National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

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D.C.M. Glandorf

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Colophon

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The technical evaluation has been performed by order and for the account of Council of Saba.

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Synopsis

Technical evaluation of a potential release of OX513A *Aedes aegypti* mosquitoes on the island of Saba

The mosquito *Aedes aegypti* transmits viruses that cause diseases such as dengue, chikungunya and zika. Measures are taken to control the mosquito since these infectious diseases represent a significant health problem. This is the case on the island of Saba, a Dutch Caribbean island. In order to fight these diseases a British company has genetically modified the mosquito in such a way that it can suppress local mosquito populations. The modification causes the mosquitoes' offspring to die prematurely. The potential release of these mosquitoes on Saba is considered to result in negligible risks for human health and the environment.

This is the outcome of a technical evaluation of the potential release of these genetically modified mosquitoes. RIVM's GMO (Genetically Modified Organisms) Office was commissioned by the Executive Council of Saba to perform this evaluation.

Among others, this evaluation looked into effects on the food chain to determine whether an important food source would disappear if the local mosquito population were to be eliminated. It was also considered whether it is unhealthy if people accidentally swallow a genetically modified mosquito. Another element of the evaluation was whether the genetic modification would increase the efficiency of the mosquito to spread diseases.

An evaluation of the efficacy of application of the genetically modified mosquitoes was not part of this technical evaluation. The same applies to socio-economic effects or the desirability of using these mosquitoes.

Keywords: genetically modified mosquitoes, Saba, GMO, risk assessment, environment, infectious diseases, *Aedes aegypti*

Publiekssamenvatting

Technische evaluatie van een mogelijke inzet van genetisch gemodificeerde muggen op Saba

De mug *Aedes aegypti* brengt virussen over die meerdere ziekten kunnen veroorzaken, zoals dengue, chikungunya en zika. De mug wordt bestreden omdat deze infectieziekten een groot gezondheidsprobleem vormen. Dit is het geval op het eiland Saba, dat onderdeel is van Caribisch Nederland. Om de ziekten te bestrijden heeft een Brits bedrijf met behulp van genetische modificatie de mug zodanig aangepast dat lokale muggenpopulaties teruggedrongen kunnen worden. Door de modificatie sterft het nageslacht vroegtijdig. Deze toepassing blijkt op Saba verwaarloosbaar kleine risico's voor mens en milieu met zich mee te brengen.

Dit blijkt uit een technische evaluatie van de mogelijke inzet van deze genetisch gemodificeerde muggen. Het Bureau GGO (Genetisch Gemodificeerde Organismen) van het RIVM heeft deze evaluatie in opdracht van het bestuur van Saba uitgevoerd.

Bij deze beoordeling is onder andere naar de voedselketen gekeken: verdwijnt er niet een belangrijke voedselbron wanneer de lokale muggenpopulatie wegvalt? Ook is onderzocht of het ongezond is als mensen per ongeluk een genetisch gemodificeerde mug inslikken. Een ander beoordelingspunt is of de mug door de genetische modificatie niet juist beter in staat wordt om ziekten over te brengen.

Evaluatie van de effectiviteit van de inzet van de genetisch gemodificeerde muggen was geen onderdeel van deze technische evaluatie. Hetzelfde geldt voor sociaaleconomische effecten of de wenselijkheid om deze muggen in te zetten.

Kernwoorden: genetisch gemodificeerde muggen, Saba, ggo, risicobeoordeling, milieu, infectieziekten, *Aedes aegypti*

Contents

Executive summary

Request for technical evaluation
The GMO Office of the National Institute of Public Health and the Environment (RIVM) is requested by the Council of Saba to provide a technical evaluation of the environmental risk assessment (ERA) for the release of *Aedes aegypti* on Saba submitted by Oxitec Ltd. The evaluation focuses on the consequences of a potential release of genetically modified (GM) *Aedes aegypti* (strain OX513A) mosquitoes on the island of Saba. The aim of the release is to suppress the local *Aedes aegypti* population, which is a known vector for human diseases such as dengue fever, chikungunya and zika. *Aedes aegypti* is considered an invasive species in several jurisdictions and is subject to vector control on Saba.
This technical evaluation considers potential adverse effects on human health and the environment of Saba as a consequence of the release of OX513A on Saba, in case this release may take place in the future. Potential adverse effects are compared to those of the non-modified *Aedes aegypti* and are considered in the context of local vector control measures. This evaluation does not consider the efficacy of the release of OX513A *Aedes aegypti* in Saba in suppressing local populations of wild type *Aedes aegypti* and the diseases they transmit. Socio-economic aspects are also not part of the scope of this evaluation.

Proposed release
The proposed activities involve the repeated release of male (non-biting) OX513A *Aedes aegypti* mosquitoes in the inhabited parts of the island of Saba. This strain has been used for releases in the Cayman Islands, Panama and Brazil. The OX513A mosquitoes are genetically modified so that their offspring die before adulthood, due to the introduction of a repressible lethal genetic system. When these male OX513A *Aedes aegypti* mosquitoes mate with wild females in the field, offspring is produced of which over 95% express the lethality trait. This trait causes mortality in larval stages in the absence of tetracycline that is not present in the environment. OX513A also contains the fluorescent marker gene *DsRed2* in order to visualize and monitor this strain after its release. The proposed release is foreseen to take place in all inhabited parts of the island of Saba and aims to suppress the local population of *Aedes aegypti* to very low levels and potentially to elimination with demonstration of continued elimination for a period of a year thereafter. Monitoring of the local *Aedes aegypti* populations is part of the proposed activities. Earlier releases of OX513A mosquitoes in the Cayman Islands, Panama and Brazil have demonstrated a clear reduction in *Aedes aegypti* mosquito populations.

Environmental risk assessment
The environmental risk assessment is performed according to Annex II of European Directive 2001/18/EC (EU, 2001)[1]. The Guidance document on the Environmental Risk Assessment (ERA) of GM insects of the European Food Safety Authority (EFSA, 2013)[2] is used to evaluate potential risks for human health and the environment. Where relevant,

also other, guidance documents with respect to GM insects are considered.
This EFSA Guidance describes seven 'areas of concern' that are to be taken into account in the ERA of GM insects. For each of these areas of concern it is considered whether the release of OX513A in Saba could lead to adverse effects on human health and the environment, in comparison to effects of the non-modified *Aedes aegypti*. Effects are considered in the context of current vector control program.

These areas of concern are the following:

1. Persistence and invasiveness of GM insects, including vertical gene transfer
2. Horizontal gene transfer
3. Pathogens, infections and diseases
4. Interactions of GM insects with target organisms
5. Interactions of GM insects with non-target organisms
6. Environmental impact of the specific techniques used for the management of GM insects
7. Impacts of GM insects on human and animal health

The molecular characterization of OX513A together with its phenotypic and behavioral characteristics are considered to establish whether there are any intended or any unintended changes in OX513A, as a result of the genetic modification, that could lead to a potential adverse effect on human health and the environment. The result of this comparative safety assessment demonstrates that the only differences of biological relevance are the expression of the two introduced traits; the self-limiting trait (tTAV) and the fluorescent marker trait (DsRed2). Therefore the ERA focuses on potential adverse effects of these new traits in OX513A, in comparison to effects of the non-modified *Aedes aegypti.*

For all areas of concern (EFSA, 2013)[2] there is sufficient information to conclude that adverse effects on human health and the environment are unlikely. Adequate measures and controls are taken to prevent unintended environmental release of OX513A during rearing of OX513A *Aedes aegypti* before their release. Moreover, in all environmental releases to date OX513A has performed consistently with respect to parameters identified in risk assessments and no unanticipated results or unintended effects have been observed.

The GMO Office concludes that potential adverse effects on human health and the environment as a consequence of the potential release of genetically modified OX513A on the island of Saba, under the conditions as described in the documentation of Oxitec and in the context of standard vector control, are considered to be negligible as compared to effects of non-modified *Aedes aegypti*. This is in line with recent related environmental risk assessments such as from Brazil [3,4] and the United States Food and Drug Administration [5].
The GMO Office recommends post-release monitoring by an independent party, as advised by the WHO[6], on a monthly basis until populations of OX513A are below the level of detection.

Introduction

Request from Saba
The GMO Office of the National Institute of Public Health and the Environment (RIVM) in the Netherlands was requested by the Council of Saba to evaluate documentation from Oxitec Ltd for a planned release on Saba with genetically modified (GM) male OX513A *Aedes aegypti* mosquitoes. *Aedes aegypti* is a known vector for human diseases such as dengue fever, chikungunya and zika.
This request of Saba for a technical evaluation was received by the GMO Office on February 29 (2016). On April 28 (2016) the GMO Office requested from Oxitec all documentation with respect to the potential release on Saba, including raw data and an environmental risk assessment of the planned release. These documents of Oxitec were received on September 28 (2016). Additional information was requested by the GMO Office on October 24 (2016) and January 19 (2017) and this information was received on November 30 (2016), January 11 (2017) and March 13 (2017), respectively. All documents and additional data were evaluated by experts from the RIVM, Wageningen University & Research and Saba. The resulting technical evaluation was thereafter reviewed by other experts of the RIVM, Wageningen University & Research and by international experts from Belgium and the United Kingdom.

Scope of the environmental risk assessment (ERA)
This technical evaluation concerns the assessment of potential risks for human health and the environment of Saba as a consequence of the release of OX513A *Aedes aegypti*, in case this release may take place in the future. The main question therefore focusses on the potential adverse effects on the flora and fauna of Saba and on the health of human inhabitants of Saba, in comparison to effects caused by non-modified wild type *Aedes aegypti.* These effects are considered in the context of the local vector control program of *Aedes aegypti* on Saba. Potential effects of the release on the wider environment of Saba are also taken into account in this environmental risk assessment.
This evaluation does not consider the efficacy of the release of OX513A on Saba in suppressing local populations of wild type *Aedes aegypti* and the diseases they transmit, nor does this evaluation takes into account socio-economic aspects of this potential release.

Documentation sent to Saba and evaluation by the GMO Office
The documentation of Oxitec follows Annex III of the Cartagena Protocol on the Biosafety to the Convention on Biological Diversity (CBD, 2000)[7]. The evaluation by the GMO Office (RIVM) is based on the data and information included in Oxitec's documentation, but also takes into account other relevant data such as scientific literature and published risk assessments of similar releases with the OX513A *Aedes aegypti* in other countries. The evaluation of the GMO Office as reflected in this document follows the structure as given in Oxitec's documentation and conclusions of the GMO Office are indicated in bold.

The Guidance document on the environmental risk assessment) of GM insects of the European Food Safety Authority (EFSA, 2013)[2] was used to evaluate the documents of Oxitec. Other available guidance on the ERA of GM insects such as the Guidance on risk assessment of living modified organisms under the Cartagena Protocol (Part II on living modified mosquitoes) (CBD, 2000)[7], the WHO guidance framework for testing of genetically modified mosquitoes (WHO, 2014)[8] were also taken into account when relevant.
The 'areas of concern' as described in the EFSA Guidance for the ERA of GM insects were used to structure the ERA. These 'areas of concern' cover elements that are generally taken into account in the ERA of GMOs in most countries to assess potential adverse effects on human health and the environment of GMOs as a consequence of the genetic modification on a case-by-case basis.

The areas of concern for GM insects are:

- Persistence and invasiveness of GM insects, including vertical gene transfer
- Horizontal gene transfer
- Pathogens, infections and diseases
- Interactions of GM insects with target organisms
- Interactions of GM insects with non-target organisms
- Environmental impact of the specific techniques used for the management of GM insects
- Impacts of GM insects on human and animal health

Evaluation of Oxitec's documentation and review of this evaluation was carried out by experts of the RIVM, Wageningen University and Research in the Netherlands, the Biosafety and Biotechnology Unit (SBB), WIV-ISP in Belgium, Department for Environment, Food and Rural Affairs (DEFRA) in United Kingdom and the Saba Conservation Foundation on Saba (Annex 1).

Planned release
The planned release involves the repeated release of male (non-biting) *Aedes aegypti* mosquitoes (strain OX513A). This strain has been studied for over 12 years. Male OX513A mosquitoes mate with the wild type females of their own species only, leading to a reduction of the local population of *Aedes aegypti*. The mosquitoes are genetically modified so that their offspring dies before adulthood (as fourth larval instar or pupae). This is caused by the introduction of a repressible lethal genetic system based on the widely used tetracycline transcriptional activator (tTAV) into *Aedes aegypti*, which results in lethality of the offspring of OX513A males and wild type females in the absence of tetracycline (such as in the environment). When GM male *Aedes aegypti* mosquitoes mate with wild females in the field, offspring is produced of which over 95% express the lethality trait in the absence of tetracycline. The GM *Aedes aegypti* also contains a fluorescent marker gene, DsRed2, so that they can be identified and monitored.
The proposed repeated release is foreseen to take place in all inhabited parts of the island of Saba and aims to suppress the local population of *Aedes aegypti* to very low levels and potentially to elimination with demonstration of continued elimination for a period of a year thereafter. To demonstrate continued elimination, monitoring of the local population

of *Aedes aegypti* will take place until a year after the release has taken place. Earlier releases of OX513A mosquitoes have resulted in a clear reduction of local *Aedes aegypti* mosquito populations during the program. No other monitoring is foreseen.

Specific aspects of the environmental risk assessment (see Figure 1)

(1) Only male (non-biting) OX513A *Aedes aegypti* will be released in the environment. Male pupae are selected by sorting. However, sorting of the pupae results in ≤0.2% of the pupae to be female. Therefore, the ERA takes into account that 0.2% of the released OX513A population is female.

(2) Trait penetrance (probability of a gene or genetic trait to be expressed) is 95%. After mating of OX513A with wild type *Aedes aegypti* the majority (95%) of the offspring will express the lethality trait and will die. About 5% of the offspring (male and female) will survive, also in the absence of tetracycline. This aspect is taken into account in the ERA.

