With the **technique for spreading PI**, any behavioural resistance by field mosquitoes to *Wolbachia*-carrying mosquitoes would simply interrupt population modification and therefore the spread of *Wolbachia* in the field, with no particular consequences for the environment in comparison with a functional PI technique; *Wolbachia*-carrying individuals would simply be confined to breeding among themselves (it should be noted that because behavioural resistance depends on the genetic background of the released mosquitoes, it might no longer be expressed towards hybrid offspring carrying *Wolbachia* and the PI technique could then continue).

Thus potential development of behavioural resistance to released mosquitoes in field mosquitoes would not in principle entail any particular environmental risks.

b. **Risks associated with potential drift in the technique or loss of functionality**

Potential for functional drift has been identified for the RIDL and gene drive techniques as well as IIT. Would there be environmental and health risks if these techniques showed functional drift in the course of a strategy?

- In the event of loss of functionality of the **RIDL** technique owing to *tTAV* genetic drift, the technique would be halted as soon as the problem was detected, since regular in-house tests are used to check the functionality of *tTAV* in addition to field monitoring. In the field, loss of *tTAV* function would result in survival of offspring of the male RIDL mosquitoes released. These offspring would have 50% of the RIDL mosquito genotype and be hemizygous for the transgene cassette containing the non-functional *tTAV* gene and a marker. The genes of the reared strain and the transgene cassette would segregate in the following generations. Compared with the RIDL mosquitoes released, the distinctive features of these offspring in terms of environmental risk would be that females would carry the non-functional transgene cassette and the transgenic individuals

would be fertile. While no risk seems in principle to be associated with any dispersal in the environment of genes from the RIDL reared strain and the associated cassette, an appropriate assessment of the related risks (vector competence, selective advantage, toxicity, etc.) will have to be made on the basis of information from an applicant's dossier. Susceptibility of RIDL mosquitoes to insecticides used locally and to alternative vector control methods will in any case have to be verified to ensure that these mosquitoes can be eliminated if the line's lethality trait loses functionality (resumption of the RIDL strategy would be possible and effective even for dispersed transgenic mosquitoes once the transgenic strain's functionality was restored).

- Inactivation of **gene drive** by cassette mutation or methylation or by epigenetic factors would have no particular impact on the environment.
- Lastly, we have seen that **IIT** would lose efficacy if females were accidentally released, since they could then breed with field males and cause population modification rather than reduction. There would not in principle be any particular risks for the environment. As far as health is concerned, the released females would bite, presenting the risk that new pathogens might be introduced into the environment if blood used in the rearing facilities was contaminated, thus underlining the importance of quality control of blood fed to mothers of the mosquitoes released (see Section 4.3.3). They might, however, be weaker vectors than female field mosquitoes in the case of PI associated with the *Wolbachia* bacteria that they host. As a precaution, *Wolbachia*-carrying mosquitoes' vector competence for local pathogens should in any case be studied before these mosquitoes are released, even for IIT. Release of females is discussed in more detail below in a paragraph of its own in connection with the specific risks of mosquito release (point 4, b).

Thus functional drift in gene drive and *Wolbachia*-mediated spread of PI would not in theory entail any particular environmental impact in comparison with these techniques when properly functional. As for the RIDL technique, functional drift with tTAV losing its lethality trait in transgenic offspring would result in environmental dispersal of transgenic mosquitoes, albeit limited, necessitating characterisation of the strain released, particularly in terms of vector competence, to rule out the possibility of increased pathogen transmission. For IIT, the quality of the blood used in rearing facilities and the vector competence of females must be verified in order to prevent increased pathogen transmission in the event of accidental release.[76]

4. Specific risks associated with mosquito release

Prior to use of any technique involving release of mosquitoes, whether GM or not, the ecological impact and role of the mosquitoes released should be assessed with reference to volume and recurrence of releases, potential persistence and spread of the mosquitoes released, their fertility and reproductive isolation, the fitness of any offspring and their adaptability to a new environment, and other characteristics including vector competence compared with field populations. Where appropriate, the stability of a transgene or a symbiotic relationship with *Wolbachia* should be monitored over time.

a. Persistence and invasiveness

This question is relevant to all mosquito releases but raises different issues depending on whether the vector control technique's objective is population reduction, elimination or modification:

[76] This must be taken into account for any release of female mosquitoes into the environment. Specific consideration is given in this section to accidental release of females in IIT, since it would be followed by establishment of *Wolbachia* in the population and this would undermine the technique's efficacy.

- **As regards population reduction techniques** – SIT, IIT, the RIDL technique and gene drive for elimination of a population or eradication of a species – there is no dissemination, dispersal or spread of the released mosquitoes, their transgenes or the associated *Wolbachia* bacteria in the long term:

  - With **SIT**, no genetic material is transmitted or spread after death of the sterile males. In the event of residual fertility (see Section 4.3.2, '4. Actual efficacy versus theoretical efficacy'), any males remaining fertile despite irradiation could nevertheless spread random mutations. With a population elimination strategy, these mutations will be eliminated with the population. With a population reduction strategy, a number of scenarios are possible depending on the effects of the mutations:

    - If they are detrimental, they will be counterselected in the long term;
    - If they have no impact on mosquito fitness, their frequency in the population will not increase in relation to their initial frequency in the residual mosquito population;
    - If they confer a selective advantage on mosquitoes, their frequency will increase in the population and their effect will be counterproductive for vector control, which explains why the IAEA recommends complete sterilisation of released males. As for specific health risks, the possibility of a stable combination of mutations conferring a selective advantage on mosquitoes with mutations conferring increased vector competence might be taken into consideration, even though it seems unlikely.

  - With **IIT**, *Wolbachia* cannot spread from the males released, since *Wolbachia* transmission is solely maternal.[77] *Wolbachia* spread should be considered in the event of accidental release of females (see point (b) below). In the event of residual fertility, i.e. incomplete CI (see Section 4.3.2, '4. Actual efficacy versus theoretical efficacy' and Ross et al., 2017), the released males might have offspring with field females. *Wolbachia* would still not be transmitted, but the genetic material of the released males might spread in the population, which would have no impact if the strain used for IIT was local. If this is not the case, it should be ascertained that the strain employed does not have greater vector competence than the field strain.

  - With the **RIDL** technique the transgene does not spread, owing to its lethality in offspring of the males released. The 2% residual survival rate caused by incomplete trait penetrance does not lead to transgene persistence in the long term, although the genetic material of the laboratory strain minus transgenes spreads to some extent.

    Indeed, if they reproduce, these 2% of survivors, which are hemizygous for the transgene, will transmit their genetic material to a small proportion of the natural population. Unless a random mutation has made it non-functional, the transgene itself will soon disappear, since its lethality will operate in subsequent generations. Thus of the few transgenes persisting beyond the first generation of males released in the field, 98% will then disappear in the next generation with the death of the mosquitoes carrying them, and so on and so forth. On the other hand, offspring not inheriting the transgene will be viable and will carry a quarter of the genetic material of the laboratory strain, whose genetic variations (polymorphisms) may be absent from the release area. This should have no consequences other than to introduce a certain degree of genetic variability into natural populations (influx of laboratory-strain haplotypes into areas where these haplotypes were previously absent). One consequent recommendation would be that the laboratory strains used for releases should not be more competent for transmission of human pathogens than the field strains in the release area. It would therefore be advisable to undertake regular assessment of the transgenic strain's

[77] Experimentally verified by O'Connor et al. (2012).

vector competence, which should not be greater than that of field mosquitoes, in order to avoid the risk of introducing traits that might increase the species' pathogen transmission rate.

By contrast, this recommendation must be qualified in the case of *tTAV* genetic drift, in which the non-functional transgene persists (see above analysis of consequences of a loss of efficacy).

- Lastly, with **gene drive** for elimination or eradication, the gene drive cassette spreads initially before eventually disappearing because of the sterility of the females homozygous for the transgenes. If the gene drive is inactivated, hemizygous transgenic individuals could persist temporarily; the transgenes could then be regarded as harmful recessive alleles in the females, which will gradually be eliminated from natural populations.
- It is emphasised that **the question of the released mosquitoes' persistence and invasiveness, their transgenes or associated *Wolbachia* bacteria does not arise for SIT, IIT and the RIDL and gene drive techniques cited above when they are used for population elimination and this objective is achieved.**[78]

- **As regards population modification techniques** using gene drive or *Wolbachia*-mediated spread of PI, the persistence and invasiveness of the modification making the population resistant to pathogen transmission (*Wolbachia* for the PI technique and the genetic cassette for gene drive) are constituent characteristics of these vector control techniques. Furthermore, the genetic background of the released mosquitoes is not transmitted through super-Mendelian inheritance, unlike the *Wolbachia* bacteria or gene drive cassettes themselves. The *Wolbachia* bacteria or the gene drive cassette could in any case be introgressed into a local mosquito strain before being released. The corresponding releases are also on a smaller scale than the mass releases required for reduction or elimination strategies. It should nevertheless be noted that the *Wolbachia*-mediated technique to spread PI may necessitate a substantial release to achieve the initial relative density needed to counteract the fitness costs associated with *Wolbachia*.

  A risk assessment must therefore take into consideration the evolution and long-term effect of gene drive transgenes for population modification on the one hand and of *Wolbachia* bacteria for the technique to spread PI on the other, as well as their potential for vertical transfer to any interfertile (sub)species or the potential for horizontal transfer to other organisms or microorganisms (the probability and consequences of such transfers are set out below, in the specific sections on *Wolbachia* techniques and gene drive).

Thus the question of the risks associated with persistence and invasiveness of the mosquitoes released and the modifications that they carry raises different issues depending on the techniques themselves and the strategies for which they are used. (1) Regarding release of sterile or sterilising males, whether GM or not, this question theoretically should not arise. However, in the actual application of these techniques there may be deviation from the theory, seen in the residual fertility of some of the individuals released (see Section 4.3.2). This would have no impact in the case of population elimination but should be taken into account for population reduction. (2) Regarding mosquito release for population modification (reduction of vector competence), these characteristics are an integral part of the corresponding techniques and will necessitate an appropriate risk assessment beforehand and a long-term monitoring mechanism.

[78] If the objective of elimination is not achieved, the population reduction argument applies.

b. Release of females

It is also necessary to consider specific risks associated with release of females, whether intentional, for the PI technique, or accidental, in the case of SIT, IIT and the RIDL and gene drive techniques (even though gene drive techniques require only small-scale releases and could therefore allow accurate sorting of males).

Accidental release of females may arise out of imperfect sexing (see Section 4.3.3). Female mosquitoes bite, are more or less fertile, more or less competent for vector transmission of local pathogens and could carry new pathogens acquired by vertical transmission from blood used in rearing facilities (a preventable risk, see Section 4.3.3):

- Mosquitoes' mere ability to bite reflects their nuisance characteristic;
- The vector competence of any released females in comparison with that of wild females must be assessed in the light of the techniques used;
- The females' fertility depends on the technique used;
- The need to sterilise and monitor the quality of blood used in rearing facilities to prevent introduction of pathogens into the environment is treated at greater length in Section 4.3.3.

The characteristics of females that must be taken into account in the event of release, whether accidental or not, depending on the techniques used, explained above in the relevant sections, are summarised in Table 4.

Table 4. Characteristics of females to be taken into account in the event of release, whether accidental or intentional, for vector control techniques based on mosquito release.

Vector control technique	Characteristics of released females			
	Nature of release	**Nuisance**	**Vector competence**	**Fertility**
SIT	Accidental	Biting	Unchanged	Sterile
RIDL	Accidental	Biting	Unchanged	Non-viable offspring
IIT (unidirectional CI)	Accidental	Biting	Theoretically reduced because PI likely[1]	Fertile
IIT (bidirectional CI)	Accidental	Biting	Theoretically reduced because PI likely[1]	Sterile with wild populations owing to CI (fertile with released males)
IIT-SIT	Accidental	Biting	Theoretically reduced because PI likely[1]	Sterile because irradiated
Spread of PI	Intentional	Biting	Reduced for target pathogens owing to PI[2]	Fertile (intentional spread of PI)
Gene drive for elimination/eradication	Accidental	Biting	Unchanged	Homozygotes sterile, Hemizygotes fertile (partly sterile in the published example; the technique requires optimisation)[3]
Gene drive for population modification (e.g. anti-*Plasmodium*)	Accidental	Biting	Nil for *Plasmodium*	Fertile

[1] PI is not systematic and should be verified in this case. [2] PI ought to be established in the case of a technique based on PI spread. [3] Partial sterility of hemizygous females is a problem in terms of gene drive efficacy but not in terms of risk.