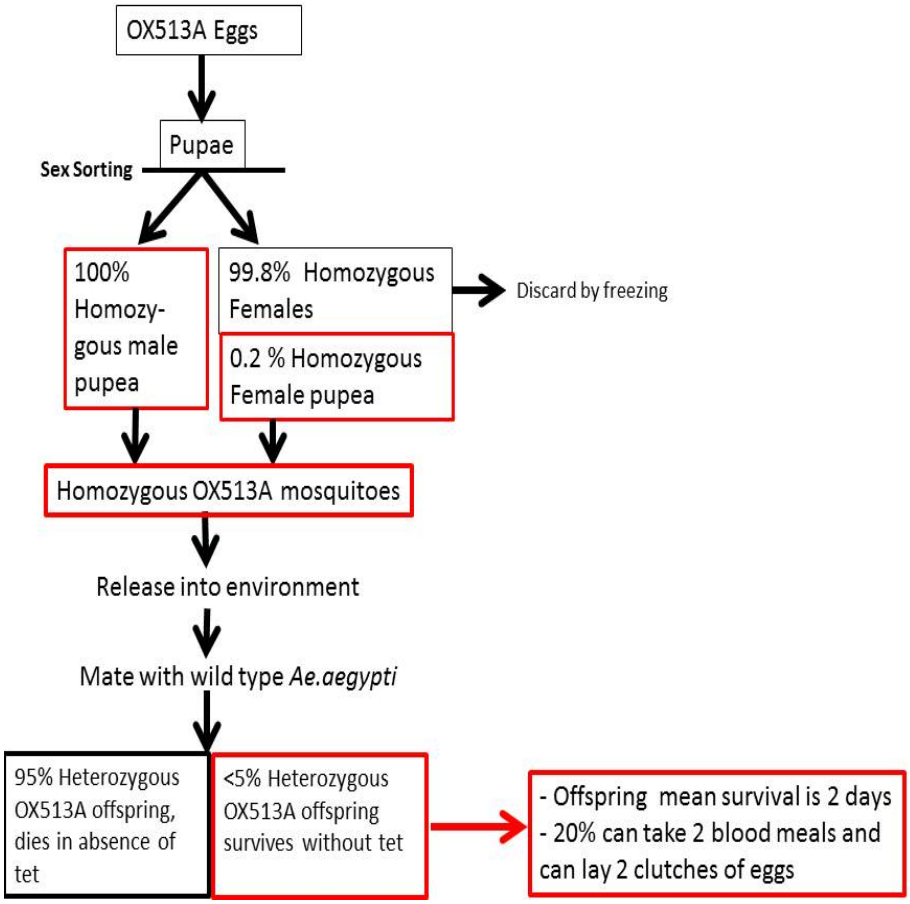

Figure 1. Schematic representation of the process of sorting, environmental release and survival of male and female OX513A Aedes aegypti (drafted by the GMO Office).

Technical evaluation of Oxitec's documentation

This technical evaluation summarizes relevant parts of Oxitec's documentation and follows the same numbering is used in this documentation. Conclusions of the GMO Office are indicated in blue boxes. For further details, reports and references, the reader is referred to the full document and the additional information, as supplied by Oxitec, which can be accessed through the links below.

Submission to RIVM, September 28, 2016
http://www.rivm.nl/bibliotheek/rapporten/2017-0087_Submission
Additional information November 30, 2016
http://www.rivm.nl/bibliotheek/rapporten/2017-0087_AI1
Additional information January 11, 2017
http://www.rivm.nl/bibliotheek/rapporten/2017-0087_AI2
Additional information January 11, 2017
http://www.rivm.nl/bibliotheek/rapporten/2017-0087_AI3
Additional information March 13, 2017
http://www.rivm.nl/bibliotheek/rapporten/2017-0087_AI4

Part A Characterisation of OX513A

Molecular characterization

1 Recipient organism *Aedes aegypti*

The recipient organism is the *Aedes* (*Stegomyia*) *aegypt*i (L.) mosquito that belongs to the genus Diptera: Family Culicidae, Genus Aedes.
Aedes aegypti is a (sub)tropical species of mosquito found between 15°N and 15°S, typically in Africa and parts of South America but has not been reported to have a cosmopolitan habitat extending from 40°N and 40°S latitude. It is considered as an invasive species in several jurisdictions.
Aedes aegypti originated from Africa and now has a worldwide distribution in all tropical and subtropical habitats.

Aedes aegypti is closely associated with human habitations. Breeding is tied to artificial water containers, such as potted plant holders, water tanks, tires, discarded plastic and metal containers such as soda cans, drains and roof guttering as well as ephemeral containers, such as puddles. Once eclosed, the adult *Aedes aegypti* mosquitoes live in and around houses where females have easy access to the blood meals necessary for egg development. The mosquito eggs are laid individually by females in the damp walls of both natural and artificial containers that can hold water. Eggs are the long-term survival structures of these mosquitoes, surviving, on average, up to 6 months. The larvae and pupae prefer relatively clean water typically found in containers such as water storage containers, flowerpots and waste materials such as tires, cans and bottles. In tropical countries waste material containers are year-round sources of mosquitoes. The duration of the larval stages is approximately 7-9 days and of pupae 2-3 days but these developmental rates are temperature dependent. The preferred sites for adults are domestic urban environments in sheltered dark spaces within houses/ apartments.

Aedes aegypti is a day biting mosquito with two peaks, one mid-morning and one mid-afternoon. *Aedes aegypti* bites humans and birds. The average adult lifespan for mosquitoes in nature (outside of the laboratory) is estimated to be 8-15 days.
Spontaneous flight of adults is limited to around 200m depending on availability of breeding sites, and hosts from which to take a blood meal. However the species can be dispersed by passive transport on boats, trains and modes of long distance transport. International Sanitary Regulations require ports and airports to be clear of *Aedes aegypti* up to 400m from the site.
Climate and the availability of breeding sites are the two main factors that regulate the populations of *Aedes aegypti* in urban environments. The effect of temperature on larval development of *Aedes aegypti* has been well studied, and has an ecological temperature range of 14-30°C, at which the larval development is a function of temperature. Temperature also affects adult size, dry weight and ovariole number all of which fall as the temperature rises. Temperature at different altitudes

is thought to affect distribution, with an elevation of 1800-2400 m likely to be limiting to the species and lower levels in temperate latitudes.

2 Donor organisms and general description of inserted genetic elements

The genetic elements in the recombinant DNA construct inserted in OX513A and their function, along with the origin of the DNA sequence are detailed in Table 1 of the documentation. Further information on each of the elements and the organisms where DNA sequences originated from is given below:

2.1 *Trichoplusia ni (Cabbage looper moth)*

The transposable element piggyBac, used as a carrier to introduce the intended genes into *Aedes aegypti*, was isolated from a cell culture of cabbage looper (*Trichoplusia ni).* Cabbage looper is a pest that feeds on the leaves of cruciferous plants but does not have any known toxic or pathogenic properties.
The piggyBac transposon is non-autonomous transposon that has been well studied and used to transform insects from a range of taxa: Diptera, Lepidoptera, Coleoptera and Hymenoptera.

2.2 *Drosophila melanogaster (Vinegar fly)*

Several regulatory elements of the inserted DNA are derived from *Drosophila melanogaster. D. melanogaster* is not known to have toxic or allergenic properties*.* Due to their short generation time they make excellent model organisms for developmental biology and other disciplines and have been well studied in laboratories for over a century.

2.3 *Discosoma spp.*

The *DsRed* marker gene is derived from *Discosoma spp. Discosoma* species are also known by their common name of mushroom corals and are found throughout many marine environments. *Discosoma spp* have particular fluorescence proteins that are similar to the green fluorescent protein (GFP) family of proteins. A mutation of DsRed enabled the generation of a close variant, DsRed2, which has improved expression and solubility, assisting its use as a marker. The fluorescent DsRed2 has been used extensively as a marker in a wide variety of organisms from viruses, to fungal species and mammals.

2.4 *Escherichia coli**

The *tTAV* gene (coding for lethality) and the non-coding binding site for the tTAV protein are derived from *Escherichia coli.* This is an intensively studied bacterium which serves as a model organism across a range of disciplines. The *E. coli* strains used in the generation of the tetracycline-repressible system are all laboratory strains that are non-pathogenic.

2.5 *Herpes simplex virus type 1**

VP16, used as a transcriptional activator of the *tTAV* gene, is derived from Herpes simplex virus type 1. Herpes simplex virus type 1 (HSV-1) is a human virus usually associated with infections of the lips, mouth,

** In OX513A VP16 is used in a fusion protein with domains from E. coli and known as tTAV. Activating regions derived from the HSV-1 have been coupled to control elements derived from E. coli in order to develop the conditional lethal tetracycline-repressible transactivator element, tTA, widely used as the tet-repressible control system.*

and face. It is the most common herpes simplex virus and many people develop it in childhood. VP16 is a virion phosphoprotein of HSV and a transcriptional activator of viral immediate-early (IE) genes and requires an acidic transcriptional domain. If absent, VP16 is impaired in its capacity to support the infectious cycle.

2.6 Small synthetic linking sequences

Synthetic linking sequences are used to connect genetic elements within the construct. They do not have any function.

3 Plasmid used in the transformation of OX513A

The plasmid used is pOX513, containing the piggyBac transposable element. This transposable element is only capable of integrating into DNA flanked by an open reading frame (ORF) within the element when its inverted terminal repeats (ITRs) are intact. In the construct used for transformation the transposase gene of the piggyBac element was irreversibly destroyed by deletion of a section of that gene. Transformation is done by using a helper plasmid that supplies the piggyBac transposase activity but that is in itself unable to transpose into other DNA. One of the ITR's that flank the wild type piggyBac transposase has been removed in the helper plasmid so that the helper plasmid itself cannot integrate.

Transformation of *Aedes aegypti* to obtain OX513A was achieved through microinjection of individual eggs of the Rockefeller background strain. The microinjection consisted of the vector plasmid, pOX513 (Figure 2) co-injected with a piggyBac 'helper plasmid' as the source of piggyBac transposase. Once a stable transformed line of laboratory reared *Aedes aegypti* was identified it was made homozygous. The OX513A strain has been continuously maintained since 2002, representing over 115 generations.

After transformation the inserted DNA in strain OX513A was sequenced and proven to be identical to the sequence as present in plasmid pOX513A.

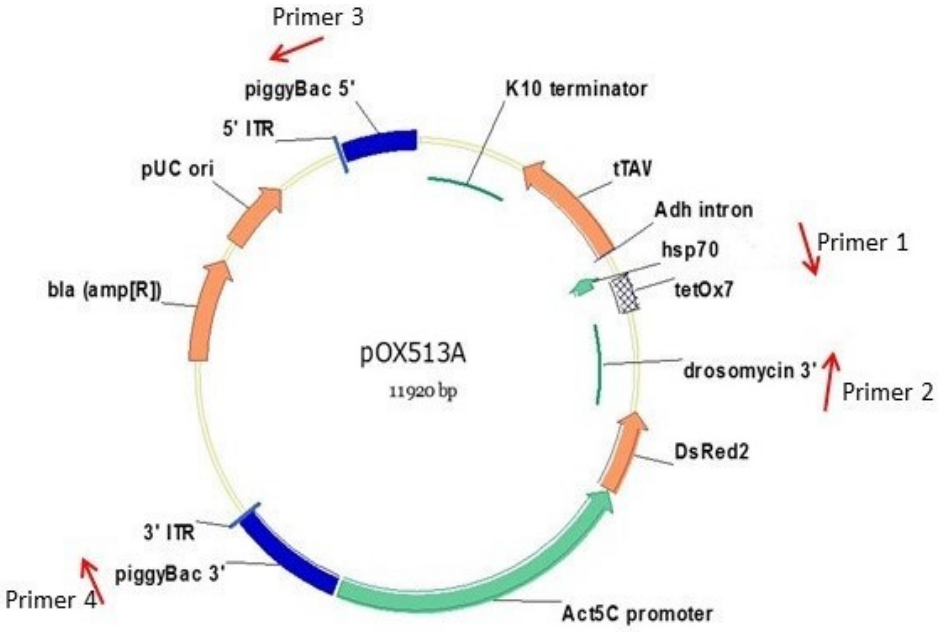

Figure 2. Map of the plasmid used in the transformation of OX513A. Primer locations are a schematic representation intended to represent the general regions of the plasmid amplified as described in Section 4.1 of part A.

4 rDNA insert and characteristics of modification

4.1 *Detecting the absence of plasmid backbone in transgenic lines*

To check the absence of the vector backbone (including the *bla* gene and the pUC origin of replication) primers 3 and 4 were used to amplify a fragment of 4045 bp that contained the complete vector backbone (Figure 2). No signal was found in the genomic DNA of OX513A, whereas a positive signal was found in the pOX513A plasmid used to construct the strain. Proper controls were used to verify that the genomic DNA of strain OX513A was of sufficient quality for PCR. In addition, analysis of the flanking sequences of the insert indicated the absence of the vector backbone.

The absence of the *bla* gene in strain OX513A was demonstrated by Southern analysis, using the *bla* gene as a probe (additional info March 13, 2017). No signal could be detected in the OX513A strain, whereas the *bla* gene was detected in the pOX513A plasmid DNA and DNA obtained from an *Aedes aegypti* strain known to contain the vector backbone. The *tTAV* gene was detected in all samples except for the wild type sample, demonstrating the presence and integrity of the DNA used in the assay. These results demonstrate the absence of the vector backbone in OX513A.

4.2 *Number of copies inserted and insert stability*

Three restriction enzymes (*Age*I, *Bgl*II, and *Sal*I), each cutting once in the insert present in strain OX513A were used to digest genomic DNA of OX513A. Southern analysis using AC5/*DsRed* and *tetR* as a probe, demonstated the presence of one single insert. Moreover, analysis of the ratio of numbers of fluorescent progeny to non-fluorescent progeny, confirmed the expected Mendelian ratios for a single insertion.

OX513A has been maintained in continuous culture in the laboratory since 2002 (>115 generational equivalents) with no observation of genetic or phenotypic instability.

4.3 *Verifying the insertion site and sequencing the regions flanking the gene*

Inverse PCR was used to identify the genomic sequence adjacent to the insertion site of OX513A. DNA flanking sequences of 307bp and 315bp on either side of the insertion site were obtained. The *Aedes aegypti* genome has been fully sequenced, assembled, and annotated with respect to known genes, ESTs and transcripts. The combined flanking sequence of 622bp was compared with the *Aedes aegypti* genome sequence, transcript and EST databases using the BLAST tool on the Vectorbase website (www.vectorbase.org). Both Blastn and Blastx functions were used to compare the sequence in both orientations at the nucleotide level (Blastn) and translated sequence level in all 6 reading frames, to deposited amino-acid sequences (Blastx). The flanking sequence shows 94.6% identity across its entire length with a single genome sequence contig (1.859), showing an unambiguous match with this contig in *Aedes aegypti* genome sequence.
No homology to known open reading frames was identified, so no genes appear to be disrupted in *Aedes aegypti* by the insertion. In addition, results indicate the nearest gene/EST hit to be 30.5kb away and it is therefore unlikely to be affected by the insertion.

4.4 *Nature of the inserted traits*

Aedes aegypti OX513A is biologically similar with respect to its life-history characteristics to the non-modified populations of *Aedes aegypti* except for the introduction of two traits.

4.4.1 Fluorescent marker DsRed2

The fluorescent marker protein (DsRed2) enables the detection of OX513A in the field, and allows evaluation of the dissemination of OX513A *Aedes aegypti* and its genes resulting from the release of OX513A males. The DsRed2 is from Clontech Laboratories and was artificially developed from DsRed to enhance the fluorescence and improve the solubility, which in turn increases the sensitivity. In OX513A, there are three additional amino acids (MAR) at the N-terminus, which are from a cloning linker sequence. The DsRed2 protein is expressed constitutively in the developmental stages (larva stage) of the OX513A mosquito and results in a fluorescent phenotype when viewed with diagnostic equipment (Figure 3).

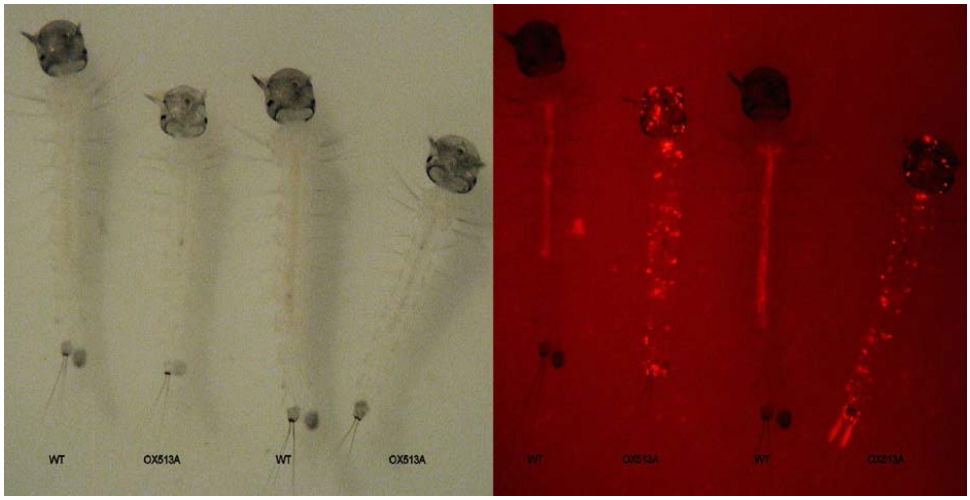

Figure 3. Expression of fluorescent marker (DsRed2) in OX513A Aedes aegypti under diagnostic fluorescence microscope. The fluorescent marker is strongly expressed in a characteristic punctate manner, allowing easy identification of OX513A individuals.

4.4.2 Self limiting trait tTAV

An insect-optimized tetracycline repressible transactivator protein (tTAV) is integrated into OX513A to produce a phenotype whereby the offspring after mating has increased mortality. tTAV acts as a tetracycline-regulated switch (Figure 4) which confers conditional cell death and thus enables the mass rearing of the mosquito in the laboratory when tetracycline is supplied.

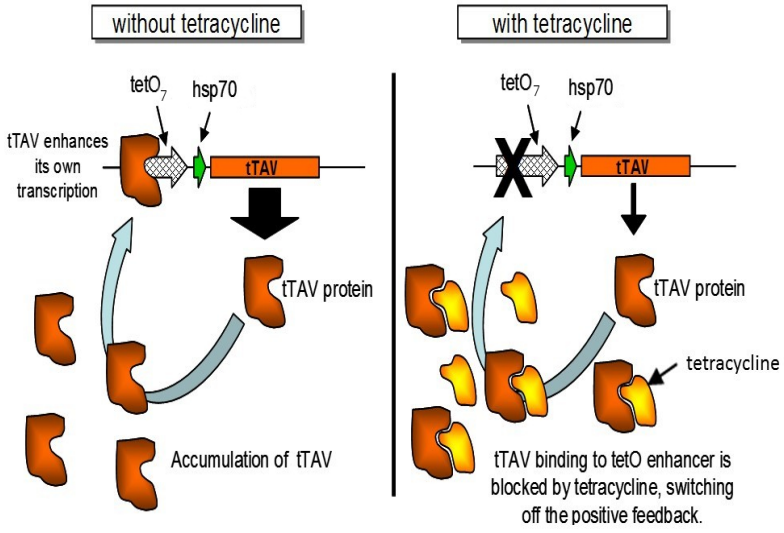

Figure 4. Schematic representation of the tTAV system. In the absence of tetracycline (left panel), small amounts of tTAV protein generated by the effect of the hsp70 promoter (hsp70) can bind to the tetO binding sites ($tetO_7$), creating a positive feedback loop that enhances expression of tTAV. When the tTAV protein accumulates in sufficient quantities it affects cellular function, resulting in cell death in the developing larvae. In the presence of tetracycline (right panel), tTAV is prevented from binding to the tetO sites and can therefore not enhance the expression from the hsp70 promoter. This prevents the accumulation of tTAV.

The self-limiting trait in OX513A works via a tTAV system (a variant of tTA) which elicits cell death. High-level expression of tTA is deleterious to cells as it can repress normal transcription. tTAV is a tTA variant sequence optimized for expression in *D. melanogaster* and other insects. tTA and its variants, such as tTAV, have been used in fungi, rodents, plants, and mammalian cultures.

4.5 Potential for toxicity and allergenicity of the introduced proteins

To assess whether the tTAV or DsRed2 protein produced by OX513A contain sequences that may be toxic or allergenic to humans or animals as a result of biting by genetically modified females or after (incidental) ingestion, bioinformatic analyses were conducted. Results do not indicate any significant homology of the tTAV and DsRed2 protein sequences to known toxins and allergens. In addition, potential toxicity and allergenicity of the inserted proteins tTAV and DsRed2 was studied based on reviews of the scientific literature and other relevant studies with these proteins. Additionally, a literature search was performed on additional elements of the OX513A rDNA construct. None of these studies indicated any significant risk for human and animal health resulting from the newly expressed proteins in OX513A.

4.6 Conclusions regarding the molecular characterization of the insert in OX513A

The GMO Office concludes that the molecular characterization of *Aedes aegypti* OX513A does not indicate any safety issues for human health, animal health and the environment.
This is based on the following observations:

- The sequence of the insert in OX513A is similar to the sequence in pOX513A, based on sequence similarity between the introduced plasmid and the insert in OX513A
- OX513A does not contain vector backbone sequences from the plasmid used for transformation, based on PCR and Southern analysis
- The insertion does not disrupt endogenous gene functions in OX513A as demonstrated by sequencing of the flanking regions and subsequent sequence comparison with *Aedes aegypti* genome sequence
- The insert consists of an intact single copy, as demonstrated by Southern analysis and Mendelian inheritance
- The inserted sequences has been shown to be stable over many generations
- No sequences have been inserted that code for toxins or allergens as evidenced by bioinformatic analyses and literature searches

5 Further characterisation of OX513A

5.1 Life table parameters

Several life table parameters have been examined in the laboratory for the OX513A strain in comparison with its non-modified *Aedes aegypti* comparator strain: larval mortality, developmental rate (i.e., time to pupation), adult size, and longevity. Larvae were grown in the presence of tetracycline. Only two statistically significant differences were found:

the OX513A larval survival was 5% lower than that of the non-modified strain and there was a reduced longevity of OX513A.

OX513A was also compared to laboratory reared *Aedes aegypti* of wild origin (wild caught) originating from two regions of India. They did not significantly differ with respect to the following parameters under laboratory conditions: blood meals per female; oviposition events per female; eggs laid per female; hatch rate; pupation rate and adult emergence. In these tests only developmental time from first instar to adult emergence was slightly longer for OX513A than for wild type.

5.1.1 Reproductive capacity

Several parameters regarding reproductive capacity have been measured for both the non-modified and the OX513A strain in two independent laboratory studies. These studies indicate that only minor life table differences of limited biological relevance exist between OX513A and wild type *Aedes aegypti* populations.

5.1.2 Insemination capacity

The insemination capacity of males (i.e., the number of females a male is capable of inseminating over the course of his lifetime) of a non-modified strain of Malaysian origin and OX513A was evaluated. Results show that OX513A males inseminated just over half as many females compared to the wild type males during their lifetime, indicating a slight fitness penalty in OX513A compared to the non-modified strain.

5.1.3 Mating competitiveness

Extensive testing of the OX513A strain mating competitiveness in a range of environments has been carried out. This includes studies in laboratory cages and in regulated environmental releases in the Cayman Islands and Brazil. A summary of the results is given below.

5.1.3.1 Mating competitiveness in the laboratory

Mating competitiveness studies for OX513A against wild-type strains from around the world have been carried out in a wide variety of international laboratories. The OX513A strain performed successfully against all the *Aedes aegypti* wild type strains tested regardless of the genetic background.

5.1.3.2 Mating competitiveness in semi field conditions

Mating competitiveness in a purpose-built field house in Kuala Lumpur (Malaysia) showed that approximately 50% of the OX513A males found mates. This is equivalent to a fully competitive strain.

5.1.3.3 Mating competitiveness in regulated environmental releases

Regulated environmental releases resulted in mating competitiveness estimates of 0.56 (Cayman Islands) to 0.0004 to 0.059 (Brazil), indicating that mating competitiveness depends on many factors such as environmental factors, timing of release and immigration of wild *Aedes aegypti*.

5.2 Response to abiotic factors

5.2.1 Temperature response of OX513A

The temperature response of heterozygous OX513A has been evaluated in the laboratory in order to determine:

- If the penetrance of the phenotype of OX513A heterozygotes varies when reared at temperatures different than that the laboratory standard (penetrance is the probability of a gene or genetic trait being expressed)
- If OX513A has altered survival at temperatures outside of *Aedes aegypti's* natural range.

Experiments in the absence of tetracycline demonstrated that penetrance of the phenotype is independent of the temperature and that survival rates of OX513A and the wild type strain are similar at different temperatures, also outside the natural temperature range of *Aedes aegypti*.

5.2.2 Dose response to tetracycline and its analogues

The response of OX513A heterozygous larvae to different doses of tetracycline, chlortetracycline, oxytetracycline, and doxycycline has been evaluated to identify the lowest concentrations which allow for greater survival of heterozygous OX513A as compared to larvae reared in the absence of tetracycline or its analogues.
The experiments show that concentrations at and below 3 ng/mL tetracycline, 1 ng/mL chlortetracycline, 10 ng/mL oxytetracycline and 0.1 ng/mL doxycycline do not increase survival of OX513A larvae, i.e. do not increase the proportion of functional adults. These levels are higher than the mean published concentrations of each analogue found in environmental bodies of water.

5.2.3 Susceptibility to chemical insecticides

OX513A and its non-modified wild type strain with similar background were found to be equally susceptible to four commonly used insecticides (temephos, permethrin, deltamethrin and malathion) and showed similar significant survival to bendiocarb. Two *kdr* mutations associated with pyrethroid and DDT resistance were absent in the OX513A strain.

5.2.4 Behavioral responses of OX513A to insecticides

Behavioral responses of OX513A male mosquitoes were overall similar to those displayed by the wild type strain, including significant contact irritancy to pyrethroids and significant spatial repellence to DDT.

5.2.5 Tetracycline loaded blood study

It was demonstrated that the penetrance of the OX513A phenotype in hemizygous offspring of female *Aedes aegypti* is not influenced by the presence of tetracycline in blood. This indicates that tetracycline present in human or animal blood, for example as a consequence of antibiotic treatment, would not affect the percentage of OX513A offspring that expresses the conditional lethality trait.

5.2.6 Trait penetrance

Penetrance is the probability of a gene or genetic trait being expressed. Under laboratory conditions the observed penetrance of the self-limiting trait in OX513A is always found to be over 95%. This means that less than 5% of the offspring of a cross between OX513A males and wild

type *Aedes aegypti* females will survive if reared without tetracycline in the rearing water or the environment.

5.2.7 Non-penetrant OX513A progeny: longevity and fecundity

The survival and fecundity (fertility) of the small observed proportion (<5%) surviving heterozygous OX513A offspring has been investigated in the laboratory.
Median survival of both OX513A males and females in the absence of tetracycline was 2 days compared to the non-modified males and females that have a much higher median survival under laboratory conditions. In contrast to the wild type, substantial mortality was observed in the surviving OX513A progeny. A small fraction (~20%) does survive long enough to take two blood meals and some produced two clutches of eggs. The OX513A strain lays a statistically significant larger egg clutch (69,9) during the first gonotrophic cycle, compared to the wild type (54,8). Hatching rate is somewhat higher for OX513A eggs, but this is not statistically significant.

5.2.8 Field penetrance

Penetrance of the trait in the field was studied in the Cayman Islands (East End) and in two release sites in Brazil (Itaberaba and Mandacaru). Overall estimates of the percentage of incomplete penetrance ranged from 0-4.28%, falling below the ~ 5% reported in laboratory studies.

5.3 Dispersal and longevity - regulated environmental releases of OX513A

In a typical *Aedes aegypti* urban habitat in Brazil, the mean dissemination of OX513A genes (as determined from eggs in ovitraps) was estimated to be 64 m and 79 m from the place where the mosquitoes were released for the two periods evaluated, which corresponds with the dispersal of OX513A males and the wild type comparator strain observed at the same site.
In Malaysia, in an uninhabited forested area, maximum dispersal distances of OX531A and the wild type comparator strain were similar (220 m), but mean distance travelled by the OX513A strain was lower (52 vs. 100 m). Life expectancy of the OX513A males and the wild type comparator strain used in this study in Malaysia was similar (2.0 vs. 2.2 days).
In the Cayman Islands results demonstrated an average life expectancy of OX513A ranging between 0.1 to 1.6 days. No wild type comparator was released in this study.
This indicates that dispersal rates and longevity of OX513A are not greater than that the wild type.