Thus the females released have different fertility and vector-competence characteristics according to the technique used, with no specific features relating to GM traits. The potential risks associated with release of females must be carefully assessed for the *Wolbachia*-mediated technique for spreading PI owing to the deliberate nature of the release, the females' fertility and the uncertainty associated with evolution of the PI phenotype. Moreover, all the released females bite, which takes us back to the need to ensure, at an earlier stage, that there are no pathogens in the blood supplied to the rearing facilities.

5. Additional risks specific to transgenic mosquitoes

Released mosquitoes expressing novel products must be assessed on a case-by-case basis in terms of direct impact on predators and indirect consequences for the food web.

With regard to the effects on human and animal health, the presence of any novel products in the saliva of transgenic mosquitoes will have to be assessed on a case-by-case basis in terms of toxicity and allergenicity for mosquitoes that are likely to bite.

In the absence of the specific data needed to conduct this type of assessment for the examples of GM mosquitoes presented in this opinion, we can only stress that it should be carried out on the basis of the assessment criteria for transgenic mosquitoes set out in Section 5.

6. Additional risks specific to the RIDL technique

Specific point relating to the use of tetracycline in this technique

The RIDL technique is based on a system that depends on supply of tetracycline in the rearing facilities to suppress the lethality conferred by the gene construct. Could the bacteria in the microbiota of the reared mosquitoes develop tetracycline resistance and, if so, what would be the environmental consequences? While some papers have suggested that the midgut bacteria of mosquito larvae are eliminated during their metamorphosis into adults (DeMaio et al., 1996; Gaio et al., 2011; Moll et al., 2001), more recent work shows that the midgut is not fully sterilised upon metamorphosis (Gimonneau et al., 2014; Tchioffo et al., 2016). Even if the adults released are not treated directly, their gut flora may therefore be enriched in tetracycline-resistant species, which could be verified by analysis of these mosquitoes' microbiota. There might thus be a risk of dispersal of tetracycline-resistant bacteria into the environment from the mosquitoes released. Since tetracycline-resistant bacteria already exist in the environment (Nesme et al., 2014), this dispersal would not in principle constitute a specific environmental risk. It might, however, be worth studying the specific routes of exposure, through mosquitoes, to any such tetracycline-resistant bacteria. Furthermore, water from mosquito rearing facilities must be treated in sewage treatment plants. Whatever the level of treatment applied locally, water from healthcare and veterinary establishments will always contain greater quantities of antibiotics than water used in mosquito rearing facilities. Use of tetracycline in the RIDL system would therefore theoretically have no particular consequences for the environment.

7. Additional risks specific to gene drive

The issues specific to gene drive derive from the fact that the genetic modification it confers is invasive (intentionally but uncontrollably) and concerns a whole population (or, conceivably, a whole species). They are also connected with the characteristics of the CRISPR-Cas9 system currently used for this approach. Gene drive techniques necessitate release of mosquitoes and can be used either for population elimination, and even eradication of a species, or for population modification.

Before we consider the specific risks associated with gene drive, it should again be stressed that the most likely 'risk' at present is that gene drive approaches will be less effective than expected (see Section 4.3.2). We have seen in point 3 above that the risks of loss of efficacy during implementation

of gene drive techniques have not been associated with any particular environmental risk but that a health risk might have to be considered if pathogens selected for being insensitive to the resistance mechanism spread by gene drive turned out to be more virulent for humans or animals. Other possible consequences have been raised with reference to natural gene drive systems (see point 3 above (Lindholm et al., 2016)).

a. CRISPR-Cas9 collateral (or off-target) mutagenesis activity and its consequences

The possibility of off-target cleavages by the CRISPR-Cas9 system used in gene drive cannot be ruled out. What would be its consequences in a gene drive strategy?

Could off-target cleavages affect or deflect the gene drive mechanism itself? Gene drive cannot work unless the sequence targeted by the guide RNA is bordered by sequences homologous to the sequences flanking the gene drive cassette in the genome. Thus an off-target cleavage could in all likelihood lead to a mutation through non-homologous end joining rather than insertion of the gene drive cassette owing to lack of homology between the cassette's flanking regions and the sequences bordering the off-target site.[79] The gene drive cassette would not be more likely to be inserted at the cleavage site by non-homologous recombination than any other DNA sequence. If the intention is to target several genes of the same family, the guide RNA would have to be specifically designed for this purpose with particular attention to conservation of critical areas of the sequence (PAM and seed). Off-target cleavages not anticipated on the basis of this type of sequence homology should therefore lead not to insertion of a gene drive cassette but to non-homologous end joining, which could result in mutations in the off-target sites.

What would be the impact of these off-target mutations in terms of risk? Most of them are unlikely to have any effect on the mosquitoes. Off-target mutations with a detrimental effect would lead to disappearance of the mutated individuals by natural selection. Off-target mutations conferring on mosquitoes a selective advantage, improved vector competence or greater toxicity for their predators would be extremely unlikely. In this case, what would be the associated risks? The risks pertaining to these rare mutations would differ according to the type of gene drive strategy: (1) In the case of gene drive for elimination, these mutations would not interfere with elimination of mosquitoes and would disappear with them; (2) in the case of gene drive for population modification, they might persist in the new population and interfere with the intervention. To forestall this possibility before implementing a vector control strategy of this kind, a population of mosquitoes should be exposed to Cas9 and the corresponding RNA guide activity over several generations in contained conditions, their phenotype (particularly vector competence) should be characterised, and a sample of individuals should be sequenced.

Collateral mutagenesis activity can be minimised by choosing an endonuclease with higher fidelity than Cas9 and designing RNA guides that are as specific as possible. New versions of the Cas9 protein have been developed to reduce collateral mutagenesis, such as SpCas9-HF1 (Kleinstiver et al., 2016) and eSpCas9 (Slaymaker et al., 2016). Other nucleases that are functionally equivalent to Cas9, such as Cpf1 (Zetsche et al., 2015), could also be used in its stead if they proved superior in terms of specificity and efficacy. Conversely, a mutation leading to increased expression of the endonuclease could raise the frequency of off-target cleavages.

Thus Cas9 collateral mutagenesis activity will not interfere with spread of a gene drive cassette but could generate off-target mutations. These mutations will generally be eliminated with a gene drive strategy for population elimination but could persist with a gene drive strategy for

[79] The length over which there must be homology with the sequences flanking the gene cassette in order to initiate homologous recombination has not been clearly ascertained but is estimated to be several hundred base pairs. In practice, researchers use between 500 bp and 1.5 kb at present. The degree of imperfect homology tolerated is not known. A few mismatches spread along the sequence should not prevent recombination. Small insertions or deletions would doubtless also be tolerated. It may be assumed that efficacy of recombination will decline in proportion to an increase in size of deletions/insertions and a decrease in homology.

population modification. The possibility of undesirable mutations could be partly anticipated by preliminary studies in contained conditions. Nucleases less prone to collateral mutagenesis are being developed.

b. Risk of gene-drive cassette transfer and its consequences

Horizontal transfer of a gene drive cassette to another insect through a retroelement or another type of transposon is conceivable, although the likelihood is virtually nil. In evolutionary terms, horizontal transfer in insects is a reality. In principle, on this scale of millions of years, a gene drive cassette has no higher probability than any other gene of transferring between species. Two cases may be considered:

1. After horizontal transfer and random insertion in a new host species, a gene drive cassette would be non-functional. Functional gene drive depends on two conditions: (1) homology in the recipient genome to the specific guide RNA sequence for targeting DNA cleavage, and (2) homology between the flanking sequences of the guide RNA recognition site and flanking sequences of the gene drive cassette to allow its insertion at the cleavage site by homologous recombination. Following a random insertion of the gene drive cassette, there would be no relation between the guide RNA sequence carried by the cassette and the sequences flanking the cassette's new insertion site, thus preventing the cassette from spreading through gene drive. In the hypothetical new host species, the guide RNAs could then either fail to find homologous sequences or else, in areas of homology, induce some DNA cleavage leading to mutations but no gene drive cassette insertion.
2. If horizontal transfer allowed expression of the guide RNA and Cas9 protein prior to insertion of the gene drive cassette into the genome of the new species, and on condition that the latter had the requisite homology, the gene drive cassette could be functional, i.e. be copied by homologous recombination. Indeed in this case the guide RNA could target insertion of the cassette into a site enabling its spread. It should nevertheless be noted that the gene drive would not continue to spread in subsequent generations unless the transfer took place in gamete precursor cells (see Section 5 for assessment of risks associated with horizontal transfer).

Vertical transfer of a gene drive cassette to other species is possible provided that they can cross-breed with the original species. Interfertile species are uncommon among mosquitoes. The best-known example is the interfertility between the *An. gambiae s.s.* subspecies and the *An. coluzzii* or *An. arabiensis* subspecies of the *An. gambiae s.l.* complex (see Section 2.3.2.1). After vertical transfer, the gene drive may or may not be functional in the new (sub)species, depending on the homology of the targeted sequences. The relevant strategy can be planned and designed in advance in two ways:

1. If the interfertile species concerned is also a vector, as in the case of *An. arabiensis* and *An. coluzzii*, which are both interfertile with *An. gambiae* and vectors of *Plasmodium*, and if analysis of these species' functions in the ecosystem suggests that it would not entail any significant risks, extension of the gene drive would be desirable. The guide RNA could be designed to target a gene common to these species. The risks associated with modification of each of the species must be taken into consideration.
2. Conversely, if extension of gene drive to an interfertile species is not desirable, the guide RNA could be designed more specifically, depending on the state of knowledge of the allelic diversity of these genes in a given complex of interfertile species.

The allelic diversity of *Anopheles* species that are major malaria vectors is well known thanks to genome sequencing of 16 species (Neafsey et al., 2015). For other species complexes that might possibly be targeted, knowledge of their diversity will seldom be available beforehand; it would be advisable to use sequencing to verify the allelic diversity of a target sequence in any interfertile subspecies complex. The risk of a gene drive extending to other species whose elimination is not intended is extremely low because of the reproductive barriers (species barriers). If hybridisation

occasionally occurs and produces fertile individuals (in closely related species or entities hitherto wrongly considered to be separate species), gene drive will spread only if the guide RNA target sequence and its flanking sequences are sufficiently similar to those in the target species to allow cleavage and homologous recombination, which can be forestalled by a good knowledge of the genomes.

Thus horizontal transfer of a gene drive cassette is possible, although highly unlikely on the scale of single individuals and generations. In most cases a gene drive cassette will not be functional after horizontal transfer to a new species. Vertical transfer into interfertile related (sub)species is more probable. Extension of spread of a gene drive cassette to an interfertile related species can be planned and designed beforehand, if it is desirable, when the genomes of both species are known.

c. Specific question of eradication of a species

From a technical point of view, it is highly unlikely that the theoretical objective of eradicating a species by gene drive will be achieved at present owing to the strong probability of resistance development or functional drift when current techniques are applied (see Section 4.3.2). With its present design, it is likely that gene drive will at best eliminate a local population. Strategies are nevertheless being developed to reduce impediments to gene drive spread. Conversely, other sophisticated techniques are being designed purposely to limit the spread of gene drive, thus making it possible to avoid forcing a technique on communities that have not deliberately chosen it by reducing the objective to local elimination (Min et al., 2017b; Noble et al., 2016).

Assessment of risks associated with local elimination of a mosquito population was addressed in point 2 of this section. The question of a species' role in the ecosystem will have to be considered at the global level in order to assess the consequences of its hypothetical eradication.