5.4 Potential toxicity - oral exposure studies

5.4.1 *Toxorhynchites* spp (predatory mosquito)

Toxorhynchites larvae feed on small aquatic organisms. No significant impact was found on the development, fecundity and longevity of two predatory T*oxorhynchites* species (*Tx. amboinensis* and *splendes*) that were fed exclusively with OX513A larvae, compared to the same species that were fed with a diet consisting of wild type larvae. These results indicate no toxicity as a consequence of the genetic modification.

5.4.2 *Poecilia reticulate* (guppy fish)
To determine the effects of OX513A on the guppy fish *Poecilia reticulate,* a 14-day feeding study was performed. No significant differences were observed with regard to mortality, fish length, weight, appearance and behavior between fish fed with a diet of OX513A and fish fed with a diet of wild type *Aedes aegypti*. These results indicate no toxicity as a consequence of the genetic modification.

5.5 OX513A morphology
There are no genes introduced into the male mosquitoes that are intended to alter the morphology of the insect and no morphological differences between OX513A males and the wild type males have been observed.

5.6 Analysis of expression of the introduced proteins in female mosquito saliva
An analysis was conducted to detect the presence of the tTAV and DsRed2 proteins in OX513A female mosquito saliva using Western blot analysis. Using the proper controls, the tTAV and DsRed2 proteins could not be detected in female OX513A saliva.

5.7 Vertical transmission of dengue and chikungunya viruses in OX513A
The passage of a disease causing agent or pathogen from an infected female to its offspring is known as vertical transmission. To assess whether OX513A females were more competent for vertical transmission of dengue and chikungunya viruses than females from a wild type strain, vertical transmission of dengue (serotypes 1-4) and chikungunya viruses in OX513A females and a wild type comparator strain have been evaluated.
No differences were found between the homozygous OX513A strain and the non-modified comparator strain, nor were there significant differences in fecundity observed.

5.8 Stability of the insert in OX513A
See section 4.2 of part A.

5.8.1 OX513A quality control
Regular strain integrity quality control assays are carried out on the OX513A colony with respect to:

- Colony genotyping;
- Penetrance, stability and tetracycline dose response;
- Mating competitiveness.

5.9 Conclusions regarding the phenotypic characterization of OX513A
The GMO Office confirms conclusions of Oxitec that no phenotypic changes were detected in OX513A relative to the comparator(s) that suggest unintended effects as a consequence of the genetic modification with respect to:

- Life table parameters, including mating competitiveness
- Response to abiotic factors including temperature and insecticides
- Adult dispersal and longevity
- Potential toxicity by oral exposure studies

In addition, the GMO Office concludes that data demonstrate the

following:

- Mean reported concentrations of tetracycline and its analogues in water are well under the concentrations necessary to allow phenotype rescue in OX513A
- Trait penetrance in progeny produced by male OX513A and wild type females is observed to be around 95% or higher, both under laboratory conditions as under field conditions. This means that less than 5% of the offspring will survive in the absence of tetracycline and thus in the environment
- About 20 % of this surviving progeny is able to survive for about 2 days, long enough for the females to take two blood meals and produce two clutches of eggs
- Vertical transmission of DENV and CHIKV is not changed in OX513A
- Female OX513A do not contain tTAV and DsRed2 proteins in the saliva

6 Detection and identification of OX513A

6.1 Methods and sensitivity for detecting OX513A Aedes aegypti in the environment

There are two primary detection methods available: fluorescence-based detection and DNA sequence-based detection.
Fluorescence-based detection is possible by microscopic detection of fluorescent larvae and pupae as a consequence of the production of the DsRed2 protein in OX513A. This marker has proven to be stable over 115 generation equivalents since 2002. This marker is not visible in eggs and adults of *Aedes aegypti* OX513A. Eggs of OX513A are therefore required to be hatched under controlled laboratory conditions for visualization of the DsRed2 marker gene expression (additional information November 30, 2016).
DNA sequence-based detection visualizes a PCR-amplified unique genetic fragment within OX513A, based on the genomic regions flanking the insertion site. Adults require molecular analysis by PCR to detect whether they are genetically modified or not.

6.2 Monitoring the Aedes aegypti population in the environment

The trapping methods used in an OX513A program to monitor *Aedes* mosquitoes are well established. There are two principle trapping methods used either in combination, or in isolation depending on the phase of the program and the intent of the monitoring.
Ovitrap (egg catch) surveys are the principal monitoring tool deployed, mimicking natural breeding sites in which females lay eggs and have been adopted as a standard monitoring tool for *Aedes aegypti* populations. The World Health Organization (WHO) recommends use of ovitraps for *Aedes aegypti* surveillance.
The *BG-Sentinel adult trap* was developed specifically targeting *Aedes (aegypti* and *albopictus)* mosquitoes. These traps use a combination of visual and host mimic olfactory cues to attract adult (male and female) *Aedes* mosquitoes that are captured in a suction trap.

7 Regulated environmental releases of OX513A

7.1 *Previous Aedes aegypti vector control projects using OX513A*

Regulated environmental releases of OX513A males have been conducted since late 2009 in collaboration with partners in both vector control programs and academia.
Releases in 2009 in Grand Cayman served principally to demonstrate that released OX513A males could successfully compete with wild males in their natural urban setting and mating is not compromised by insertion of the OX513 recombinant DNA (rDNA) construct. Additionally, in Malaysia the 2010 releases also served to demonstrate that the insertion of the rDNA construct has not altered the dispersal range of in the OX513A strain. Subsequent releases of OX513A conducted to date in Cayman Islands, Brazil and Panama demonstrated the efficacy of the release of the OX513A strain in the context of a vector control program.

In sections 7.1.1 to 7.1.5 of part A of the documentation of Oxitec descriptions are given of the releases in Cayman islands, Brazil and Panama including the number of OX513A introduced and the level of suppression of local *Aedes aegypti* populations. These are not reported here, since they are not considered to be relevant for the environmental risk assessment.

7.1.5.1 Environmental persistence

In Panama a post release environmental monitoring survey was performed to assess the persistence of the OX513 genetic construct in the environment. Final releases of OX513A took place on 31 October 2014. Environmental monitoring in control and release sites continued 138 days post-release through a network of 60 ovitraps and fluorescence screening of larvae for the DsRed2 marker. Over 20,000 *Aedes aegypti* larvae where individually screened. Prior to the final release, OX513A fluorescent larvae comprised 100% of the trapped larvae in the treated area, 25 days post-release it was 5%, 84 days post-release it was 0%. Although the absence of OX513A genes could only be confirmed from 12 weeks post-release onward due to a disruption in data collection, the available data suggest that OX513A genes are unlikely to occur in the environment 6-8 weeks post-release.

7.1.1 Conclusions drawn from previous vector control projects using OX513A

The GMO Office confirms the conclusions of Oxitec with respect to data supplied on environmental releases in Cayman Islands, Malaysia, Brazil and Panama performed since 2009 on:

- mating competitiveness
- adult dispersal and longevity
- dissemination and persistence of OX513A genes into the environment

The GMO Office also remarks that no unintended effects on human animal health and the environment have been observed in any of the releases. However, there was no dedicated environmental monitoring in place during these releases.

Part B Intended use of OX513A *Aedes aegypti* on Saba

1 Details of the proposed release on Saba

General overview

Eggs are shipped in regular shipments throughout the course of the program to the facility on Saba near the release site where they are reared to obtain pupae, sex sorted to select male pupae and where the males are matured to adults for release. Sexually mature OX513A males are released from specialized release devices in a grid-like pattern from predefined release points to ensure even coverage of the area.

OX513A integrated into the Latin-American wildtype will be used for the release on Saba. The Latin-American strain used is the lead strain subject to regular quality testing as described in the Standard Operating Procedures (SOPs) and is used in field programs around the world. There is a high degree of confidence of the genetic integrity of the insert in this particular background strain and its mating competitiveness (additional information November 30, 2016).

The OX513A program can be divided into three sequential phases: (1) a preparation phase, (2) an intervention phase and (3) a maintenance phase. Release rates are informed by ongoing monitoring and evaluation of various metrics.

1.1 Anticipated outcomes for Saba Island

The proposed OX513A *Aedes aegypti* suppression program on Saba aims to suppress the local population of *Aedes aegypti* to very low levels and potentially to elimination with demonstration of continued elimination for a period of a year thereafter
Aedes aegypti is considered an invasive pest on Saba.

Sustained releases of OX513A on Saba aim to have several measurable effects on the wild *Aedes aegypti* population, including, in temporal order:

1. An increase of the overall male-to-female ratio for *Aedes aegypti;*
2. Wild type *Aedes aegypti* females mating with OX513A males;
3. Suppression of the target *Aedes aegypti* population leading to potential elimination of *Aedes aegypti* on the island;
4. Continued elimination of *Aedes aegypti* for a period of one year.

The OX513A program proceeds with an ongoing evaluation of the population dynamics of the wild *Aedes aegypti* population, and release rates are adapted based on monitoring throughout the release period as control/elimination is achieved. Once control is gained and the island is effectively free from wild *Aedes aegypti,* continued close monitoring and low level releases of OX513A at points at risk of potential re-introduction will be required to maintain the wild *Aedes aegypti* free status.

1.2 Location of releases

The proposed release on Saba is island wide throughout the four principle human populated areas of The Bottom, Windward side, Zion’s

Hill and St. Johns, and any minor inhabited areas in between (Figure 5). Releases will also take place in the areas of the port at Fort Bay Harbor, and Juancho E. Yrausquin Airport. Releases of male OX513A are done typically up to three times a week at predetermined geo-referenced locations generally not more that 100m apart to ensure coverage of the release area.
The island may be subdivided into areas with different release rates depending on the heterogeneity of inhabited areas and corresponding *Aedes aegypti* infestation but the broad areas of release will be aligned with road patterns in the predicted habitat of *Aedes aegypti* on Saba (i.e. the areas inhabited by humans) as illustrated in Figure 5 below.

Figure 5. Principle habitat of Aedes aegypti on Saba represented as urban areas including an approximately 100m distance from human habitation (shaded in red). Total shaded area is equivalent to approximately 3.7 km^2.

1.3 Determination of release rates - phased approach

As described earlier, OX513A releases are administered in a phased program and release rates are informed by ongoing monitoring and evaluation of various metrics.

1.3.1 Preparation phase

This phase will be used to evaluate the initial densities of *Aedes aegypti* mosquito populations in the treatment areas and optimize the OX513A rearing methodology to local conditions on Saba. Initial densities are monitored by ovitraps and adult trapping methods as well as using best available information including historical surveillance data, seasonality,

epidemiology records, existing mosquito abatement and qualitative factors such as housing type and proliferation of breeding sites.
Based on the densities of wild *Aedes aegypti* determined, an initial release rate of the OX513A males is chosen. An initial release rate (IRR), is typically between 100 and 300 male OX513A per person per week in most projects to date.

1.3.2 Intervention Phase

1.3.2.1 Mosquito release and dispersal

During the intervention phase, OX513A is released in a systematic manner from a predetermined georeferenced grid of release points at regular time intervals, for even and consistent coverage of the treatment area. Release points will be spaced no more than 100m apart, and releases will occur up to 3 times per week. In past projects, substantial suppression was observed 4-6 months following initiation of releases, but depending on local conditions and mosquito densities, this could be up to 12 months.
The key factor governing the rate of *Aedes aegypti* population reduction is the fraction of local wild *Aedes aegypti* females mating with released OX513A: this mating should be in a greater proportion with OX513A than with local wild *Aedes aegypti* males.
The release rate necessary to achieve a given mating fraction is proportional to the local *Aedes aegypti* population. The greater the OX513A male: local *Aedes aegypti* male ratio achieved, the greater the mating fraction and likelihood of having an impact on local *Aedes aegypti* population.

1.3.2.2 Adaptive management of release rate

Release rates are dynamically adjusted in response to local *Aedes aegypti* populations which are expected to fall during the period of the intervention. OX513A male releases will continue as the local *Aedes aegypti* population is suppressed, but at a reduced level chosen to maintain the mating faction at >0.5. The release rates will be evaluated every 6-8 weeks during the suppression phase and adjusted based on the mating fraction.
Significant suppression of the local *Aedes aegypti* population is typically expected within 4-6 months of initiation of releases. However, to achieve effective elimination of *Aedes aegypti* from Saba it is expected to take longer and may tend towards 12 months.

1.3.3 Maintenance Phase

When the local *Aedes aegypti* population drops sufficiently to demonstrate convincing suppression or elimination, the program enters a maintenance Phase, where releases and monitoring will continue, but without estimating the mating fraction. Resurgence of the local *Aedes aegypti* population, as monitored by ovitraps, will trigger an increase in release rates.
Resurgence is categorized as 4 consecutive weeks with an ovitrap index >10 %. An ovitrap index defined as the traps in which one or more eggs confirmed as *Aedes aegypti* is found, divided by the total amount of traps. Once the wild *Aedes aegypti* population has been effectively suppressed (<10% ovitrap index) the program enters the maintenance phase designed to stop resurgence of *Aedes aegypti* population and to sustain the attained goals. This approach can be applied to contiguous

subareas within the program as they become well controlled, even while other areas remain in the intervention phase. Resurgence could arise from small pockets of residual *Aedes aegypti* populations, egg bank and/or immigration.
The maintenance phase protocol is similar to that used in the intervention phase – with releases and/or use of other vector management tools being planned and targeted based on monitoring data. The areas of high risk and the loci of re-infestation will be principally the transport hubs at the port at Fort Bay Harbor and Juancho E. Yrausquin Airport, which will be further characterized through the monitoring during the intervention phase. These areas are at a minimum proposed for sustained releases on an ongoing basis, with program objectives assessed annually in consultation with the Saba Public Health officials and appropriate government and other stakeholders.
In the remaining areas, treatment is anticipated to stop as the *Aedes aegypti* population is eliminated, and be subject to ongoing monitoring, with targeted OX513A releases if reinfestation is detected.

Estimated numbers of OX513A Aedes aegypti to be released on Saba
Estimates for release numbers in the context of the proposed Saba project can be made with a reasonably high degree of confidence based on experience in suppression projects to date (add. info. November 30, 2016).
Taken on average over the first 12 months, releases are anticipated to average 160 OX513A males/person/week. For a population of approximately 1800 on Saba this would mean that up to approximately 15 million OX513A males would be released during the initial 12-month period. After the first 12 months, averaged over the whole island, the release rates are not expected to exceed on average 50 OX513A males/person/week. This would equate to up to approximately 4.7 million OX513A males during the subsequent 12 months (additional information November 30, 2016).

1.4 Containment measures prior to release

Eggs of OX513A will be produced in the UK under the Genetically Modified Organisms (Contained Use) Regulation 2014 and the production is handled under Containment Level 1 (CL1) conditions. Upon export to Saba, OX513A eggs (amounts to be stated in a separate advanced notification) will be packaged in three layers of shatter-proof containment, and shipped by air by a commercial courier service transiting through St. Maarten.

1.4.1 Mobile Rearing Unit Overview

OX513A will be imported to a locally established Mobile Rearing Unit (MRU). MRUs are insect production laboratories fabricated within standard shipping containers which conform with relevant ISO (International Organization for Standardization) standards for shipping containers, and are adapted as mobile insectaries that provide compliance up to and including ACL2 (Arthropod Containment Level 22). Standard Operating Procedures (SOPs) are provided that detail all measures that are relevant to containment.

The MRU for Saba is proposed to be installed in the area of the port at Fort Bay Harbor in close proximity to electrical and fresh water supply from the desalination plant.
Wastewater disposal from the MRU will be consistent with that of other commercial buildings in the area of Fort Bay harbor.

1.4.1.1 Design and construction

MRUs are pre-fabricated in the UK to a generic design, which is tailored before shipping according to different project localities and requirements. An assessment of all relevant building codes and installation requirements will be completed for Saba to inform the MRU construction process. To ensure a safe working environment, Oxitec MRUs have a minimum specification that is compliant with respected codes for structural, mechanical, plumbing, electrical, fire safety and disability access. Oxitec will work with local planning officials on Saba to assess applicable code requirements.

1.5 Rearing of OX513A from egg to adult mosquitoes

The process of rearing OX513A eggs to adults is well established in SOPs.
All SOPS are provided and are proven to be sufficient. However, SOPs are not adjusted yet for use on Saba. It is indicated (additional information March 13, 2017) that final adjustment to existing SOPs and the development of final project-specific SOPs for Saba will be undertaken as a matter of priority once a confirmation of the project is established through contractual agreement between Oxitec and the government of Saba.

Conformance with SOPs, training and supervision during project

Oxitec will send an accountable local project manager to reside on Saba and oversee implementation of the project throughout the initial community engagement and local set-up stage, plus the entire duration of mosquito releases (12 months anticipated). Oxitec will also have someone present in the 12 months of maintenance that immediately follows, if deemed necessary. Any non-conformance with SOPs will be recorded and reported to UK central operations, and corrective actions will be taken under the direction of the Oxitec Quality Assurance Manager. Activities require local staff to be supplied by the Saba Department of Agriculture, Hygiene & Vector Control. Four local staff members will be required during the first year during the preparation and intervention phases, and two staff members in the following year for maintenance and monitoring. Full training and supervision will be given by Oxitec who will work in close partnership with the Saba Department of Agriculture, Hygiene & Vector Control, to ensure all participants are trained in Standard Operating Procedures relevant to their function. Training records will be retained on file as part of standard procedure. After the initial proposed two year project has finished (set-up plus one year elimination plus one year maintenance), activities to maintain the local *Aedes aegypti* population under control such as ongoing supply of OX513A eggs, and technical support visits will be available subject to contractual agreements (additional info November 30, 2016).

1.5.1 Tetracycline use

The conditional lethality is repressible in the presence of the antibiotic tetracycline or its analogues. Chlortetracycline at a final concentration of 30mg/liter final concentration is used in the water of the larval rearing trays. For the number of larvae needed for the population of Saba (around 2000 people), a total of 36 grams of chlortetracycline/week is needed for the rearing of the larvae.
A study was conducted to analyze how the concentration of tetracycline changes in rearing water and in mosquitoes during their aquatic life stages. This study demonstrated that the expected amount of chlortetracycline in the output waste water from the rearing facility is estimate to be 21 times lower that the input amount. For Saba the expected amount would be 36 gram divided by 21= ~ 1.7 grams/week in output waste water.

1.6 Transport and adult release

OX513A adult males are transported in release devices, typically with 500 or 1000 males, using an adapted van or open-box truck. While different release methodologies are used in larger scale projects underway (e.g. Brazil), the small scale of the Saba project is amenable to release from an open truck or van.

1.7 OX513A program monitoring

Monitoring of *Aedes aegypti* is required to evaluate the change in the *Aedes aegypti* population to inform the adaptive management of release rates and program decisions. Ovitraps are the primary tool for monitoring changes in *Aedes aegypti* abundance and to assess the mating fraction, while adult traps can be used to provide an additional metric for the evaluation of wild *Aedes aegypti* populations. It allows also for estimating the ratio of OX513A and wild male *Aedes aegypti*. The primary focus of monitoring will be *Aedes aegypti.* Due to the close related nature and behavior of *Aedes albopictus*, the same monitoring tools apply for *Aedes albopictus* (ovitraps and adult trap) where it is present. For operational programs, routine ovitrap monitoring is conducted for both *Aedes aegypti* and *Aedes albopictus* populations. *Aedes albopictus* populations are not yet observed on Saba. Depending on the trap type, other mosquito species may be captured and data recorded if deemed relevant to the project.

1.7.1 Ovitrap monitoring

Ovitraps provide a measure of the *Aedes aegypti* population and of the mating fraction of wild *Aedes aegypti* with OX513A males.

1.7.1.1 Ovitrap density, location and servicing

Ovitraps are to be located predominately by domestic dwellings, although non-residential
sites such as commercial or industrial buildings may be included. A minimum number of 30 traps, with a minimum trap density of 30 per km^2 will be deployed for each assessment area.

1.7.1.2 Species identification

Ovitraps are selective for mosquitoes that breed in clean water containers. In some geographic areas this will be limited to *Aedes aegypti*, while other species may be present that lay eggs which cannot

easily be distinguished from *Aedes aegypti* (e.g. *Aedes albopictus*). Identification of different species at the egg stage is not feasible; therefore eggs collected from ovitraps will be hatched for species identification at larval and/or adult stage using appropriate taxonomic keys. Larvae in which the DsRed2 fluorescent marker is expressed will be scored as *Aedes aegypti*, without need for further rearing and identification by taxonomy.

1.7.1.3 Species Composition
Ovitrap collections from all sites will initially be processed assuming presence of non-*Aedes aegypti* species, with confirmation at species level, until 100 positive ovitraps are assessed. Thereafter, presence of non-*Aedes aegypti* species will be checked every 4 months for the first year and is continued on an annual basis, as foreseen, at least for the year thereafter.

1.7.2 Ovitrap analysis
Following egg maturation and hatching, ovitrap catches will be analysed, including species identification as described in section 1.7.1.3.

1.7.3 Estimating mating fraction
Following egg maturation and hatching, larvae will be visually screened and the fraction of larvae with the fluorescent marker is scored by trained staff. The mating fraction is calculated as the number of fluorescent larvae divided by the total number of *Aedes aegypti* larvae (fluorescent and non-fluorescent).

1.7.4 Adult Trapping
Direct sampling of adults has the advantage that adults are readily identifiable to species and sex. The number of female *Aedes aegypti* can be used as metric for assessing the local population of *Aedes aegypti.* To differentiate between male OX513A and wild type male *Aedes aegypti* molecular tools are necessary.
Traps will be located predominately by domestic dwellings, although alternative sites such as schools and shops may also be included. A minimum of 15 BG Sentinel traps is anticipated to be needed for adult monitoring on Saba. As a minimum, these traps will normally be installed for the duration of the intervention phase and will be checked weekly.

2 Receiving environment

The human population on Saba as of January 2015 was 1811, and was between 1811 and 1991 in the five consecutive years to 2015.

2.1 Geography and Climate

2.1.1 Geography
Saba belongs to the group of the Windward Islands of the Dutch Caribbean. The Windward group is about 900 kilometers north east of the Virgin Islands, and consists of the islands St. Martin (of which half is French territory, Dutch part is named St. Maarten), St. Eustatius and Saba. Saba is located about 50 km south of St. Maarten and rises steeply from the sea. Saba is the youngest island of the Windward group and is considered to be a ‘sleeping’ volcano, rather than a ’dead’ one.

The highest point, Mount Scenery, rises 840 m above sea level.
Saba has no natural harbor. Only at a few locations it is possible to land with small boats. Fort Bay and Ladder Bay, respectively to the south and the west coast, are the only natural landing spots on the island. Large ships have to remain several hundred meters offshore.
The vegetation of Saba is mainly composed of woodland forest with ferns and damp soil, and many introduced fruit trees like the mango (*Mangifera indica*). Saba's terrestrial park stretches from Great Hole on the northeastern shoreline and the Pirate Cliffs in the northwest, up to the cloud forest at the peak of Mount Scenery. The park contains everything from arid coastal vegetation to rich cloud forest as well as the culturally important site of the island's former sulphur mine.

2.1.2 Climate

Saba has an equatorial monsoon climate with mean temperatures above 18 °C in every month of the year and clear wet and dry seasons.
The climate is characterized by a relatively dry season (January-April) and a rainy season (August-December) with moderate to fresh east to north easterlies.
Monthly rainfall and temperatures on Saba, key abiotic factors for the survival of *Aedes aegypti,* from 1971-2000 are indicated in the dossier.
Average daily maximum temperatures are near 27°C with August as the warmest month. Cooler conditions are common in the higher elevations of the island.

2.1.2.1 Weather conditions

Saba, St. Eustatius and St. Maarten (SSS) are located within the hurricane belt and almost every year at least one tropical cyclone occurs within a range of 100 miles of the SSS islands and on the average once every 4-5 years hurricane conditions are experienced.
From January 1, 2016, the Royal Netherlands Meteorological Institute (KNMI) has been responsible for Bonaire, St. Eustatius and Saba to prepare the weather forecasts and warnings for the general public as well as services to the aviation and marine sectors in Bonaire. In the event adverse weather is anticipated, steps will be followed as described in a site- specific Hurricane Preparedness Policy for Saba (SOP supplied).

2.2 Aedes aegypti on Saba

2.2.1 Habitat of *Aedes aegypti* on Saba

Aedes aegypti is a peri-domestic species closely associated with human habitations.
Breeding is tied but not exclusively to artificial water containers, such as potted plant holders, water tanks, tires, discarded plastic and metal containers such as soda cans, drains and roof guttering. Once eclosed the adult *Aedes aegypti* mosquitoes live in and around houses where females have easy access to blood meals necessary for egg development.
On Saba, qualitative drinking water is supplied by desalination facilities in Fort Bay and is considered costly. Rain water collection in urban areas means that there is an abundance of cisterns serving both private households and larger shared public areas. Local vector control services have identified cisterns as principal *Aedes aegypti* breeding sites and vector control activities target cisterns among other water containers.
The *Aedes aegypti* habitat on Saba (and other small Caribean islands)

thus has the unique addition of cisterns, not typically as abundant in other urban areas with less costly municipal water supply.

2.2.2 Functions of *Aedes aegypti* in the ecosystem of Saba

Aedes aegypti has been identified as invasive species on Saba and a high priority for both control and research. *Aedes aegypti* is not native to Saba and existing interventions attempt to control *Aedes aegypti* populations through biological control approaches involving the use of larvivorous fish in breeding site such as guppy fish, as well as the application of products based on *Bacillus thuringiensis*. Suppression or elimination of the wild *Aedes aegypti* population is thus consistent with current protection goals for vector control, and is thus not expected to alter population dynamics of non-target organisms as it is not considered to be a keystone species in the local food chain.

2.3 Flora and Fauna

Agriculture and cattle breeding

There is currently only small-scale agriculture on Saba principally due to the geology of the island; only about 216 of the 1300 hectares can be used for agriculture and cattle breeding.

Natural flora and fauna

- *Plants*
  On Saba 520 species of plants are identified.
- *Birds*
  Saba's fauna is noted as having relatively few species. Among the vertebrates, birds form the largest group, represented by 26 species. Five species of seabirds are reported to nest on Saba, and additionally 36 migrating species are reported to be present every year on a temporary basis.
- *Mammals*
  Bats are the only mammals on Saba that were not introduced by humans. Five species have been reported.
- *Reptiles* and *amphibians*
  Reptiles and amphibians a are the second largest group of vertebrates with 10 species of reptile and one amphibian (the piping frog *Eleutherodactylus Johnstonei*).
  There is one island endemic among the vertebrates: the lizard *Anolis sabanus*. Two protected, charismatic and valued species that may occur in the potential release areas of OX513A on Saba are the the Red-bellied Racer (*Alsophis rufiventris*) and Saban Anole (*Anolis sabanus*).
- *Insects*
  References to butterfly and other insect species observed on Saba are given in Oxitec's documentation.
- *Other organisms*
  A high species richness on Saba was noted for spiders: 18 families, representing 76 species.

2.4 Tetracycline in the environment on Saba

The primary routes of exposure to tetracycline in the environment are agriculture and wastewater.

In an *agricultural setting*, the most likely sources of tetracycline are from application of manure contaminated with tetracyclines used in

prophylactic or therapeutic veterinary applications (e.g. cattle), or through application of tetracycline-containing pesticides that in various jurisdictions are approved for application on certain fruit trees like pear trees. However, as mentioned earlier there is currently only small-scale agriculture on Saba principally due to the geology of the island, so there is almost no agricultural use of tetracyclines on Saba. Another source could be the use of tetracyclines in companion and other animals maintained by humans, through shedding in urine and faeces: up to 72 % of the antibiotic is being excreted in faeces and urine within 2 days of antibiotic application.

Another potential is the presence of *waste water* (e.g. irrigation, sewage) which has been contaminated with veterinary or human therapeutic tetracyclines. A review of environmental antibiotic degradation indicated that in general the highest sources of environmental tetracylines (in the µg/L range) were from hospitals and municipal wastewater, whereas surface waters, sea and ground waters were in the ng/L range. Tetracylines are well known to degrade rapidly in sunlight.
A study was performed in Brazil in which water samples were collected from *Aedes aegypti* breeding sites. Results indicated that the concentration of tetracycline was below the limit of quantification for each of the field samples.