With regard to the lessons of area-wide population elimination, it should be noted that the elimination of New World screwworm, an invasive species, in an area covering some 40,000 km^2 around Tripoli in Libya in 1992 by SIT was a success for livestock farming and protection of large mammals (Vargas-Teran et al., 2005). The consequences for the environment have not been reported. Similarly, for elimination of *Ae. aegypti* in America[80] and Europe[81] in the twentieth century there are no reports of adverse environmental consequences associated with the disappearance of this mosquito species from these areas. Attempts to reduce populations of malaria-carrying *Anopheles* in Africa have not been followed by any documented adverse consequences. Elimination would clearly be desirable from the point of view of health; the environmental consequences would have to be studied.

For the invasive species *Ae. albopictus* and *Ae. aegypti*, an area-wide elimination approach should raise relatively few ecological concerns in colonised regions given that these mosquitoes do not belong to the native ecosystem, although it will be necessary to take account of any local ecological role acquired by these species once they have become established. By contrast, the ecosystem role of a species such as *An. gambiae* is worth studying in greater detail in order to determine whether control should be for the objective of elimination rather than population modification. Is the species essential to survival of certain predators that help to limit proliferation of other vector mosquito species or agricultural pests? To the best of our knowledge, no scientific papers have reported the existence of specialist predators of a mosquito species in Africa, or in the French territories of Réunion and Mayotte, and the ecological niche left vacant should easily be filled by other species

[80] An *Ae. aegypti* eradication programme was launched in Latin America in 1947. By 1962, 18 continental countries and a number of Caribbean islands had eliminated the species from their territories, followed by three more countries after 1962. Gradual reinfestation then took place from countries that had not managed to eliminate the mosquito (Anonymous, 1997).

[81] *Ae. aegypti* reached Europe before the end of the seventeenth century. It was present in almost all Mediterranean countries up to the 1950s, after which populations rapidly declined without the causes having been clearly identified (improved water-supply systems; indirect effect of insecticide spraying, particularly to control malaria vectors). Since 1960 it has been detected only sporadically, and nowadays only Turkey has a limited number of residual populations (Schaffner and Mathis, 2014).

since mosquito species in the tropics number in the hundreds ((Foster and Walker, 2002); VectorMap http://vectormap.si.edu/). More generally, a case-by-case and region-by-region assessment, according to the vector control objective, would be necessary to understand the ecological impact of a mosquito population elimination strategy.

d. Risk of insertion of an 'undesirable' gene into the gene drive cassette

Might there be a risk of an 'undesirable' gene (for insecticide resistance, pathogen susceptibility, etc.) jumping into the gene drive cassette and 'riding along' with it?

Unlike some viruses and transposons, which are endowed with specific molecular machinery enabling them to excise themselves from one genome region and insert themselves into another, other genetic elements are unable to move autonomously in the genome. Thus for an undesirable gene to conceivably end up in a functional gene drive cassette, a transposon or a virus having first 'acquired' this undesirable gene would have to jump into the gene drive cassette without inactivating it. Two successive events would be necessary: (1) The undesirable gene would have to to somehow end up by chance in a transposon or virus, and (2) this transposon or virus would have to jump into the gene drive cassette without inactivating it.

The first event would be extremely unlikely, since it presupposes that such a gene would excise itself from the genome and copy itself intact into a transposon or virus. While mechanisms of this type (conjugative plasmids and transposons transferring antibiotic resistance genes, virulence factors, etc., from one species to another) are documented for bacteria, this is not the case for mosquitoes. It is, however, worth noting that the genomes of some mosquitoes, particularly *Aedes*, abound in transposons (70% of the genome in *Aedes*, 23% in *Anopheles* (Chen et al., 2015)) and that some DNA viruses may sometimes acquire genomic fragments of their hosts.

The second event would presuppose that such a recombinant virus or transposon carrying the undesirable gene had jumped into a gene drive cassette without inactivating it and into germline cells, an event that would be exceptionally rare. The germline is physiologically resistant to exogenous viral infections: germline cells are physically isolated from somatic cells and possess specific mechanisms for silencing parasitic nucleic acids, such as the piRNA pathway (Senti and Brennecke, 2010).

The likelihood that an 'undesirable' gene would 'ride along' with a gene drive cassette is therefore extremely low. This risk could, however, be taken into account for evolution of a population of several million individuals.

e. Unpredictable risks and the means of pre-empting them

Because of the molecular originality of gene drive, in a situation where we have only limited knowledge of the biology of genomes it is difficult to anticipate all the risks and imagine everything that might cause a gene drive intervention to turn out unfavourably. Some scientists, mindful of the need to establish barriers to gene drive, are working on reversal drive techniques. One of these techniques, already tested in fruit flies, consists in introducing a transgene carrying a guide RNA specific to the Cas9 gene and able to inactivate it (Wu et al., 2016). This approach is a workable way of countering the spread of a gene drive cassette without automatically precluding subsequent use of a new gene drive cassette with a Cas9 gene recoded in order not to be targeted by the 'antidote' guide RNA or with a gene encoding a protein functionally equivalent to Cas9 derived from species of bacteria other than *S. pyogenes*. It seems unlikely that it would be necessary to resort to such 'technology stacking', but it might be advisable to have an antidote in advance. Another possible approach is to create specific gene drive systems whose spread within a population would be genetically limited (Min et al., 2017b) and that would allow the population to be restored to its wild state by releasing wild-type mosquitoes in excess (Min et al., 2017a).

f. Precautions to be taken for research

Given the invasive potential of gene drive, the precautions to be taken for research have been discussed in a number of papers (Akbari et al., 2015; Esvelt et al., 2014; Oye et al., 2014). They are based particularly on multiple containment and restricted access to experimental insects. One of the best recommended safeguards is to carry out laboratory gene-drive experiments in a geographical area where the species studied cannot develop (the case of *An. gambiae*, requiring a tropical climate, in northern countries), which would ensure that any individuals accidentally escaping containment would be unable to become established or find reproductive partners. Preliminary experiments to test a transgene's usefulness for control can also be carried out without the gene drive components (Cas9 and guide RNA).

8. Additional risks specific to Wolbachia*-mediated techniques*

a. Risk that Wolbachia *bacteria introduced into the population may confer increased vector competence for transmission of local pathogens*

In some, fairly rare, cases mosquito/*Wolbachia* interactions result in greater viral or parasitic intensity. This is the case for *w*AlbB in *Cx. tarsalis*, which increases susceptibility to West Nile virus (Dodson et al., 2014), and for *w*Flu in *Ae. fluviatilis*, which increases the *Plasmodium gallinaceum* oocyst burden (Baton et al., 2013). In the latter case, the increase was demonstrated in only two of the four experiments conducted. The mechanisms responsible for the rise have not yet been identified. Abiotic environmental factors may influence the nature and intensity of pathogen interference (PI). Thus, in the case of *An. stephensi* transinfected with *w*AlbB, a high temperature (28°C) has the effect of blocking *Plasmodium yoelii* whereas a lower temperature (24°C) instead increases the mosquito's parasitic burden (Murdock et al., 2014). The effects of the environment on PI should therefore be studied for other *Wolbachia*/host/pathogen combinations before their use is considered.

We have also seen various possibilities for loss of efficacy of the PI technique (mosquitoes may evolve and lose their *Wolbachia* infection; mosquitoes' *Wolbachia*-mediated protection may diminish over time; PI-insensitive pathogens may be selected) (Section 4.3.2). In terms of risk, it has been concluded that it is important for the properties of viruses newly exposed to *Wolbachia* to be tested regularly under different controlled environmental conditions in order to ensure that they have not become more virulent (see above).

b. Potential for horizontal gene transfer to, from or via Wolbachia

It should be noted with regard to evolution that *Wolbachia* infections are a possible factor in horizontal gene transfer (through lateral transfer, which is DNA transfer between a symbiont and its host). At least two phenomena have been recorded:

- Incorporation of *Wolbachia* genome fragments into the host arthropod's genome

  Lateral gene transfer from *Wolbachia* to their hosts has been reported in a number of cases, including ones in which the genes transferred have been expressed in the host's genome, where they seem to have acquired a new function (Hotopp et al., 2007). This phenomenon has also been reported in *Ae. aegypti* mosquitoes expressing genes from a former *Wolbachia* transfer, although this mosquito species is not currently infected with *Wolbachia* (Klasson et al., 2009). These transfers can concern very large genome segments, as has been demonstrated in *Drosophila ananassae* (Klasson et al., 2014).

- Incorporation of arthropod host genes into the *Wolbachia* genome through a *Wolbachia*-specific phage

  For example, the paper by Bordenstein and Bordenstein (2016) shows that WOCauB2/3 and WOVitA1 phages, found in *Wolbachia* of moths and parasitoid wasps respectively, carry eukaryotic genes (including one associated with black widow latrotoxins, reflecting changes in *Wolbachia* and *Wolbachia*-phage hosts in the course of evolution) (Bordenstein and Bordenstein,

2016). The eukaryotic genes co-opted by the phage seem to give it functional advantages. When the phage is incorporated into the *Wolbachia* genome (in the form of a prophage), these eukaryotic genes are therefore integrated into the bacterial genome. Co-infections with several *Wolbachia* strains are an opportunity for genetic exchange between *Wolbachia* through phages (Bordenstein and Bordenstein, 2016). These genes could also, in theory, be transferred to the hosts.

These examples illustrate the potential for genetic exchange between species through evolution. It will be hard to assess statistical frequency and the consequences in the case of artificial transinfection of mosquitoes with *Wolbachia*, since the rareness of these events prevents them from being studied in the laboratory. Whereas evolution of insecticide resistance in mosquitoes owing to human activity (vector control, farming) is an example of direct, fast-acting and measurable human influence, the possible effect of transinfection on evolution of mosquito genomes through horizontal transfer can be expected to be relatively minor.

c. Potential for Wolbachia *transfer between mosquito species*

Wolbachia bacteria are transmitted by female mosquitoes to their offspring. It may therefore be assumed that these bacteria are specific to a given species of mosquito.

However, *Wolbachia* genomes carry signatures of transfer between *Wolbachia* strains known to colonise different species at present (Baldo et al., 2006). The underlying mechanism is not understood, but the findings suggest that these different *Wolbachia* strains have cohabited in the same host. However, the mechanism for transfer of *Wolbachia* bacteria between different species is so far unknown.

To identify specific features of techniques using GM mosquitoes (Oxitec's RIDL technique and gene drive techniques for population elimination or population modification), a cross-cutting assessment of different vector control techniques has been carried out. This assessment has taken account of techniques representative of the main categories of vector control, both conventional (chemical, biological, physical and environmental) and emerging (based on release of irradiated mosquitoes (SIT), *Wolbachia*-carrying mosquitoes (IIT, spread of PI) or GM mosquitoes). The assessment has focused on the various objectives that these techniques can achieve, their efficacy and sustainability, their technical constraints and the environmental and health risks with which they may be associated.

Although the assessment has been conducted in sufficient detail to bring out the specific features of each of the techniques studied, it has nevertheless been possible to identify the following broad trends.

The GM mosquitoes considered in the assessment have one characteristic in common: they are all transgenic and express novel products, necessitating appropriate risk assessment, specific to each gene construct. This expression of novel products means that they have another common property: the ability to express a molecular marker, which is useful for monitoring released mosquitoes and their transgenic offspring.

Apart from this characteristic, the assessment shows that for most of the points considered, GM mosquito techniques do not constitute a homogeneous group distinct from other techniques but have characteristics in common with subsets of these other techniques.