The waste treatment system on Saba is essentially via private cesspits in residential areas. There is the possibility that individuals receiving therapeutic doses of antibiotics may contribute to concentrations of tetracyclines in isolated individual residential cesspits. The A.M. Edwards Medical Center hospital on Saba in The Bottom could be expected to generate waste containing a higher concentration of tetracyclines than private residences, however waste cesspits are not the preferred habitat of *Aedes aegypti,* as it is well characterized as preferring clean standing water in an around human habitation.
Reports have suggested that *Aedes aegypti* can breed in septic tanks, usually where they are cracked or broken but this tends to be in the clear water at the top of the tank, whereas tetracyclines tend to bind to the sediment which collects at the bottom (thereby making any tetracyclines less accessible in the clear surface layer of water). Damaged or cracked septic tanks or cracked covers on cesspits do exist.

Part C OX513A Environmental risk assessment

Introduction

This environmental risk assessment (ERA) has been carried out consistent with the general principles and methodology as described in Annex II to Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (EC, 2000)[1]. Additional direction has been taken from Commission Decision of July 24, 2002 (EC, 2002)[9], establishing guidance notes supplementing Annex II to Directive 2001/18/EC. Interpretation specific to genetically modified insects has been taken from the EFSA Guidance on the environmental risk assessment of genetically modified animals (EFSA, 2013)[2] as well as the EFSA Guidance on the environmental risk assessment of genetically modified plants (EFSA, 2010)[10].
The scope of this ERA is for the deliberate environmental release of OX513A in the context of a vector control project for *Aedes aegypti* on Saba, a special municipality of the Netherlands.

1 Approach of the environmental risk assessment

This environmental risk assessment (ERA) forms part of the overall risk analysis process in order to make informed decisions regarding the deliberate release into the environment of OX513A in the receiving environment of the island Saba. The ERA is carried out using published data, study reports and other data generated through evaluations both in the laboratory and through regulated environmental releases. Additionally, scientific literature reviews and independent expert analysis have been considered in order to develop scientifically sound assessment of the overall risk. A structured and systematic approach has been taken following the six steps of ERA described in Directive 2001/18 EC to enable an individual case assessment of the potential effects of the deliberate release into the environment of OX513A. The six steps of ERA are represented in Figure 6.

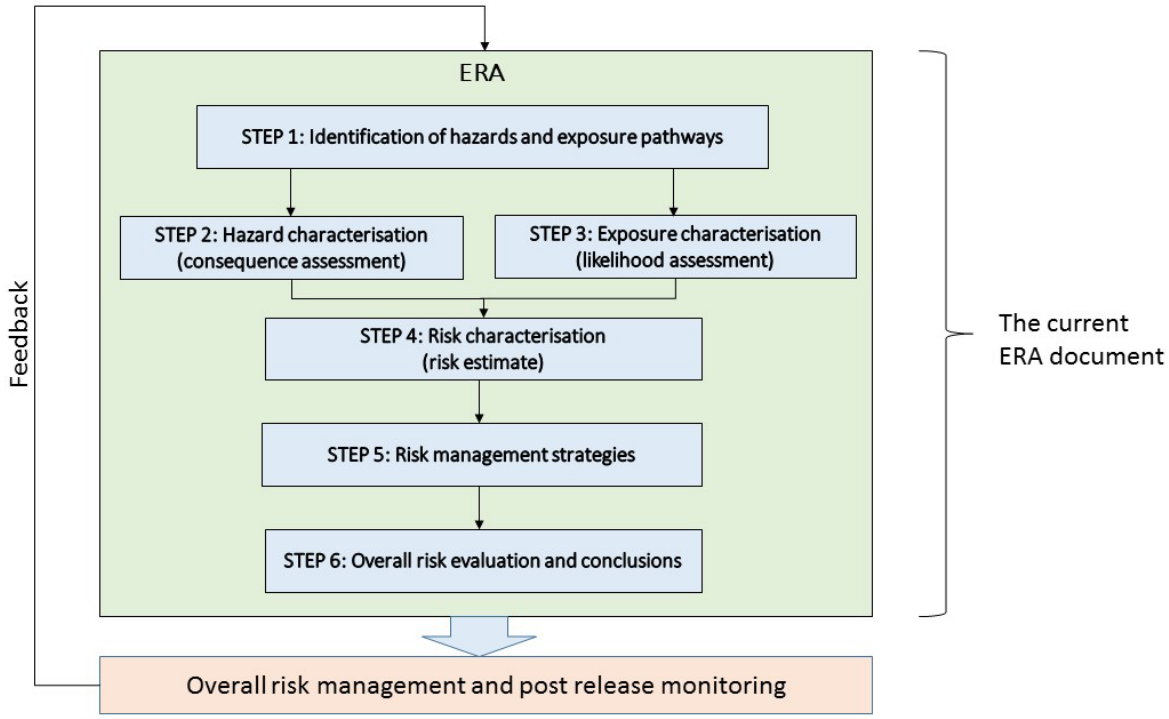

Figure 6. Six steps of environmental risk assessment (ERA) as presented in (EFSA, 2013)[2] and interpreted from Directive 2001/18/EC (2001)[1].

Using the six steps of the ERA, potential environmental impacts as identified in Directive 2001/18/EC Annex II D1 (EC, 2001)[1] have been evaluated for OX513A, as interpreted in the EFSA guidance for GM insects (EFSA, 2013)[2] by means of seven areas of concern:

1. Persistence and invasiveness of GM insects, including vertical gene transfer
2. Horizontal gene transfer
3. Pathogens, infections and diseases
4. Interactions of GM insects with target organisms
5. Interactions of GM insects with non-target organisms
6. Environmental impact of the specific techniques used for the management of GM insects
7. Impacts of GM insects on human and animal health

Choice of comparators

EFSA guidance deems it appropriate to draw on previous knowledge and experience with non-GM animals (e.g. irradiated sterile insects; mark release recapture (MRR) of wild type) and from previous applications for similar GM and non-GM traits and GM events.

Accordingly, this ERA for the deliberate release of OX513A has been conducted using appropriate comparators relevant to the specific area of risk under evaluation. Comparators may include one or several of the following:

a) Wild type *Aedes aegypti* (unmodified laboratory strains of similar genetic backgrounds to the modified strain)

b) Wild *Aedes aegypti* (wild local populations)
c) Existing control measures for *Aedes aegypti*

1.2 Molecular characterization and phenotypic characterization

The genetic transformation of OX513A involved the stable integration of two genes coding for two intended traits: a self-limiting trait (as conferred by expression of the *tTAV* gene) and a fluorescent marker trait (as conferred by expression of the *DsRed2* gene).
In sections 4.6 and 5.9 of part A of this evaluation, it was concluded that both the molecular characterization and the phenotypic characterization of OX513A did not demonstrate any unintended effects as a consequence of the genetic modification.

2 Specific areas of concern

Seven specific areas of concern associated with the deliberate release of OX513A have been assessed according to the six steps adapted from Directive 2001/18/EC (EC, 2001)[1].

*2.1 **Persistence and invasiveness, including vertical gene transfer***

The hypothesis tested is that OX513A or its progeny is not more persistent or invasive in semi-natural or natural habitats than the existing wild type population.

The key considerations for this area of concern are (based on EFSA 2013)[2]:

1. The potential of OX513A to persist in or invade the receiving environment;
2. Whether the traits introduced into OX513A confer increased fitness to the resulting population that could allow it to persist or invade more than wild *Aedes aegypti;*
3. The potential for the introduced traits to alter the habitat and/or geographic range of OX513A or hybrid populations;
4. The extent to which OX513A can reproduce and hybridize with non-GM insects of the same or different species under conditions in the receiving environment to produce viable and fertile offspring.

In addressing these considerations, the fitness effect associated with the genetic modification of the GM insect itself or its hybrid offspring is evaluated in comparison to that of the non-modified comparator.
In this context, two considerations must be noted.

a) The purpose of the self-limiting trait in OX513A is functional genetic 'sterility', whereby OX513A males are released and mate with wild females and their offspring die before reaching the adult stage. The effect is intended to reduce the target wild *Aedes aegypti* population, and by design ensures that OX513A or offspring carrying the OX513 genes cannot establish in the environment.
b) *Aedes aegypti* is a disease vector, already subject to vector control measures on Saba. Vector control aims to effectively reduce the incidence of vector borne disease. *Aedes aegypti* is considered as an invasive species on Saba.

Ad 1) Potential of OX513A to persist in or invade the receiving environment
Ad 2) Potential of the introduced traits to confer increased fitness to OX513A
To address these first two key considerations, several aspects were studied that - as a consequence of the genetic modification- could change and thereby contribute to an increased fitness, or could contribute to persistence or invasiveness of OX513 on Saba. A comparison was made between OX513A and non-modified comparator/wild type strains with respect to the following aspects: dispersal range, temperature response, life table parameters, mating competitiveness and potential for dispersal. Since the expression of the lethality trait in OX513A is dependent on the presence of tetracycline in the environment, this aspect was also studied on Saba. Trait penetrance and lifespan of the non-penetrant heterozygotes were also assessed.

Dispersal range
Spontaneous flights of adult *Aedes aegypti* are limited to around 200 m depending on the availability of breeding sites and hosts from which to take a blood meal. Under field conditions dispersal of OX513A has been assessed in both an urban habitat (Brazil), typical to *Aedes aegypti*, as well an uninhabited forested area representing a non-typical *Aedes aegypti* habitat in Malaysia. In both experiments, a similar dispersal range was observed for OX513A males in comparison to wild type *Aedes aegypti*.
The results indicate that OX513A does not have an extended range of dispersal in comparison to the non-modified comparator under field conditions

Temperature response
The temperature response of heterozygous OX513A has been evaluated in the laboratory through rearing OX513A at different temperatures and evaluating survival in comparison to the non-modified counterpart in the absence of tetracycline. This demonstrated the following:

- OX513A does not survive at temperatures outside its normally reported range under controlled laboratory conditions (9°C and 37°C).
- Temperature does not affect trait penetrance

The results suggest that OX513A does not have an extended temperature response in comparison to a non-modified comparator and that penetrance of the trait is not affected by temperature

Life table parameters
Various life table parameters have been examined for OX513A compared to *Aedes aegypti* of different genetic backgrounds including (1) laboratory reared wild type strains (i.e. established strains for laboratory use), and (2) laboratory reared wild-caught *Aedes aegypti*. Larvae were grown in in the presence of tetracycline. Results indicate that:

(1) OX513A strain in comparison with the non-modified *Aedes aegypti* laboratory strain demonstrated a 5% lower OX513A larval survival and a reduced adult longevity.
(2) OX513A in comparison to the laboratory reared *Aedes aegypti* of wild origin (wild caught) originating from two regions of India, demonstrated no differences except for a slightly longer

developmental time from first instar to adult emergence for OX513A.

The results of the examination of specific life table parameters indicate that OX513A does not have increased fitness compared to an unmodified comparator

Mating competitiveness
Successful mating competitiveness of OX513A compared to wild type *Aedes aegypti* of various genetic backgrounds has been demonstrated under laboratory conditions with no statistically significant differences observed. Also semi-field studies have been conducted in a purpose built field house in Malaysia and during environmental releases in the Cayman and Brazil. The latter releases were performed in urban sites with different housing density and site isolation and indicated no higher mating competitiveness of OX513A compared to the non-modified *Aedes aegypti.*

The results indicate no difference in mating competitiveness of OX513A compared to the non-modified *Aedes aegypti*

Penetrance of the self-limiting trait
In the absence of tetracycline, it has been consistently observed that >95% of the hemizygous progeny of a mating between OX513A and wild type die through expression of the tTAV trait. Trait penetrance of >95% was confirmed in the field in the Cayman Islands and two release sites in Brazil. Data from an environmental release in 2014 in Panama indicated that OX513A is unlikely to persist in the environment 6-8 weeks post-release.

The results indicate that the trait penetrance is as expected under field conditions and is considered to be sufficient to prevent the persistence of OX513A in the environment

Longevity of non-penetrant OX513A under laboratory conditions
A laboratory assessment of non-penetrant OX513A indicated that the lifespan of these non-penetrant OX513A hemizygotes was found to be significantly reduced relative to wild type comparators. A fraction (~20%) do survive long enough for females to take two blood meals and some females produced two clutches of eggs; this however did not result in a longer lifespan than the wild type comparator.

These results indicate that longevity of non-penetrant OX513A is reduced in comparison the non-modified wild type

Dose response to tetracycline and its analogues
As a function of penetrance of the self-limiting trait, survival of the hemizygous OX513A progeny is greatly reduced (to < 5%) in the absence of tetracycline(s). Hence, the response to tetracycline or its analogues in the environment can affect survivability and thus persistence of OX513A in the receiving environment. It was demonstrated that mean reported concentrations of tetracycline and its analogues in water bodies, as reported in scientific literature, are well under the concentrations necessary to allow phenotype rescue.
On Saba, main routes for tetracycline occurrence in the environment would be through agriculture or wastewater. However, there is only small-scale agriculture on Saba and wastewater is not the preferred

breeding habitat of *Aedes aegypti,* as they prefer clean, still water such as rainwater filled vessels near human habitation.
These data indicate that OX513A larvae will not encounter concentrations of tetracyclines in the environment high enough to increase survival of OX513A

Tetracycline loaded blood
There is a potential for small numbers of female OX513A to exist in the environment after a release (Figure 1).
A study was conducted to test the hypothesis that providing high doses of dietary tetracycline to adult female *Aedes aegypti* (such as in blood) has no effect on the penetrance of the OX513A phenotype in their hemizygous offspring. No significant differences were observed between the control group and the experimental groups by using concentrations of tetracycline approximately ten times higher than the highest dose found in humans treated with tetracycline, and five times higher than the highest dose found in the blood of animals treated with tetracycline.
The results suggest that trait penetrance will not be significantly altered in OX513A offspring due to the presence of tetracycline in blood (human or animal)

Potential for active and passive dispersal from Saba
Aedes aegypti is reported not to survive in seawater at salinity levels around 35 g/l. As Saba is an island surrounded by ocean, the release site is effectively isolated from any other landmass within the dispersal range of *Aedes aegypti.* The genetic modification has been demonstrated not to affect the dispersal range of *Aedes aegypti*.
Dispersal by human activities such as passive transport on e.g. boats, trains or automobiles is possible. Currently around the ports of entry on Saba, the harbor and airport, methods used in vector control efforts for *Aedes aegypti* are consistent with that used island wide. In addition to regular surveillance and breeding site control, this includes the use of lethal ovitraps (http://www.in2care.org), biological control approaches such as the use of larvivorous fish in breeding sites and the application of larvicidal products based on *Bacillus thuringiensis*. Due to the relatively low *Aedes aegypti* population on Saba, insecticidal fogging for adult *Aedes aegypti* has not been deemed necessary. No spraying inside airplanes or ferries, or fogging for adult *Aedes aegypti* currently takes place under the direction of the Saba Department of Agriculture, Hygiene & Vector Control (additional information, November 30 2016).
These data indicate that dispersal of OX513A -as for wild type *Aedes aegypti*- from the island of Saba is possible. In case passive transport would take place, OX513A males or it hybrid offspring will exhibit only limited survival

Ad 3) Potential for the introduced traits to alter the habitat and/or geographic range of OX513A or hybrid populations
For this key consideration aspects that could alter the habitat and/or geographic range for OX513A or its hybrid populations as a consequence of the genetic modification were studied. Aspects considered were - among others- temperature response of OX513A, effect of temperature on trait penetrance and dispersal range. Results are described above.

Ad 4) Extent to which OX513A can reproduce and hybridize with non-GM insects of the same or different species under conditions in the receiving environment to produce viable and fertile offspring

Aedes albopictus is the most closely related species to *Aedes aegypti* which is likely to be encountered in the receiving environment. Despite multiple barriers to mating, interspecific mating between *Aedes aegypti* and *Aedes albopictus* has been observed under caged conditions and at a very low frequency in the field but is not reported to result in viable offspring.

No evidence for successful interspecific hybridization of OX513A of Malaysian background and wild type *Aedes albopictus* under laboratory conditions was found.

These results indicate that OX513A does not have an altered capacity for interspecies mating with *Aedes albopictus*

Conclusions

From the above the following conclusions can be drawn:

1. OX513A does not have the potential to persist in or invade in the receiving environment
2. OX513A homozygous adults and OX513A hemizygous offspring do not have increased fitness that could allow it to persist or invade more than wild *Aedes aegypti*
3. OX513A does not have introduced traits likely to alter the habitat and/or geographic range of the OX513A mosquito or hybrid populations
4. OX513A is not able to reproduce successfully with mosquitoes of a different species in the receiving environment

The GMO Office confirms the above conclusions that increased persistence or invasiveness of OX513A is considered to be unlikely in comparison to that of the wild type *Aedes aegypti*. This is based on the data on OX513A with respect to:

- survival
- dispersal
- temperature response
- life table parameters
- lack of successful hybridization with closely related *Aedes albopictus*
- mating efficiency of OX513A

The GMO Office also confirms that tetracycline is unlikely to be present in breeding sites of *Aedes aegypti* on Saba and tetracycline concentrations in human or animal blood are unlikely to support survival of OX513A.

2.2 Horizontal gene transfer

The hypothesis tested is that the newly introduced genes in OX513A (tTAV, DsRed2) will be transferred to other organisms via a process called horizontal gene transfer (HGT). These organisms will -as a consequence of these acquired genes- not pose adverse effects in semi-natural or natural habitats.

Horizontal gene transfer (HGT) is defined in EFSA (2013)[2] as "any process in which an organism incorporates genetic material from another organism into its genome without being the offspring of that organism", or stated otherwise, the heritable transfer of a functional genetic element from one organism to another without mating.

The key considerations for this area of concern are (based on EFSA 2013)[2]:

1. The probability and frequency of HGT, and the heritability of insect DNA in the potential recipient organism, which considers:
   - The amount and size of insect DNA exposed to various recipient organisms;
   - The presence of germline cells in multicellular organisms, or single celled organisms that are susceptible to direct DNA or DNA vector exposure;
   - The presence of mechanisms enabling such cells to take up recombinant insect DNA;
   - The existence of genetic recombination/integration processes by which translocated DNA could be incorporated and heritably stabilized in the germline cells, or replicating units.

2. The biological relevance of HGT events which may occur at low frequencies, which is directly dependent on the likelihood of further vertical transmission, which considers:
   - The presence of conditions leading to positive selection of the recipient of an HGT event such that the trait will propagate in the population;
   - The presence of gene drive systems in the recombinant DNA leading to the possibility that an HGT event will increase in frequency during subsequent vertical transmission.

Ad 1) Probability of HGT

Literature review

To study the probability of HGT, literature reviews have been conducted across potential routes of exposure by HGT in the receiving environment. HGT from multicellular (eukaryotic) organisms, such as plants or insects, to other organisms is remarkably rare, occasionally being detected under optimized laboratory conditions. Current scientific knowledge supports the idea that non-sexual gene transfer of non-mobile DNA fragments between unrelated organisms (such as from insects to microorganisms) is extremely unlikely to occur under natural conditions and if it does happen it occurs on an evolutionary time span. The same accounts for HGT from orally ingested organisms to the bacteria in the gut of human, animal and or other organisms like predators or parasitoids of *Aedes aegypti.*

Literature reviews indicate that the probability of HGT between insects to other unrelated organisms is extremely unlikely to occur under natural conditions.

This above conclusion is confirmed by the United States Department of Agriculture (USDA) that reviewed the potential for horizontal gene transfer from genetically engineered fruit flies. (https://www.aphis.usda.gov/plant_health/ea/downloads/eis-gen-pbw-ff.pdf) (Accessed 20/09/2016).

Remobilization of the transposon
The newly inserted genes are located on a transposon, the *piggyBac* vector, which is highly stable in the *Aedes* genome even when exposed to exogenous transposase under a wide variety of conditions (additional information November 30, 2016). No instability in the transformed line OX513A been observed to date in over 115 generation equivalents. Data indicate that the vector in OX513A is highly stable in *Aedes aegypti*

Ad 2) Biological relevance of HGT
Although the probability of HGT between insects and other organisms is extremely unlikely to occur, it can never be excluded completely. If this would occur, this would lead to the transfer of the *tTAV* and *DsRed2* genes. Proteins encoded by these genes were demonstrated not to be toxic or allergenic based on feeding assays and on bioinformatic analyses, and are therefore unlikely to lead to adverse effects. Moreover, the tTAV and DsRed2 traits confer no known selective advantage to insects, and it is unlikely that this would be the case for other organisms. The tTAV is in fact intended to confer a selective disadvantage by design (i.e. a self-limiting trait) and after potential HGT most probably lead to a fitness reduction of the receiving organism. Even if HGT would occur, this would not lead to adverse effects

Conclusions
Based on literature review and data on stability of the insert in OX513A, the GMO Office concludes that the probability of HGT from OX513A to other organisms is extremely unlikely. If such HGT may occur, this is not considered to result in adverse effects on semi-natural or natural habitats.

2.3 Pathogens, Infections and diseases

The hypothesis tested is that OX513A or its progeny will be no more efficacious for disease transmission than a non-GM comparator.

The key considerations for this area of concern are (based on EFSA 2013)[2]:

1. Whether the rearing and release of OX513A could lead to an altered transmission range or frequency of pathogen transmission;
2. Whether the rearing and release of OX513A could lead to the introduction/emergence/selection of new pathogens or pathogen strains with increased virulence;
3. The potential for OX513A to release metabolites that alter the pathogen population;
4. The possibility for OX513A to introduce pathogens to environments where wild *Aedes aegypti* is not present and become a new source of disease;
5. Changes in the interactions with pathogens which result in an altered phenotype in OX513A that leads to increased transmission of pathogens.

Specific areas examined in addressing these considerations are: *vertical transmission, vectorial capacity, potential release of homozygous females, emergence of a non-target vector species, susceptibility to*

chemical insecticides, and *behavioral responses of OX513A to insecticides.* The potential for expansion of geographic range due to temperature response may have implications in this area of risk and has been covered in Section 2.1 of part C.

Ad 1) Altered transmission range or frequency of pathogen transmission due to rearing and release of OX513A

Rearing of the OX513A and potential release of homozygous females

To separate sexes and collect male pupae, mechanical size separation will be used as female pupae are larger than males. Data from previous OX513A regulated environmental releases demonstrate that a sorting accuracy of >99.9% is routinely achieved in operational scale projects. SOPs require a sorting efficiency of 0.2% or less. This means that ≤ 0.2% of the released OX513A will be females. During a suppression program, release rates as high as ~300 OX513A males/person/week are foreseen, this will result in a maximum (worst case) of about ~1 OX513A females/ person /week (0.2% x 300=0.6). OX513A females do not have a greater vectorial capacity including longevity and temperature range, nor a greater capacity for vertical transmission of virus than the wild type *Aedes aegypti* (see below) therefore no increase in virus transmission is expected.

The inadvertent co-release of OX513A mosquito females in the environment is not expected to increase virus transmission under the conditions encountered in the receiving environment

Vertical transmission of potentially released homozygous females

The passage of a disease causing agent or pathogen from an infected female to its offspring is known as vertical transmission. The capacity for the vertical transmission of dengue (DENV) 1,2,3,4 and chikungunya (CHIKV) was examined for OX513A and wild type comparator *Aedes aegypti* females. For both OX513A and the wild type, positive vertical transmission was observed for DENV 1, 3 and 4 but none for DENV 2 and CHIKV; no significant differences were observed between OX513A and the wild type.

Although studies did not include vertical transmission of the zika virus, there is no evidence to suggest that the capacity for vertical transmission of zika virus (ZIKV) should differ from that of other *Flaviviruses* (i.e. DENV) (additional information November 30, 2016).

Data indicate that OX513A does not have an increased capacity for vertical transmission of the viruses tested compared to its wild type counterpart

Vectorial capacity of heterozygous females

In addition to vertical transmission, the ability to transmit disease (vector capacity) has other entomological components including the vector-biting rate, vector density, vector survival and the duration of the virus extrinsic incubation period (EIP) in the mosquito. Hence the longevity of the mosquito is an important component of vectorial capacity as the shorter the lifespan, the less likely the transmission of disease will be. In order to transmit disease, the female mosquito must live long enough to pick up the virus via a blood meal, survive the extrinsic incubation period and then pass on the virus during a subsequent blood meal. Consequently, the longer-lived the females are the more likely they are to transmit pathogens. The average EIP

depends on temperature and is estimated to be 15 days at 25^0C and 6.5 days at 30^0C. A potential exists for hemizygous OX513A female progeny to survive due to the reported incomplete penetrance of the self-limiting trait. However the median survival of non-penetrant females is significantly reduced (2 days vs. 68 days). Approximately 20% of these females do survive long enough to take two blood meals and some produced two clutches of eggs. Survival of non-penetrant OX513A hemizygous females is less than that of the wild type *Aedes aegypti* counterpart. Therefore, disease transmission ability is expected to be reduced compared to wild type mosquito.

The longevity and therefore the disease transmission ability of female heterozygotes surviving in the environment due to incomplete penetrance of the tTAV trait are not increased relative to their wild type counterpart

Ad 2) Introduction/emergence/selection of new pathogens or pathogen strains with increased virulence due to rearing and release

Rearing of OX513A and rearing management practices

Rearing management practices, including blood-feeding females for egg production, and release procedures are well described in SOPs and are highly unlikely to contribute to the introduction of pathogens into the receiving environment or would lead to any altered transmission rate. Quality management procedures are in place to ensure OX513A quality for use in suppression programs, and routine quality control testing is done to ensure conformance with product specifications.

Rearing practices are unlikely to contribute to introduction of pathogens into the receiving environment

Ad 3) Potential for OX513A to release metabolites that alter the pathogen population

The only new proteins that are expressed in OX513A as a consequence of the genetic modification are the tTav and DsRed2 proteins. These proteins were not detected in the saliva of OX513A females.

There are no metabolites released that can alter the pathogen population

Ad 4) Potential for hazards related to disease transmission to derive from a potential failure of the OX513A rearing and release program.

Potential failure of the rearing and release program could lead to the introduction of a large number of female OX513A into the environment. It is very unlikely that this would lead to hazards related to disease transmission, since OX513A females do not have a greater vectorial capacity including longevity and temperature range, nor a greater capacity for vertical transmission of virus than the wild type *Aedes aegypti*.