Thus the set of techniques based on release of mosquitoes, whether GM or not, have in common:

- **The same type of technical constraints, specific to mosquito rearing and release, with variations according to the biology of the mosquito species concerned (adaptation of rearing protocols) and the vector control strategies of which these techniques form part (adjustment of scale and operating time of rearing facilities; possible need for sexing, a major technological barrier for most of these techniques);**

- **The specificity of action of the vector control technique, confined to the species of mosquito released and any interfertile species, thus reducing direct impact on health and the environment to a minimum but entailing as many interventions as there are non-interfertile species transmitting the same pathogen in a given region;**
- **Issues associated with potential persistence and invasiveness of the mosquitoes released and of the modifications that they carry, raising different questions according to the technique's objective (population reduction or population modification). In the case of population reduction, possible residual fertility in released males will have to be considered;**
- **Issues associated with potential release of females (biting capacity, vector competence, fertility, etc.), with varying analyses according to the objective and specific features of the technique used;**
- **Efficacy depending on the field competitiveness of the mosquitoes released;**
- **Higher efficacy at low densities of target mosquitoes, requiring combination of these techniques with conventional vector control methods that are effective at high densities, such as biocides, or else use of these techniques outside the seasons when endemic mosquito populations explode or prior to establishment of invasive species;**
- **An action time of several months to achieve the intended objective, meaning that these techniques cannot be used in a health emergency and entail integrated vector management with techniques having an almost immediate effect, such as biocides.**

Other characteristics independent of the GM trait are defined not by particular techniques but by the possible objective (reduction (to varying degrees) or population modification). As regards objectives, just like conventional vector control techniques, Oxitec's RIDL technique can be used for population reduction, but, unlike these techniques, it could in theory allow this reduction to continue until the local population was eliminated, as could other emerging control techniques based on mosquito release (SIT, IIT). Gene drive techniques can be used on the one hand to eliminate a local population and in theory eradicate a species (although the ability to obtain eradication, specific to this technique, is not achievable at present, owing to foreseeable development of resistance) and, on the other, to modify a population, as with the emerging technique of *Wolbachia*-mediated spread of PI.

Thus <u>all techniques for reducing a mosquito population density</u>, whether they use GM mosquitoes (RIDL and gene drive for elimination) or not (SIT, IIT and biocide, biological, physical and environmental control techniques) have in common:

- **An environmental impact associated with reduction of target mosquito population density and depending on the target species' role in the ecosystem. This impact varies according to, amongst other factors, whether the relevant species is autochthonous or invasive, whether its habitat is urban or natural, whether specialist predators exist, the extent to which the population is reduced (simple reduction, local elimination, or eradication of the species, although the latter objective is theoretical at this stage and would be a specific feature of gene drive techniques for elimination), the duration of a technique's effects (depending on how isolated the treated area is and whether the technique is self-sustaining or self-limiting, see below) and the specificity of the technique (techniques involving mosquito release being the most specific, see above). Environmental impact will therefore require appropriate assessment according to the technique used, the target species and the region to be treated;**
- **The potential for unintended replacement of the target population by the population of another vector species, which increases the more the target population is reduced and the more this reduction persists over time;**

- **Loss of immunity in previously exposed human populations, to be weighed against the benefit from disappearance of the disease for a time.**

On the other hand, techniques for modifying a population's vector competence (namely gene drive for population modification, using GM mosquitoes, and *Wolbachia*-mediated spread of PI) have in common:

- **The characteristic of modifying the target population's vector competence without reducing its density (the intended objective, at least), entailing less of an impact, in principle, with regard to environmental and health risks but requiring a comprehensive impact assessment over the long term;**
- **The persistence and varying invasiveness of the modifications induced, with the need to consider the evolution and long-term effect of the factors responsible for these modifications (*Wolbachia*, transgenes), including their potential for transfer to other species.**

Two other subsets of techniques have opposing characteristics, although both include techniques using GM mosquitoes: self-limiting techniques (with effects that are limited in space and time unless application of the technique is maintained) and self-sustaining techniques (whose effects spread across space and last over time without calling for any maintenance), with a two-dimensional (space/time) continuum of techniques between these two extremes (see Section 4.3.2). The RIDL technique belongs to the self-limiting techniques, as do SIT and IIT and most conventional vector control techniques (apart from permanent physical modifications and some biocontrol techniques characterised by persistence in the environment). Gene drive techniques belong to the self-sustaining techniques, as does, in varying degrees depending on the *Wolbachia* strain used, the *Wolbachia*-mediated technique for spreading PI.

Thus self-limiting techniques (including the RIDL technique) have in common:

- **effects that are limited in space and time unless application of the technique is maintained,**
- **the advantage of being controllable and adjustable in the light of monitoring data,**
- **the drawback of calling for demanding maintenance in the long term.**

Conversely, self-sustaining techniques (including gene drive techniques) have in common:

- **effects that spread across space (to varying degrees) and last over time,**
- **the advantage of not calling for maintenance or large-scale infrastructure,**
- **the drawback of being fairly inflexible or even theoretically uncontrollable if the spread of *Wolbachia* or the gene drive cassette encounters no obstacles.**

These characteristics may vary for a given technique depending on the vector control strategy of which it forms part (reduction or elimination) and the properties of the *Wolbachia* strains used.

With regard to their evolving aspects in terms of loss of efficacy over time through (1) development of resistance to their modes of action, (2) development of behavioural resistance, or (3) functional drift, and the consequences of potential evolution in terms of environmental and health risks, the techniques are less easy to classify into sets, since these developments result from the specific operating mechanisms of each technique.

Attention may nevertheless be drawn to two new sets of techniques with opposing characteristics in terms of resistance development, although they both include techniques using GM mosquitoes. Techniques operating through a genetic target are conducive to development of resistance to the technique's mode of action. They include gene drive techniques and some biocides. This is not the case for the other techniques, including the RIDL technique but also SIT, IIT, *Wolbachia*-mediated spread of PI and other conventional vector control techniques, whose mechanisms of action do not

operate through genetic targets or else involve too many targets for such resistance to develop easily.

Conversely, given the characteristics specific to each technique, development of behavioural resistance is conceivable for the RIDL technique and *Wolbachia*-mediated techniques but unlikely for gene drive techniques. Lastly, the risk of functional drift is a possibility for RIDL and gene drive techniques as well as for *Wolbachia*-mediated techniques. SIT differs from the other control techniques in that it does not seem to show any loss of efficacy over time.

Thus this assessment shows that, for most of the points considered, techniques using GM mosquitoes do not have specific features associated with their GM character but are comparable to other vector control techniques in different subsets depending on the aspects considered. An assessment of the benefits and limitations (including risks) of these techniques will therefore also be partly valid for techniques not necessarily using GM mosquitoes.

Apart from certain similarities with other vector control techniques, gene drive techniques are distinguished by their unique invasive potential, made possible by ad hoc use of the CRISPR-Cas9 system. While the technical limitations of current gene drive developments are likely to interrupt the spread of gene drive cassettes, assessment of improved applications in the future will have to take into account the particular invasive potential of these techniques and the uncertainties associated with their evolution in the environment. The following section deals with risk assessment criteria for GM mosquitoes and will, amongst other things, address the relevance of the current risk assessment framework for the specific features of gene drive techniques.

5. Risk assessment criteria for genetically modified mosquitoes

The referral specifically requests HCB to 'detail the criteria applying to health and environmental risk assessments of these insects [genetically modified mosquitoes] nationally (including French overseas departments, regions and collectivities), at the European level and internationally'.

To address this request, it is first necessary to look at the regulatory framework applicable to release of GMOs in the different territories concerned. Given the breadth of the request, the Scientific Committee has focused on the various territories of metropolitan and overseas France, as, depending on their status, they are covered by EU and/or international legislation.

5.1. Preliminary remarks concerning the regulatory framework for GM mosquitoes

5.1.1. Regulatory framework applicable to French territories

European Union rules apply in metropolitan France and what are known as the outermost regions, either directly or after having been transposed into domestic law. Outermost regions (OMRs) are European Union territories situated outside continental Europe. The French OMRs comprise all the overseas departments and regions (Guadeloupe, French Guiana, Martinique, Réunion and Mayotte) and the Saint Martin overseas collectivity. The rules applying to the other overseas territories are determined locally. These rules are discussed at greater length in the report by the working group of the Economic, Ethical and Social Committee.

France and the European Union are signatories to the Cartagena Protocol on Biosafety to the Convention on Biological Diversity, which, amongst other things, lays down measures applying to transboundary movement of 'living modified organisms' (LMOs, the Cartagena Protocol term that corresponds to the concept of GMOs, with a few minor differences from the EU definition (see WG report, Appendices 13 and 14)). French territories that are not part of the European Union will therefore have to apply the Cartagena Protocol at the very least.

5.1.2. Regulatory framework applicable to vector control techniques using GM mosquitoes

Use of genetically modified mosquitoes in the environment is regulated by Directive 2001/18/EC, at least when their GMO status is clear in terms of the directive's definitions: for example, if they are transgenic. Some of the genetic engineering techniques applicable to mosquitoes are among techniques whose regulatory framework is currently being debated by the European Commission (CRISPR-Cas9 site-directed mutagenesis). Moreover, genetic modifications resulting in gene drive raise radically new questions in terms of risk. Drafting of specific rules for gene drive is being discussed at the international level.

5.2. Proposed risk assessment criteria for GM mosquitoes

In defining 'the criteria applying to health and environmental risk assessments' of GM mosquitoes for vector control, the approach has been to consider the assessment criteria for release of GMOs into the environment under Directive 2001/18/EC and the Cartagena Protocol with two questions in mind:

(1) What could these criteria cover regarding risks associated with GM mosquitoes?

(2) Are these criteria sufficient to assess the risks associated with the GM mosquitoes concerned, and, if not, what new criteria should be added in principle?

5.2.1. Principles applying under Directive 2001/18/EC

Principles for environmental risk assessment under Directive 2001/18/EC, Annex II:

1. Case-by-case assessment.
2. Comparison to a non-GM equivalent.
3. Risk assessment steps: identification of characteristics that may have adverse effects, their potential consequences, the likelihood of each effect occurring, estimation of the risks (the likelihood of the adverse effect occurring and the magnitude of the consequences if it occurs), and definition of risk management strategies where appropriate.
4. Information to be provided, as appropriate, to assist in drawing conclusions regarding the environmental risk of releasing a GMO other than a higher plant (Annex II, D.1):
   - *i. Persistence and invasiveness*
   - *ii. Selective advantages or disadvantages*
   - *iii. Potential for gene transfer to other species*
   - *iv. Interactions with target organisms and potential environmental impact*
   - *v. Interactions with non-target organisms and potential environmental impact*
   - *vi. Effects on human health*
   - *vii. Effects on animal health*
   - *viii. Effects on biogeochemical processes*
   - ix. *Environmental impact of techniques used for management of the GMO*

Regarding the health aspect, it should be noted that criteria for health assessment of GMOs for non-food purposes are included in the environmental risk assessment criteria of Directive 2001/18/EC.

Risk assessment for the purposes of the Cartagena Protocol (Annex III) follows the same broad principles as Directive 2001/18/EC; individual criteria are not specified, although points to consider

are provided (recipient organisms, donor organisms, insert, etc.). The criteria listed in Directive 2001/18/EC have therefore been taken as the basis for discussion.

5.2.2. Application of the Directive 2001/18/EC criteria to use of GM mosquitoes for vector control

In 2013, at the request of the European Commission and on the basis of Directive 2001/18/EC, EFSA drew up risk assessment guidance for release of GM animals in the European Union, including a section on GM mosquitoes (EFSA, 2013). In addition, in accordance with the provisions of the Cartagena Protocol on Biosafety, assessment criteria for living modified mosquitoes have been provided by the Convention on Biological Diversity in its guidance on risk assessment of living modified organisms (BCH, 2012). These reports have been taken into account in the drafting of this section.

1. In the assessment principles set out in Directive 2001/18/EC, the principle of case-by-case assessment is wholly relevant to use of GM mosquitoes for vector control, enabling the following points to be considered amongst others:
   - Objective and method of vector control,
   - Bio-ecological characteristics of target mosquito species,
   - Molecular, genetic and phenotypic characteristics of released mosquitoes and any offspring, together with their impact, intended or unintended, on the ecosystem,
   - Receiving environments, intended scale, and natural or artificial barriers that could limit the spread of a mosquito population.
2. The principle of comparison to a non-GM equivalent is also relevant, allowing not only the non-GM parental line of the modified mosquito but also the target mosquito in the release area to be taken as comparators. The comparative approach to risk assessment must then be adapted to the particular contexts and objectives of the different types of vector control technique:
   i. If the objective is population reduction, the impact of sterile or sterilising GM mosquitoes can be compared to that of other control methods for reduction, such as chemical or biological control and some physical and environmental control methods;
   ii. If the objective is population modification, the impact of the new GM mosquito population can be compared to that of the target mosquito together with its usual management system in each environment where the new population could be expected to become established.
3. The standard risk assessment steps are also relevant to assessment of GM mosquitoes: assessment of the genetic and molecular characteristics of GM mosquitoes will usually include analysis of the genetic construct and method of modification, the number of insertion sites and transgene copies, the insert structure and sequence, the flanking regions, bioinformatic analysis of ORFs,[82] stability and heritability of the transgene(s) and associated trait(s), and transgene expression. In addition, it must be specified whether new proteins are present in the mosquitoes' saliva. For gene drive, additional verifications are required: it is necessary, for example, to examine the endonuclease version used and the specificity of gRNAs employed for the target-species genome in order to assess the likelihood of collateral mutations and, where appropriate, estimate the consequences; it is also necessary to assess whether the gRNAs could recognise sequences in genomes of other non-target (sub)species in the event of interfertility. Comparative assessments will include, in particular, comparison of GM mosquitoes' susceptibility to different

[82] ORF: Open reading frame, detected by bioinformatic programs and consisting of a DNA sequence that can, once transcribed, encode a peptide or a protein.

pathogens and their associated vector competence to that of non-GM mosquitoes and target mosquitoes in the release area.

4. The criteria listed in Directive 2001/18/EC make it possible to cover the following aspects of risk assessment for use of GM mosquitoes for vector control, provided that they are refined on a case-by-case basis for individual dossiers:

   i. Persistence and invasiveness

   Persistence and invasiveness of the GM mosquitoes released and any offspring of the same species or an interfertile (sub)species (where appropriate) are evidence of the spread of the genetic modifications. The following points may here be considered:

   - Bio-ecological characteristics of the released (and target) mosquito species and the modification's impact on these characteristics:
     - Species' biogeography and potential impact of the modification,
     - Species' potential for survival, reproduction and dispersal and potential impact of the modification.

   The specific features of the target release area and any natural or artificial barriers given the target species' bio-ecology can be covered in this assessment.

   - Potential for vertical gene transfer:
     - within the same species (giving consideration to the modification's impact on reproductive biology),
     - with interfertile (sub)species (giving consideration to the reproductive isolation of the target mosquito species and any impact that the modification may have).

   The characteristics of persistence and invasiveness are not considered risks per se: their consequences will have to be assessed in terms of environmental risk.

   ii. Selective advantages or disadvantages

   The selective advantage or disadvantage conferred on the GM mosquito by the modification must be assessed for each parameter in the list of criteria.

   iii. Potential for gene transfer to other species (horizontal gene transfer)

   This criterion covers the potential for transgene transfer to organisms in the surrounding environment other than offspring of the GM mosquitoes released. The following points may here be considered:

   - Transgene transfer to other insects, including other (sexually incompatible) mosquito species,
   - Transgene transfer to microorganisms in the mosquito's microbiota or in the surrounding environment.

   This assessment must cover:

   - The transgenes' molecular characteristics, including presence of any mobile elements or homology with bacterial genes,
   - Likely mechanisms of horizontal transfer through biological intermediaries between insects or by homologous recombination in bacteria,
   - Exposure of potential recipient organisms to GM mosquitoes.

   If potential for horizontal transgene transfer is identified, the environmental and health consequences must then be assessed, taking into account:

- Potential for subsequent vertical transfer in offspring of the recipient organism, i.e. the possibility that horizontal transfer might reach the germ cells of the recipient organism,
- Potential for persistence in recipient bacteria, i.e. the possibility that the transgene transferred might not confer a selective disadvantage on these bacteria,
- Potential for transgene expression in recipient organisms (e.g. specificity of action of a gene drive mechanism, promoter specificity, codon usage, intron presence, etc.),
- Prevalence of the trait in recipient organisms and the receiving environment.

iv. Interactions with target organisms and potential environmental impact

Target organisms here being defined as the mosquitoes targeted by vector control using GM mosquitoes and, indirectly, as the pathogens they transmit, the following points may be considered in this section:

- Consequences of release of GM mosquitoes for the target population in the case of:
  - release achieving the desired objective (population reduction, elimination or modification, or even eradication of a species),
  - genetic drift in the population of GM mosquitoes released,
  - unintentional release of females,
- The risk, for non-target organisms, ensuing from intentional modification or reduction of the target population (this point is discussed further in the section below on interactions with non-target organisms),
- The risk of development, in target mosquitoes and pathogens, of resistance to the vector control mechanism used, whether the objective is population modification or reduction. This possibility would lead to a loss of efficacy of the control technique, whose possible environmental impact would have to be assessed.

v. Interactions with non-target organisms and potential environmental impact

Non-target organisms cover all organisms other than the intended targets of GM mosquitoes. The following points may here be considered:

- GM mosquitoes' indirect impact through the food web on direct and indirect prey and predators of the target organisms if the latter are modified into a non-equivalent population or if their population is reduced or eliminated (or their species eradicated), and any potential consequences for ecosystem services (pollination, biological control, etc.). This assessment should include consideration of:
  - whether the target mosquitoes are autochthonous or invasive allochthonous, since local elimination of an invasive species might result in a return to the ecosystem state prevailing prior to its introduction,
  - whether there are specialist predators of the target mosquitoes in the area targeted by vector control,
  - whether there are non-specialist predators of target mosquitoes whose survival may depend temporarily on these mosquitoes (in the wet season, for example) in the area targeted by vector control,
  - whether the target environment is natural or largely artificial or urbanised,
  - whether the desired or actual impact is temporary or permanent,
- The impact of potential ecotoxicity of GM mosquitoes and any offspring for non-target organisms,

- The risk of unintended replacement of the target population, if its ecological niche is rendered partly or wholly vacant by vector control, by a non-equivalent population, and the associated consequences (e.g. emergence of potentially harmful secondary vectors),
- The impact on any host populations of pathogens transmitted by the target mosquitoes.

vi. Effects on human and animal health

Given the specific nature of mosquitoes as nuisance insects and vectors of disease-causing pathogens, the following factors may be considered in this section:

- Impact of the genetic modification on nuisance from released mosquitoes and any offspring in comparison with nuisance from their local wild targets for humans and animals,
- Impact of the genetic modification on vector competence of released mosquitoes and any offspring in comparison with the vector competence of their local wild targets for different pathogens,
- Risk of selection of new pathogen strains that GM mosquitoes would be able to transmit and that would be more virulent than the strains originally targeted owing to selection pressure from the technique applied,
- Risk of an increase in the population of biting females that are more or less fertile and more or less vector competent in the environment following unintentional release of females for a strategy requiring release of males only, and its consequences,
- Risk of loss of efficacy by the vector control technique, and its possible consequences in terms of a health risk,
- Risk of introduction of new pathogens into the environment owing to (intentional or unintentional) release of female mosquitoes infected during rearing,
- Indirect risk of loss of immunity of a human population following reduced incidence of a disease caused by a pathogen transmitted by the target mosquitoes (the intended objective).

Other typical health risks may be considered:

- Toxicity of GM mosquitoes and any offspring in comparison with their non-GM comparators for their predators and for humans by likely routes of exposure (e.g. bites or accidental ingestion),
- Allergenicity associated with GM mosquitoes and any offspring in comparison with their non-GM comparators through biting of hosts or exposure of employees working in GM mosquito rearing facilities.

vii. Effects on biogeochemical processes

Microorganisms involved in biogeochemical processes may be considered and treated as non-target organisms (see criterion above). The following specific points may also be considered:

- The insects' greater or lesser contribution, upon death, to decomposition of organic matter in soil and water,
- Potential for impact on composition of soil microflora,
- Potential for impact of elimination of a target mosquito population on the microbiotic balance of its habitat (particularly at the larval stage for endemic species).

viii. Environmental impact of techniques used for management of the GMO

The environmental impact of the management methods required by vector control techniques using GM mosquitoes can be compared to the environmental impact of the management methods needed for the vector control techniques that they are intended to complement/replace.

5.2.3. Analysis and conclusion

Are the criteria laid down in Directive 2001/18/EC sufficient to assess the risks associated with the mosquitoes concerned, and, if not, which new criteria should be added in principle?

While each new dossier must be assessed on a case-by-case basis, the HCB Scientific Committee, in the wake of the working group study, has not in principle identified any particular environmental risks that could not be covered by the general criteria listed in Directive 2001/18/EC. In addition to the criteria laid down in Directive 2001/18/EC, treated at greater length by EFSA in its above-mentioned guidance (EFSA, 2013), and the assessment criteria defined in accordance with the Cartagena Protocol by the Convention on Biological Diversity in its guidance on risk assessment for living modified organisms (also mentioned above) (BCH, 2012), WHO has published a guidance framework for testing of genetically modified mosquitoes that includes a section on risk assessment (WHO, 2014). According to the members of the working group, this report includes no environmental risk assessment criteria that are not already covered by Directive 2001/18/EC.[83]

While the general risk assessment criteria laid down in Directive 2001/18/EC are sufficient to assess the risks associated with GM mosquitoes, the HCB Scientific Committee, following the working group, nevertheless notes that gene drive has given rise to some radically new issues, given the deliberate invasiveness of the desired modification, which in theory has the potential to reach all individuals of a species in the environment, whether to eradicate or to modify it. By design, release is not limited in space or time. Risk assessment for gene drive must therefore be adapted to this change in scale and possible objectives. The relevant risk management must also be adjusted. It should be noted that no gene drive technique has yet reached the stage of technical development at which release into the environment could be contemplated: most discussions are still at the stage of defining good practice for research in contained conditions, having due regard to the precautions to be taken to prevent a gene drive cassette from being accidentally released into the environment. Techniques also being developed to reverse or limit gene drive could prevent any undesirable spread of such a mechanism for modifying populations.

In response to the question in the referral, it may therefore be concluded that **the criteria listed in Directive 2001/18/EC, officially applicable to environmental risk assessment for release of GM mosquitoes in the European Union, are scientifically relevant and in principle sufficient for assessment of the risks associated with use of GM mosquitoes for vector control. As provided for in the case-by-case approach of the directive, the specific information required for assessment of GM mosquitoes capable of gene drive must be determined and outlined.** This might be further developed in a more comprehensive report on gene drive.

Over and above the referral's question concerning risk assessment criteria, it should be noted that the potential for cross-border dispersal of GM mosquitoes raises a problem in terms of management: at present, intentional and unintentional transboundary movements of living modified organisms have to be notified on the basis of the Cartagena Protocol, since each party (or signatory) to the Protocol is required to take appropriate measures to prevent unintentional transboundary movements. Because this is impractical for GM mosquitoes able to spread a modification, this question should be addressed at the international level and lead to regulatory harmonisation at the supranational level.

[83] It should be noted that the assessment recommended in the WHO report includes benefits as well as risks.

5.3. Widening reflection to other emerging control techniques

SIT does not appear to be regulated at the EU level.[84] Release in Italy of mosquitoes sterilised by irradiation (Bellini et al., 2013) did not require authorisation or prior risk assessment (Bellini, pers. comm.). The paper on these trials refers to the International Standards for Phytosanitary Measures produced by the FAO and International Plant Protection Convention (IPPC), which deal with release of sterile insects in the guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms (Bellini et al., 2013; FAO, 2016). Mosquitoes sterilised by irradiation would therefore be considered biological control agents and SIT would be treated as biological control. The specific regulatory framework for France would have to be clarified.

Lastly, the question of the regulatory status of insects artificially infected with *Wolbachia* has not yet been raised in the European Union, since no applications for release have been submitted there so far. It is possible that, according to the regulatory definitions of Directive 2001/18/EC, mosquitoes artificially infected (transinfected) with endocellular bacteria may be considered genetically modified. In this case, use of such mosquitoes could either be subject to the corresponding regulatory requirements or be exempted from the scope of the directive after a debate and decision at the EU level. It is also possible that a legal analysis may find that *Wolbachia* thus used for vector control have the status of a biocide. It should be noted that, at the international level, use of these mosquitoes is not at present considered to come under the Cartagena Protocol because of the way in which the LMO definition is interpreted (Marshall, 2011). It is worth noting that *Wolbachia* are regulated as a biopesticide in the United States (Dobson et al., 2016) and a veterinary chemical product in Australia (De Barro et al., 2011; Dobson et al., 2016).

Whatever the regulatory status of insects artificially infected with *Wolbachia* bacteria in the European Union,[85] the Scientific Committee believes that assessment using criteria adapted from Directive 2001/18/EC would be relevant.

6. Benefits and limitations of use of genetically modified mosquitoes in France

The fourth question in the referral concerns 'the potential risks and benefits of using genetically modified mosquitoes for France, including overseas territories, particularly from the social, economic and ethical angles'. The Scientific Committee here proposes to highlight the benefits and limitations of use of GM mosquitoes with respect to the scientific and technical aspects.

Assessment of the specific features of GM mosquito techniques compared with other vector control techniques (Section 4) has made it possible to identify their benefits and limitations in terms of possible objectives, efficacy and sustainability, technical constraints, and environmental and health risks. Rather than going back to the comprehensive assessment of characteristics set out in the previous sections, we shall here focus on the salient points.

[84] According to Directive 2001/18/EC, mosquitoes released as part of a standard SIT strategy are sterile and, as such, do not fit the regulatory definition of an organism and therefore a GMO. Any irradiated mosquitoes that have retained their fertility are considered to be GMOs because their genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; they are nevertheless exempt from the directive as products of mutagenesis that has not involved use of recombinant nucleic acid molecules.

[85] HCB questioned the European Commission on this subject in a letter dated 2 June 2016.

6.1. Benefits and limitations of Oxitec's RIDL technique

It should be noted that HCB has not been formally requested to assess the risks associated with deliberate release of RIDL mosquitoes. No application dossiers have been submitted. The general aspects developed here are intended for guidance.

The RIDL technique is a variant of the standard sterile insect technique (SIT) for local elimination or reduction of a mosquito population. The technique's benefits and limitations are discussed in relation to characteristics of techniques that can be used for the same objectives, including existing vector control techniques and other emerging techniques involving release of sterile or sterilising mosquitoes without gene drive, namely SIT and incompatible insect technique (IIT).

The RIDL technique is based on release of mosquitoes from a stable and well-characterised transgenic line, with a well-defined induced dominant lethality mechanism. By comparison, SIT is based on release of a diverse mix of mosquitoes sterilised by irradiation, while IIT is based on release of mosquitoes from lines transinfected with *Wolbachia*, which are sterilising because of the cytoplasmic incompatibility conferred by the bacteria.

Relevance of possible objective for French territories

OX513A, the only line currently developed to an operational level using Oxitec's RIDL technique, is a line of *Ae. aegypti*, a vector of several arboviruses including the dengue, chikungunya, Zika and yellow fever viruses. This species of mosquito is not present in metropolitan France. The French territories that could benefit from *Ae. aegypti* vector control are the French West Indies, French Guiana, New Caledonia, French Polynesia and Wallis and Futuna (where it is a secondary vector to *Ae. polynesiensis*) as well as Mayotte and Réunion. The fact that *Ae. albopictus* is also present in the latter two territories means, however, that vector control for *Ae. aegypti* must be coupled with an equally effective control technique for *Ae. albopictus* in order to avoid replacement of *Ae. aegypti* by *Ae. albopictus*. The possibility that *Ae. aegypti* could be replaced by *Ae. polynesiensis* might also be considered prior to applying the RIDL technique in French Polynesia or Wallis and Futuna.

In comparison, SIT is not limited by a specific line of a given mosquito species. It could theoretically be used for any local mosquito species, including the *Ae. albopictus* mosquitoes in metropolitan France and the *Anopheles* vectors of malaria and lymphatic filariasis in some overseas French territories such as Mayotte. As for IIT, mosquito lines transinfected with different *Wolbachia* strains have been developed not just for *Ae. aegypti* but also for *Ae. albopictus* and *Ae. polynesiensis*, with promising trials having taken place for *Ae. polynesiensis* in French Polynesia.

Technique's potential and consequences

Like SIT and IIT, the RIDL technique can have two different objectives: reducing vector population density or eliminating a vector population. While the first objective can be achieved with existing techniques, this is not the case for the second. For control of vector-borne diseases, elimination may be preferable because it is more lasting, but this will depend on how isolated the release area is. It may, however, carry a greater risk for the environment, depending on the target species' role in the ecosystem. The risk of unintended replacement by another vector will be higher. Human populations in the region treated might become more susceptible to new epidemics following a loss of immunity, a risk that would, however, have to be weighed against the benefit from disappearance of the disease for a time.

Specificity of action and consequences in terms of vector-control efficacy and risks

The RIDL technique, like SIT and IIT and every technique that works through mating between released mosquitoes and target wild mosquitoes, has a specificity of action that is confined to the species concerned and any interfertile species. *Ae. aegypti* is characterised by a strong reproductive barrier. No other mosquito species is known to be sexually compatible with it. The RIDL OX513A line will therefore specifically target reduction or elimination of *Ae. aegypti* mosquito populations, with

no direct impact on other organisms in the environment or on human health. This must be compared with the variable, but usually greater impact, of other control techniques on health and the environment. Deltamethrin, for example, the most commonly used adulticide for controlling mosquitoes in France, has a non-selective mode of action. It is highly toxic to aquatic organisms as well as to bees and pollinators, which has led to significant restrictions on and precautions for its use.

However, while a vector control technique's specificity of action can reduce its environmental impact, it will not necessarily promote efficacy of control for a particular disease: a specificity that is too narrow may adversely affect this efficacy. For example, several viruses transmitted by *Ae. aegypti* are also carried by *Ae. albopictus* and *Ae. polynesiensis*. In regions where these species coexist, control by release of *Ae. aegypti* mosquitoes will preserve *Ae. albopictus* and *Ae. polynesiensis* mosquitoes. It will therefore have to be combined with another technique targeting *Ae. albopictus* or *Ae. polynesiensis*.

Persistence and invasiveness

The OX513A mosquito transgene does not persist in the environment owing to the lethality that it induces in offspring of released males. The sporadic transgenic individuals arising from incomplete trait penetrance are well characterised and do not lead to long-term persistence of the transgene. By comparison, in the case of SIT, mutations do not persist in the environment because of the sterility of the mosquitoes released. The random mutations spread in the event of residual fertility in irradiated individuals are not known; a risk associated with their segregation in the population is, however, unlikely. With IIT, *Wolbachia* cannot spread in the population from the released males, even in the event of residual fertility owing to incomplete CI, unless females are accidentally released.

Durability, loss of efficacy and associated risks

Whereas the risk of loss of efficacy through development of resistance to a technique's mode of action is common with certain biocides and is one of their major limitations, it is improbable with the RIDL technique or SIT and IIT, since they do not operate through genetic targets. This could give these emerging techniques a significant advantage in terms of durability.

However, development of behavioural resistance by target wild mosquitoes to RIDL mosquitoes is conceivable owing to use of a single line on the various release sites, even when the latter are remote from the geographical origin of the line's genetic background. While development of such resistance could compromise the RIDL technique's efficacy in a given region, it would not in principle be associated with any environmental or health risks. In comparison, the *Wolbachia*-carrying mosquitoes used for IIT could also encounter behavioural resistance. This resistance development would be improbable for the irradiated mosquitoes used in SIT.

Lastly, the RIDL technique might lose its functionality in the event of *tTAV* genetic drift. However, tTAV functionality is monitored; any disruption could be swiftly rectified. In the field, loss of *tTAV* function would result in survival of offspring of the male RIDL mosquitoes released. While no risk seems in principle to be associated with any dispersal into the environment of genes from the RIDL reared strain and the associated cassette, an appropriate assessment of the related risks (vector competence, selective advantage, toxicity, etc.) would have to be made on the basis of information from the applicant's dossier. IIT could also lose its functionality if there were a significant release of females. No biological mechanism entailing loss of functionality has been identified for SIT.

Self-limiting technique and consequences

The RIDL technique, like SIT and IIT and various existing vector control techniques such as biocides, is what is known as 'self-limiting' in that its effects are limited in space and time unless its application is maintained. Consequently, the RIDL technique has the advantage of being controllable and adjustable in the light of monitoring data. Conversely, it will call for demanding maintenance in the long term. It should be noted that it will be more expensive as part of a population reduction strategy

than as part of a population elimination strategy, the latter having the advantage of no longer requiring application of the technique once the objective of elimination has been achieved.

Monitoring and surveillance

As far as surveillance is concerned, RIDL mosquitoes carry a fluorescent marker associated with the repressible lethality gene cassette, which allows effective monitoring of transgenic mosquitoes in the environment and offers options for adjusting application of the technique. This monitoring system is more effective than that for SIT mosquitoes, which may be covered with fluorescent powder when they leave the rearing facilities. While the SIT mosquito marking also allows them to be monitored and their releases to be adjusted accordingly, it cannot be used to detect any offspring in the event of residual fertility or to distinguish SIT mosquito eggs in order to measure the technique's efficacy. A *Wolbachia*-specific molecular marker can be used for IIT.

Conditions of use

Like SIT and IIT, the RIDL technique requires several months to reduce or eliminate a mosquito population, with the period varying according to various parameters such as target area, initial density of target mosquitoes, number of mosquitoes released, isolation, etc. Furthermore, these techniques are not effective at high densities of target mosquito populations. They require prior reduction of mosquito density by use of conventional control techniques that are considered effective at such densities. Thus the RIDL technique, like the other techniques based on mosquito release, cannot be considered a quick answer to an emergency situation or the first solution when faced with a high-density mosquito population. It must form part of integrated management with different combinations of techniques over the long term.

Technical and logistical constraints

Significant technical constraints relating to infrastructure and logistics are associated with mosquito rearing and release for the RIDL technique, as in the case of SIT and IIT. Applied for population reduction, these techniques require long-term stable and functional mass rearing to guarantee recurrent mass releases (this statement would have to be qualified if the same techniques were used for elimination). The quality of rearing conditions is critical to ensuring that released mosquitoes are properly competitive. Sexing techniques, which are essential to avoid releasing female mosquitoes (which bite, are vectors but cannot have viable offspring), are a major technological barrier to most vector control techniques involving mosquito release. Lastly, quality control of blood used to feed reared females is imperative to avoid all risk of introducing pathogens into the environment if females are accidentally released.

Risks associated with accidental release of females

RIDL females bite and are vectors but cannot have viable offspring. Accidental release of females could carry with it the risk of introducing pathogens into the environment, a risk that can be prevented by quality control of blood supplied to the rearing facilities. In comparison, SIT females also bite, are vectors and are sterile. IIT females bite, have theoretically reduced vector competence in the case of *Wolbachia*-mediated PI and are fertile (but sterile with wild males with bidirectional CI or completely sterile in combination with SIT (IIT-SIT, i.e. IIT with low doses of sterilising irradiation), which is the approach recommended by the IAEA in the absence of a 100%-effective sexing method). Release of fertile females for IIT could furthermore compromise the control strategy.

Entomological versus epidemiological efficacy

Interest in the RIDL technique is based on its proven efficacy, in small-scale trials, for reducing populations of field mosquitoes. The technique's efficacy for preventing transmission of mosquito-borne diseases such as dengue, Zika and chikungunya, has not yet been established. This is also the case for SIT and IIT, at a less advanced stage of development.

Stage of development

The RIDL technique has undergone Phase 3 testing and is now being tested in Phase 4. The results of this larger-scale trial will be critical for testing the feasibility of the RIDL approach in actual conditions. In comparison, IIT has undergone promising small-scale trials, while SIT is less advanced in terms of demonstrating reduction of mosquito populations in the field, but a number of trials are planned.

The significant benefits and limitations of the RIDL technique resulting from this assessment are summarised in the box below.

In conclusion, the RIDL technique is closely related to SIT and IIT; all three techniques would be worth testing step by step on a precautionary basis for the purpose of contributing to vector control in French territories, depending on the vectors concerned, in combination with the conventional techniques currently being used. In particular they would help reduce insecticide use. In addition to a lesser risk of exposure for humans and the environment, lower insecticide use owing to use of techniques based on mosquito release would preserve insecticide efficacy by lessening pressure for selection of resistance. This would thus enable insecticide use to be reserved specifically for epidemics and public health emergencies. The RIDL technique is the furthest advanced but could only be used to control *Ae. aegypti* at present; for the other vectors, SIT and IIT, possibly combined with low doses of sterilising irradiation (IIT-SIT), could be envisaged.

Benefits and limitations of the RIDL technique based on the example of the OX513A line[86]

Significant benefits:

Shared by RIDL, SIT and IIT (sterile/sterilising insect techniques) in contrast to conventional techniques:

- Direct impact on health and the environment: minimal (owing to specificity of action)
- Indirect impact on the ecosystem: reduced in comparison with use of biocides (especially as the particular target species, *Ae. aegypti*, is invasive and urban, reliant on artificial sites)
- Possible objective: reduction, but also elimination in isolated areas
- Durability: development of resistance to technique's mode of action unlikely (but other mechanisms entailing loss of efficacy possible for RIDL and IIT, see 'Limitations')

Shared by RIDL, SIT, IIT, and gene drive for elimination (reduction techniques based on mosquito release) in contrast to modification techniques:

- No persistence or invasiveness (except, for RIDL, SIT and IIT, a low level of dispersal associated with possible residual fertility, with no significant consequences)

Shared by RIDL, SIT, IIT and most conventional techniques (self-limiting techniques) in contrast to self-sustaining techniques (gene drive):

- Controllable and adjustable

Specific to RIDL:

- Monitoring by means of a fluorescent molecular marker (IIT: *Wolbachia*-specific molecular marker; SIT: fluorescent powder)

Significant limitations:

Shared by RIDL, SIT and IIT in contrast to conventional techniques:

- Cannot be used for high-density target mosquito populations
- Cannot be used in public health emergencies
- Risk of unintended vector replacement
- Need to plan as many interventions as there are non-interfertile vector species (owing to specificity of action)
- Need for large-scale infrastructure and demanding maintenance in the long term (self-limiting techniques, unlike gene drive, a self-sustaining technique)
- Need for some level of isolation of the target mosquito population in order to be more efficient and longer-lasting

Shared by RIDL and IIT:

- Risk of behavioural resistance development (loss of efficacy but not associated with any environmental risk) conceivable for RIDL and IIT, unlikely for SIT
- Risk of functional drift possible for RIDL (tTAV disruption, loss of efficacy quickly detectable and rectifiable, not associated with any risk in principle, but the strain must be assessed in the event of dispersal). Risk also possible for IIT (accidental release of females) but unlikely for SIT (unless there is a problem with the irradiation source)

Limitations relating to the techniques' stage of development:

Shared by RIDL, SIT and IIT:

- Entomological efficacy proven only in the short term and on a small scale
- Epidemiological efficacy not yet proven
- Sexing technique in need of improvement

[86] The risks associated with novel products derived from expression of transgenes will have to be assessed on the basis of a notification dossier. In the absence of specific data, the HCB Scientific Committee has not considered this point in this assessment.

6.2. Benefits and limitations of gene drive for population elimination

The benefits and limitations of gene drive for population elimination are discussed on the basis of the example described in Section 3.3 and assessed in Section 4. This example, developed for the mosquito species *An. gambiae*, the main *Plasmodium* vector responsible for malaria in sub-Saharan Africa, is still at the research stage but is intended for application. Other vectors could be targeted by the same technique in future.

This technique has no equivalent to date, although it has some similarities to a self-sustaining variant of sterile insect techniques. Its original feature is its ability to spread sterility in a target mosquito population from a small number of releases of sterilising males.

Relevance of possible objective for French territories

An. gambiae, the species targeted by the application currently being developed, is present on only one French territory, Mayotte, where it is a vector of *Plasmodium* and filarial worms. The *An. arabiensis* mosquito species, still residually present in Réunion, could also be targeted by a release of *An. gambiae* mosquitoes, since they may cross-breed; however, there have been no reports of malaria or lymphatic filariasis there since the 1970s. French Guiana is still affected by malaria (*P. vivax*), but the vector species, *An. darlingi*, is sexually incompatible with *An. gambiae*.

Potentialities of the technique

Because of its invasive mechanism, the gene drive technique has the theoretical potential to eradicate a species. Its use in an isolated environment could limit elimination to a local population. At this stage of its technical development it is nevertheless to be expected that the spread of sterility would be limited by development of resistance in the target population, which would lead merely to a transient reduction in the population. The consequences of population reduction or elimination with respect to the species' role in the ecosystem and the possibility of its unintended replacement by another vector species are the same as those described for SIT, IIT and the RIDL technique. Gene drive further raises the question of the consequences of possible eradication of a species, which must be assessed globally.

The benefits and limitations of the gene drive approach for elimination have been identified on the basis of the assessment set out in the previous sections.

The benefits include:

- Theoretically unlimited elimination of vector mosquitoes of the same species,
- Possibility of treating large areas using a small number of releases,
- No need for maintenance or large rearing facilities,
- Persistence of effects (with variation in case of resistance development),
- Specificity of action, limited to the target species, thus reducing risks to non-target organisms and to health,
- No persistence of transgenes in the environment,
- Possibility of monitoring with molecular markers.

The limitations include:

- Lack of flexibility and possibility of control,
- Resistance development, likely at the current stage of the technique's development,
- Potential for unintended transfer of a gene drive cassette and its consequences,

- Impact of elimination of populations of target mosquito species on the ecosystem, particularly for an endemic species in a natural environment,
- Potential for unintended replacement of the target species by another vector species,
- Issue of loss of immunity of human populations in the area where the vector has been eliminated,
- Inability to respond to a public health emergency because of the time needed to reach a mosquito-population reduction threshold able to bring down disease transmission,
- Uncertainty associated with evolution of the gene drive mechanism in the environment.

6.3. Benefits and limitations of gene drive for population modification

The benefits and limitations of gene drive for population modification are discussed on the basis of the example described in Section 3.4 and assessed in Section 4. This example has been developed at the research stage for the *An. stephensi* mosquito, the main *Plasmodium* vector responsible for malaria in Asia. Other vectors could be targeted by the same technique in future.

Unlike the techniques considered above (Sections 6.1 and 6.2), this technique seeks to reduce mosquitoes' vector competence without affecting population density. The technique of *Wolbachia*-mediated spread of PI has the same objective and has already undergone field trials.

Relevance of possible objective for French territories

An. stephensi, the target species in the example of gene drive being developed, is not present on any French territories. The trials for *Wolbachia*-mediated spread of PI target *Ae. aegypti* mosquitoes. The French territories that might benefit from it are the same as those concerned by the OX513A line for the RIDL technique (see Section 6.1).

Potentialities of the technique

In this example, the gene drive technique is used to spread resistance to pathogen transmission in the target population by means of a transgene that prevents the pathogen from developing in the mosquito.

The benefits and limitations of the gene drive approach for population modification have been identified on the basis of the assessment set out in the previous sections.

The benefits include:

- Spread in the target population of a heritable factor reducing mosquitoes' vector competence,
- No impact, in principle, on target mosquito population density, thus lessening the risk of impact on the ecosystem,
- Possibility of treating large areas using a small number of releases,
- No need for maintenance or large rearing facilities,
- Persistence of effects (with variation in case of resistance development),
- Specificity of action, limited to the target species, thus reducing risks to non-target organisms and to health,
- Possibility of monitoring with molecular markers.

The limitations include:

- Lack of flexibility and possibility of control,
- Development of resistance to spread of the transgene, likely at the current stage of the technique's development,
- Impact of selection pressure on pathogens,
- Question of transgene persistence in the environment,
- Potential for unintended transfer of a gene drive cassette and its consequences,
- Issue of loss of immunity of human populations in the area where the vector has been modified,
- Inability to respond to a public health emergency because of the time needed to render a significant part of the mosquito population resistant to transmission of the disease,
- Uncertainty associated with evolution of the gene drive mechanism in the environment.

The two gene drive approaches discussed in this opinion are still undergoing development. Current research is concerned with, amongst other things, reducing evolution of resistance to gene drive, developing a gene drive mechanism whose spread would be limited, and designing tools able to reverse an existing gene drive. Research is also under way into procedures for assessing the long-term effects of gene drive on ecosystems. At this time it is premature to contemplate deployment of gene drive in the environment. As for the objective of population modification, the alternative approach using *Wolbachia*-mediated spread of PI is already being tested in the field even though PI mechanisms are still not properly understood.

6.4. Need for integrated vector management

A number of vector control techniques thus exist, and the choice between them should be informed by criteria relating to desired efficacy of components of vectorial capacity as well as to vector biology and behaviour, the epidemiological context (seasonal transmission, extended transmission or epidemic) and the environmental and socio-economic situation, including available human and financial resources. In addition, these methods have to be accepted by the community so that measures can be implemented as effectively as possible. They must also have minimal impact on the environment, which can translate into acceptability as well as implementation procedures (co-design of innovations with beneficiaries).

WHO suggests an integrated vector management strategy, defined as 'a rational decision-making process to optimise the use of resources for vector control' (WHO, 2012). Its object is to improve the efficacy and cost-effectiveness of interventions forming part of national vector control programmes whilst limiting their ecological impact and reducing insecticide use as much as possible. Such a strategy allows for the fact that in the same region a vector may transmit several pathogens or the same control method can be used for several vectors transmitting different pathogens. Thus, in some cases, rational choice of a limited number of interventions can make it possible to control several diseases at once. Integrated vector management is based on five key elements: (1) Close collaboration within the health sector and with other sectors, (2) an integrated approach to vector-borne disease control, (3) fact-based decision-making, (4) awareness-raising, social mobilisation and a legislative framework, and (5) capacity-building (see WG report, Section 5).

All vector control techniques can thus be considered for the purposes of integrated vector management, and the decision to apply one technique rather than another or to use a combination can be taken on the basis of specific criteria (WHO, 2012). The different situations and vector systems found on French territories should be characterised using these criteria, which must allow

for the distinction between anticipating and responding to a public health emergency. This approach should be complemented by consideration of social, ethical and economic factors (see Recommendation from the Economic, Ethical and Social Committee).

Through this guidance on vector control techniques using genetically modified mosquitoes and other emerging techniques based on mosquito release, this opinion should add to the options available to the public authorities for an integrated vector control approach. Practical integration of these options into the range of vector control tools currently used, depending on the specific contexts in the different French territories, would call for additional knowledge to complement the expertise of HCB.

The techniques developed for mosquito vector control can also be considered for other pathogen vectors responsible for diseases with a significant impact on human and animal health. For plant health, control of disease vectors or crop pests could also benefit from similar approaches.

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Appendix 1: Referral

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

MINISTÈRE DE L'ÉCOLOGIE, DU DÉVELOPPEMENT DURABLE
ET DE L'ÉNERGIE

La ministre

Paris, le 12 OCT. 2015

Madame la Présidente,

La récurrence des épidémies de maladies vectorielles transmises par les moustiques (dengue, chikungunya,...) est établie, principalement dans les DOM. Le moustique « tigre », vecteur du chikungunya, est par ailleurs désormais implanté en métropole.

La lutte anti-vectorielle appelle une combinaison d'actions de toutes natures, dont la mise en place de bonnes pratiques par les citoyens et des stratégies de destruction des larves et adultes résiduels.

La destruction des larves et des adultes s'appuie principalement sur des substances chimiques insecticides.

La palette de molécules disponibles, efficaces et ne présentant pas de danger sanitaire important pour les populations, est néanmoins réduite. Seule une molécule « larvicide » et une molécule « adulticide » sont autorisées en France, même si quelques autres molécules autorisées dans d'autres pays d'Europe sont en cours d'examen par l'Anses.

Le développement de résistances à la molécule adulticide a été d'ores et déjà constaté dans certaines régions. Le malathion a été utilisé ces derniers mois en Guyane, mais son usage a cessé suite à son classement par le CIRC (centre international de la recherche sur le cancer, dépendant de l'Organisation mondiale de la santé OMS) comme « cancérogène probable » en mars 2015.

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Dans ce contexte, le Gouvernement doit examiner avec rigueur toutes les options à sa disposition. L'une d'entre elles consiste à introduire des populations de moustiques disposant d'un patrimoine génétique modifié par rapport à celui des populations de moustiques Aedes aegypti présents sur notre territoire et principaux vecteurs de la dengue et de la fièvre jaune.

L'introduction de moustiques au patrimoine génétique modifié vise à réduire la survivance de la descendance des adultes vecteurs de maladies. Elle est testée dans différentes régions du globe.

Ainsi, il existe par exemple des moustiques génétiquement modifiés tels que ceux développés par la société Oxytec. Le génome de ces moustiques est modifié pour y insérer un gène qui sera transmis à la descendance et stoppera le développement larvaire, entrainant ainsi la diminution des populations de moustiques. La société a procédé à des essais sur le terrain au Brésil, au Panama, aux îles Caïman et en Malaisie. Elle indique disposer de résultats probants assurant une réduction de plus de 90% des populations. Toutefois certaines associations de protection de l'environnement se sont montrées critiques considérant que la technologie ne serait efficace qu'avec des nombres de lâchers de moustiques trop importants, et donc peu réalistes. Le Gouvernement de Malaisie aurait d'ailleurs abandonné l'idée de recourir à cette technologie après des essais menés en 2010, la jugeant peu efficace et trop coûteuse. Par ailleurs, la stratégie même de suppression de population est sujette à critique dans la mesure où elle pourrait entrainer le développement d'autres familles de moustiques qui pourraient également être porteuses d'agents pathogènes.

Une autre stratégie, développée par la Fondation Oswaldo Cruz basée à Rio de Janeiro, consiste à "immuniser" les populations de moustiques Aedes Aegypti en les infectant artificiellement par une bactérie Wolbachia les rendant réfractaires au virus de la dengue. Des expérimentations actuellement en cours en Australie, au Vietnam, en Indonésie et au Brésil laisseraient entrevoir des premiers résultats prometteurs. Si cette technique a l'avantage de ne pas laisser de niche écologique vacante, en remplaçant les populations vectrices de pathogènes par des populations non vectrices, le risque pourrait être que ces populations deviennent plus compétentes pour transmettre d'autres agents pathogènes.

Dans ce contexte, je souhaite disposer de votre éclairage concernant l'utilisation de moustiques génétiquement modifiés dans le cadre de la stratégie de lutte anti vectorielle.

Le Haut Conseil des Biotechnologies établira un état des lieux de la recherche et de la commercialisation de ces insectes génétiquement modifiés ainsi que des techniques de production de ces insectes génétiquement modifiés et leurs spécificités par rapport aux techniques déjà utilisées.

Le Haut Conseil des Biotechnologies précisera quels sont les critères applicables pour l'évaluation sanitaire et environnementale de ces insectes au niveau international, européen et national (y compris DROM-COM).

Enfin, le Haut Conseil des Biotechnologies déterminera les résultats des premières utilisations et expérimentations menées dans le monde et indiquera quels pourraient être les bénéfices et les risques de l'utilisation de ces insectes génétiquement modifiés pour la France, y compris les DROM-COM, notamment d'un point de vue socio-économique et éthique.

Je souhaite que vous puissiez nous faire part de vos résultats pour le mois de mars 2016.

Je vous prie d'agréer, Madame la Présidente, l'expression de mes salutations les meilleures.

Ségolène ROYAL

Appendix 2: HCB Scientific Committee and preparation of the opinion

The HCB Scientific Committee is a multidisciplinary committee consisting of scientific figures appointed for their expertise in relation to HCB missions.

The Scientific Committee's composition is shown below in alphabetical order of surname. Since it changed during HCB's work, it is given for the whole period covered by preparation of this opinion:

- Composition further to the decree of 30 December 2014 appointing HCB members and to the law of 2 December 2015:

  Claude Bagnis, Avner Bar-Hen, Marie-Anne Barny, Philippe Berny, Yves Bertheau (resigned with effect from 3 February 2016), Pascal Boireau, Thierry Brévault, Bruno Chauvel, Denis Couvet, Elie Dassa, Nathalie Eychenne, Claudine Franche, Philippe Guerche, Joël Guillemain, Guillermina Hernandez-Raquet, André Jestin, Bernard Klonjkowski, Marc Lavielle, Valérie Le Corre, Olivier Lemaire, Didier Lereclus, Rémi Maximilien, Eliane Meurs, Nadia Naffakh, Didier Nègre, Jean-Louis Noyer, Sergio Ochatt, Jean-Christophe Pagès, Daniel Parzy, Catherine Regnault-Roger, Michel Renard, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Hubert de Verneuil, Jean-Luc Vilotte.

- Composition further to the order of 10 April 2017 appointing HCB members, published on 28 April 2017:

  Frédérique Angevin, Claude Bagnis, Avner Bar-Hen, Marie-Anne Barny, Pascal Boireau, Thierry Brévault, Bruno Chauvel, Cécile Collonnier, Denis Couvet, Elie Dassa, Barbara Demeinex, Claudine Franche, Philippe Guerche, Joël Guillemain, Guillermina Hernandez-Raquet, Jamal Khalife, Bernard Klonjkowski, Marc Lavielle, Valérie Le Corre, François Lefèvre, Olivier Lemaire, Didier Lereclus, Rémi Maximilien, Eliane Meurs, Nadia Naffakh, Didier Nègre, Jean-Louis Noyer, Sergio Ochatt, Jean-Christophe Pagès, Xavier Raynaud, Catherine Regnault-Roger, Michel Renard, Tristan Renault, Patrick Saindrenan, Pascal Simonet, Marie-Bérengère Troadec, Bernard Vaissière, Hubert de Verneuil, Jean-Luc Vilotte.

All members of the HCB Scientific Committee complete a public declaration of interests every year. They are also asked to disclose any interests prior to consideration of each referral. No members of the Scientific Committee declared conflicts of interest that might have affected preparation of this opinion.

This opinion was prepared by the HCB Scientific Committee on the basis of the report by the expert working group (see Appendix 3) under the chairmanship of Dr Jean-Christophe Pagès, with Dr Pascal Boireau and Dr Claudine Franche as vice-chairs and scientific coordination by Dr Catherine Golstein.

The working group's work and the report in progress were discussed by the Scientific Committee at meetings on 24 March, 28 April and 22 June 2016. Briefer updates on the report's progress were provided at meetings on 13 July and 27 September. The report was presented at the meetings of 27 October and 15 December prior to finalisation and was discussed on 26 January 2017 with a view to preparing a Scientific Committee opinion. The draft Scientific Committee opinion was discussed at the meetings of 25 February, 28 March, 27 April and 24 May 2017. The finalised opinion was adopted electronically on 31 May 2017.

The opinion was translated into English by Sarah Brickwood in consultation with Catherine Golstein. The English translation was completed on 28 February 2018.

Appendix 3: Scientific Committee working group and preparation of the report

The Scientific Committee working group consisted mainly of experts from outside HCB, selected for their expertise in the subjects considered necessary to address the referral. Additional experts were called upon to supplement this expertise. The working group included the head of the National Centre for Vector Expertise (CNEV). The CNEV is a multidisciplinary body able to marshal the whole range of French skills and expertise quickly and effectively for decision support in the fields of medical and veterinary entomology, vector control, and the social sciences applied to vector control. HCB and the CNEV agreed to place the Scientific Committee working group under the auspices of both institutions.

Working group composition

Catherine Bourgouin, Institut Pasteur, Paris, Unité Génétique Fonctionnelle des Maladies Infectieuses (Functional Genetics of Infectious Diseases Unit), CNRS URA 3012

Jérémy Bouyer, CIRAD, UMR CIRAD-INRA Contrôle des Maladies Animales Exotiques et Emergentes (CIRAD-INRA Joint Research Unit on Control of Exotic and Emergent Animal Diseases), Head of Vector Team, Director of Research, NICETT & African Union, Ethiopia

Fabrice Chandre, CNEV and IRD, Director, CNEV, Infectious Diseases and Vectors: Ecology, Genetics, Evolution and Control (MIVEGEC), UM-CNRS 5290-IRD 224

Jérémie Gilles, FAO-IAEA, Head of Mosquito Unit at FAO-IAEA Insect Pest Control Laboratory in Vienna

Christophe Lagneau, EID Méditerranée (Entente interdépartementale pour la démoustication du littoral méditerranéen), Research and Development Director

Eric Marois, University of Strasbourg, IBMC, Réponse Immunitaire et Développement chez les Insectes (RIDI) (Immune Response and Development in Insects)

Mylène Weill, University of Montpellier 2, CNRS, CNRS Research Director, Institut des Sciences de l'Evolution de Montpellier (ISEM) (Montpellier Institute of Evolutionary Sciences)

Co-rapporteurs:

Pascal Boireau, Vice-Chair of HCB Scientific Committee, Director of Anses Animal Health Laboratory,

Jean-Christophe Pagès, Chair of HCB Scientific Committee, Director of Research and Innovation, French Blood Agency (EFS)

Scientific coordinator: Catherine Golstein, Senior Scientific Officer, HCB.

The working group called on the following additional experts for the inception seminar and to supplement its expertise as new issues required:

Pierre Gallian, French Blood Agency (EFS), Advisor, Biological Qualification of Donations & Emerging Viruses

Marianne Maquart, National Reference Centre for Arboviruses (CNR Arbovirus), French Armed Forces Biomedical Research Institute (IRBA), Assistant Supervisor

Bernard Cazelles, Ecole Normale Supérieure (ENS), MR 7625 CNRS-UPMC-ENS, Eco-Evolutionary Mathematics Team

Claudio Lazzari, University of Tours, Insect Biology Research Institute (IRBI), Professor in the Genetics and Animal Biology Department

Marie-Claire Paty, from the French Institute for Public Health Surveillance (InVS), was also consulted.

Each member of the working group completed a public declaration of interests and a confidentiality undertaking.

Preparation of the report

The Scientific Committee working group met on 2, 3 and 4 March 2016. The first day took the form of a seminar open to members of both HCB committees and the ad hoc working group of the Economic, Ethical and Social Committee. With a programme consisting mainly of contributions from experts from the Scientific Committee working group, supplemented by two contributions on the referral's socio-economic aspects, the inception seminar had a threefold objective: (1) To provide background to vector-borne epidemics and introduce vector control issues; (2) To review the current state of different vector control strategies, their specific features and possible limitations; (3) To highlight the questions and topics that should be addressed by each committee's working group.

Evidence from representatives of the British firm Oxitec (which recently became a subsidiary of the American biotechnology company Intrexon) was taken jointly on 3 March 2016 by both committee working groups.[87] The object of the hearing was to consider research and development work, risk assessments for applications for placing on the market or for field trials of GM mosquitoes, and feedback from the various trials worldwide by the first and so far only company to market GM mosquitoes for vector control.

The Scientific Committee working group completed preliminary work for its report on 3 and 4 March 2016 by giving thought to the various relevant criteria for assessing different vector control techniques and the more specific question of risk assessment for GM mosquitoes, approached in terms of Directive 2001/18/EC. An initial cross-cutting assessment of the different techniques was drafted, followed by a period of exchange of views and information, designed to answer questions from members of the Economic, Ethical and Social Committee working group.

The WG report was prepared, discussed and finalised electronically by the working group members. It also benefited, through its coordinator, from interaction with the Scientific Committee. The report is available on the HCB website.[88]

[87] Evidence was taken from Dr Hadyn Parry, Chief Executive Officer, and Dr Andrew McKemey, Head of Field Operations. Dr Camilla Beech, Head of Regulatory Affairs, was unable to attend these hearings at the last minute.

[88] http://www.hautconseildesbiotechnologies.fr/sites/www.hautconseildesbiotechnologies.fr/files/file_fields/2017/05/31/rapportgtcshcbcnevadopte170523.pdf (in French).