It is unlikely that a potential failure of the OX513A rearing and release program would lead to hazards related to disease transmission

Ad 5) The possibility for OX513A to introduce pathogens to environments where wild *Aedes aegypti* is not present and become a new source of diseases

Emergence of a non-target vector species

Aedes albopictus has not been reported yet on Saba, but has been identified as a potential public health threat to Saba and placed on the Preliminary Alert List for the Leeward and Windward Dutch Islands. If *Aedes albopictus* where to arrive on Saba, the potential exists for *Aedes albopictus* to displace existing *Aedes aegypti* in the receiving environment during the OX513A program, or become established after elimination targets have been achieved, and result in an increased hazard of transmission of arboviruses like such as dengue, chikungunya virus and yellow fever on Saba. This has been examined by evaluating:
a) If *Aedes albopictus* is likely to occupy the *Aedes aegypti* ecological niche if *Aedes aegypti* numbers are reduced through the OX513A program compared to existing control measures; and,
b) If *Aedes albopictus* represents a greater threat for arbovirus transmission than *Aedes aegypti.*

Ad a) Potential for niche replacement by Aedes albopictus were it to arrive on Saba
It is often cited that the ecological niche of *Aedes aegypti* is distinct from *Aedes albopictus*, whereby *Aedes aegypti* persists predominantly as an urban vector, breeding in artificial containers and feeding almost exclusively on humans. *Aedes albopictus* is described as predominantly associated with peri-urban and rural environments and in addition to humans, feeds on a variety of mammalian and avian species. However, this habitat segregation could be due to larval competition when both species co-inhabit a same region. In absence of *Aedes aegypti, Aedes albopictus* has been shown to readily invade urban areas.
As both *Aedes aegypti* and *Aedes albopictus* are container-breeding species, capable of feeding on human hosts, a significant degree of habitat overlap could be expected in proximity to human habitation and larval cohabitation of the two species is frequently observed. In the context of Saba, the potential for *Aedes albopictus* to occupy the *Aedes aegypti* ecological niche if *Aedes aegypti* numbers are reduced can therefore not be excluded. This may only be relevant for the future, since *Aedes albopictus* is not yet reported on Saba.
There is uncertainty about replacement of *Aedes aegypti* by *Aedes albopictus* on Saba in case *Aedes aegypti* populations are suppressed

Ad b) Potential for increased virus transmission by Aedes albopictus were it to arrive on Saba.
Both *Aedes aegypti and Aedes albopictus* can transmit arboviruses such as dengue and chikungunya virus. The WHO states that in general the vector competence of *Aedes aegypti* and *Aedes albopictus* is similar, although *Aedes albopictus* is considered to have lower vector competence than *Aedes aegypti* for transmitting arboviruses, including zika. Vector competence was assessed experimentally. Given the conclusion of the study that *Aedes aegypti* and *Aedes albopictus* exhibit similar transmission potential for zika, the presence of *Aedes albopictus* would not represent an increased threat for zika virus transmission. Although *Aedes albopictus* is not currently reported as present on Saba, were it to appear there is no evidence that *Aedes albopictus* is a more competent vector for dengue, chikungunya and zika, than is *Aedes aegypti.*

If replacement of *Aedes aegypti* by *Aedes albopictus* on Saba would occur as a consequence of the OX513A release, it is considered to be unlikely that the human disease burden caused by dengue, chikungunya and zika would be increased compared to that of *Aedes aegypti*

The GMO Office notes that *Aedes aegypti* feeds almost exclusively on humans in daylight hours and typically rest indoors. In contrast, *Aedes albopictus* is usually exophagic and bites humans and animals opportunistically. *Aedes albopictus* has also been shown to exhibit strongly anthropophilic behavior similar to *Aedes aegypti* in specific contexts. In contrast to *Aedes aegypti*, that exclusively feeds on humans, zoonotic transmission of viruses to humans through *Aedes albopictus* therefore cannot be excluded. However, the chance that this would occur on Saba is considered to be small.
If replacement of *Aedes aegypti* by *Aedes albopictus* on Saba would occur as a consequence of the OX513A release, the risk of zoonotic transmission of viruses from an animal reservoir to humans is increased. However, the likelihood that this would occur on Saba is considered to be low

It should be noted that the issues described under 5) are not related to the genetic modification of OX513A, but are a consequence of effective suppression of the wild type *Aedes aegypti* on Saba. As such, replacement of *Aedes aegypti* by *Aedes albopictus* on Saba can occur with any vector control method that effectively suppresses *Aedes aegypti*.

Ad 6) Changes in the interactions with pathogens which result in an altered phenotype in OX513A that lead to increased transmission of pathogens
There are no differences found that would result in phenotypic changes, potentially leading to an increased transmission of pathogens by OX513A

Other aspects studied
Susceptibility to chemical insecticides
Susceptibility to chemical insecticides is an important feature for OX513A, as chemical insecticides can be used as part of a risk management strategy for elimination of the OX513A strain from the environment if so desired. OX513A and a sensitive wild type strain (control) were found to be equally susceptible to four commonly used insecticides (temephos, permethrin, deltamethrin and malathion) and showed similar significant survival to bendiocarb. *Two kdr* mutations associated with pyrethroid and DDT resistance were absent in the OX513A strain.
Data indicate that OX513A is equally susceptibility to the most commonly used insecticides as the respective non-modified comparator strains

Behavioral responses of OX513A to insecticides
Behavioral responses of OX513A male mosquitoes were overall similar to those displayed by the wild type strain, including significant contact irritancy to pyrethroids and significant spatial repellence to DDT.
Altered behavioral responses to certain chemical pesticides in OX513A relative to a wild type comparator were not observed

Conclusions
The GMO Office confirms the conclusion of Oxitec that it is unlikely that OX513A or its progeny will be more efficacious for transmission of arboviruses of significance to human health, such as dengue, chikungunya and zika than the respective non-GM comparator strains. This is based on the following conclusions.

1. OX513A does not have an increased capacity for vertical transmission of arboviruses such as dengue, chikungunya and zika compared to its non-modified counterpart;
2. Increased virus transmission is not expected due to the inadvertent co-release of OX513A mosquito females in the environment
3. Rearing practices are unlikely to contribute to introduction of pathogens

The GMO also concludes that the human disease burden caused by dengue, chikungunya and zika is unlikely to be changed when replacement of *Aedes aegypti* by *Aedes albopictus* on Saba would occur as a consequence of the OX513A release.

In addition, the GMO Office concludes that if replacement of *Aedes aegypti* by *Aedes albopictus* on Saba would occur as a consequence of the OX513A release, the risk of zoonotic transmission of viruses from an animal reservoir to humans is increased. However, the likelihood that this would occur on Saba is considered to be low.

As mentioned above, the GMO Office notes that potential consequences for human health and the environment of replacement of *Aedes aegypti* by *Aedes albopictus* on Saba (if this would occur) are not related to the genetic modification of OX513A, but are a consequence of effective suppression of the wild type *Aedes aegypti* on Saba. As such, replacement of *Aedes aegypti* by *Aedes albopictus* on Saba can occur with any vector control method that effectively suppresses *Aedes aegypti.*

The GMO Office further remarks that the most commonly used chemical insecticides for control of wild type *Aedes aegypti* may be used as part of a risk management strategy for OX513A.

2.4 Interaction with target organisms

The hypothesis tested is that OX513A will interact with target organisms (Aedes aegypti) at the release site as intended and that the interaction with target organisms does not lead to adverse effect in semi-natural or natural environments in comparison to current vector control programs.

The key considerations to test this hypothesis based on EFSA (2013)[2] are:

1. Observed resistance to the conditional insect control trait;
2. Reduction in efficacy or resistance development in the target organisms against the GM insect mediated effect;
3. Changes in interactions with target organisms arising from an altered genetic diversity of a reared GM insect population that may result in adverse effects;
4. Effects on target organisms due to the release of low-quality GM insects or non-GM insects that may result in adverse effects

Ad 1) Observed resistance to the conditional insect control trait
To date, resistance to the OX513A self-limiting trait has not been observed in over 115 generational equivalents reared since 2002. In addition, vector control organizations in areas where OX513A releases have taken place continue to monitor and control wild *Aedes aegypti* populations using existing interventions as part of Integrated Vector Management (IVM) programs and resistance to the self-limiting trait has not been reported.
Resistance of wild type *Aedes aegypti* to the OX513A trait has not been observed

Ad 2) Reduction in efficacy or resistance development in the target organisms against the GM insect mediated effect
Deliberate releases of OX513A have been conducted since late 2009 in collaboration with partners in both vector control programs and academia and significant (>90% based on egg counts) population suppression has been consistently observed in vector control projects.
Reduction in efficacy of OX513A has not been observed

Ad 3) Changes in interactions with target organisms arising from an altered genetic diversity of a reared GM insect population that may result in adverse effects
Existing control programs attempt to control *Aedes aegypti,* thus population suppression is an accepted and intended consequence of any control program. *Aedes aegypti* is not native to the release area thus a reduction in population size could help to restore the environment to the state prior to the establishment of the non-native pest (EFSA, 2013).
The intended effect is suppression of wild type *Aedes aegypti* population

Variability between the laboratory reared OX513A and the wild type mosquito strain
A key variable which would have an immediate effect on the wild population is the capacity of OX513A to mate with local *Aedes aegypti* and produce offspring. An increased mating competitiveness or variability in other reproductive parameters has not been observed
No variability between the laboratory reared OX513A and the wild type mosquito strain has been observed that would result in an adverse effect

Ad 4) Effects on target organisms due to the release of low-quality GM insects or non-GM insects that may result in adverse effects

Quality of laboratory reared strain and potential consequences of the release of female mosquitoes
EFSA (2013) suggests that impacts on target organisms could occur from unintended releases of untransformed fertile reared individuals, i.e. significant proportions of females when male-only releases are intended, or from insects contaminated with parasites or pathogens.
OX513A colonies are reared in a highly controlled environment under a quality management program to ensure that males released are of optimal health and quality to compete for mating wild females. Colonies are continually monitored and routinely subject to quality control testing for multiple parameters.
In an OX513A release program, there is the potential for small quantities of two types of female mosquito to be present in the environment (Figure 1); the first is (a) inadvertent co-release of homozygous (OX513A) females with homozygous males, and the second is (b) hemizygous OX513A progeny of released males that have mated with wild females and survive as a consequence of the incomplete penetrance of the self-limiting trait.

a) inadvertent co-release of homozygous (OX513A) females with homozygous males
Sorting efficiency of males and female *Aedes aegypti* OX513A in large scale production; involves the physical separation of male and female pupae that have been proven to be very efficient >99.9%. The release of homozygous OX513A females could impact the target *Aedes aegypti* population by potentially resulting in the generation of hemizygous OX513A progeny from an OX513A female and wild male *Aedes aegypti*. This scenario represents the intended effect on the population, which would be the same as an OX513A male mating with a wild female *Aedes aegypti*.

b) Survival of female hemizygous OX513A
There is a potential for hemizygous females surviving in the field as a result of incomplete penetrance of the self-limiting trait. The longevity of both OX513A males and females was found to be significantly lower than that of wild type. Further details are discussed under 2.3 of part C.

Conclusions
With respect to the interaction of OX513A with target organisms, the GMO Office concludes that:

1. Resistance to the conditional insect control trait has not been observed before
2. A reduction in efficacy, or resistance development in the target organisms against the GM insect-mediated effect is unlikely to occur in the period of the proposed environmental release, or in the longer term in the context of an IVM program
3. No changes in interactions with target organisms arising from an altered genetic diversity of a reared GM insect population that may result in adverse effects have been observed or are expected

No effects on target organisms due to the release of low-quality GM insects or non-GM insects that may result in adverse effects are expected

2.5 *Interactions with non-target organisms*

The hypothesis tested is whether OX513A, in comparison to the wild type Aedes aegypti, has no adverse effects on non-target organisms in semi-natural and natural areas taking into account current vector control.

The key considerations, based on EFSA (2013)[2], are:

1. Effects on abundance or species composition of:
   a) natural enemies/predators and the pest regulation service they provide;
   b) competitors of GM insects and the ecological functions they provide;
   c) pollinators and the pollination services they provide;
2. Effects on biodiversity, concerning species of conservation value (rare or threatened species), or of cultural value (aesthetic value) and food chain effects;
3. Effects on other ecosystem services including *Aedes aegypti* as a decomposer and as a resource for decomposers, nutrient cycling, water regulation and purification;
4. Effects on abundance or species composition of host plants or host animals and the ecosystem services they provide;
5. Effects of toxins or allergens associated with the GM insect on insectivorous vertebrates.

Ad 1) Effects on abundance or composition of species such as natural enemies/predators, competitors and pollinators

In this section it is evaluated whether suppression of local *Aedes aegypti* populations by the introduction of OX513A males on Saba can negatively affect populations of organisms that feed on *Aedes aegypti* or the ecological functions that they provide.

Aedes aegypti is an invasive species on Saba, it has therefore not co-evolved with other organisms in the receiving environment and is unlikely to represent a keystone species on which other organisms rely on for food.

In a recent risk assessment conducted for the release of *Aedes aegypti* carrying the intracellular bacterium *Wolbachia* it was concluded that *Aedes aegypti* was unlikely to have interactions with natural ecosystems, and that it was unlikely that the other species rely heavily or even moderately on *Aedes aegypti* as a food item or provider of ecosystem services.

Predators of Aedes aegypti

Non-target predator organisms may include invertebrate species such as *Toxorhynchites spp*., dragonflies, spiders, water-borne Crustaceans such as Mesocyclops, amphibians, such as frogs, lizards and geckos, fish, insect feeding birds, and bats. It should be noted that the scientific literature frequently indicates that mosquito predators are regarded as generalized predators. As *Aedes aegypti* is an invasive species on Saba, it is unlikely that there is a keystone species that is only feeding on *Aedes aegypti*.

Mammals (bats)
Since adult mosquitoes remain indoors most of the time they would not form a large proportion of the diet of any species typically found outdoors. *Aedes aegypti* mosquitoes are active in the day whereas bats are principally active at dawn and dusk.
The American Mosquito Control Association (AMCA) reviewed the role of bats for mosquito control, indicating that although bats do eat mosquitoes, the consumption of mosquitoes by bats comprised of less than 1% of the gut contents of wild caught bats in the studies reviewed to date, and other insects, such as moths provide better nutritional value. An analysis of the diet through stomach content analysis or fecal pellet analysis showed that bats are opportunistic feeders. Therefore it is unlikely that OX513A would adversely affect predatory mammals.

Birds
Insectivorous birds of the order Passeriformes which may potentially feed on mosquitoes may be found outdoors in the release area on Saba, notably the scaly-breasted thrasher, the pearly-eyed thrasher, the trembler, the lesser Antillean bullfinch, and the blue-crowned Euphonia are reported. Perhaps the most frequently anecdotally cited bird regarding the consumption of mosquitoes is the purple martin (*Progne subis*), the largest species of martin in North America; however both the American Mosquito Control Association and the Purple Martin Conservation Association (PMCA) declare that this is erroneous and not supported by evidence. There is temporal isolation between the purple martin and the mosquito activity patterns, with the birds and mosquitoes not flying at the same times or altitudes. Mosquitoes only form a small part of the overall diet of the birds. Therefore it is unlikely that OX513A would adversely affect predatory birds.

Amphibians
The only amphibian reported on Saba is the piping frog (*Eleutherodactylus Johnstonei*).
However, in the context of potential use as biological control agents for mosquitoes, amphibian predators such as frogs and reptiles are not recognized as consumers of mosquitoes in sufficient numbers for mosquito control. Also there is unlikely to be significant habitat overlap with piping frogs in the same breeding sites as *Aedes aegypti,* as *Aedes aegypti* is more associated with human habitats and piping frogs are reported on Saba to occur in primary forest. Therefore it is unlikely that OX513A would adversely affect amphibians.

Invertebrates
Insects from the orders Odonata, Coleoptera, Diptera and Hemiptera all may opportunistically feed on mosquito adults or larvae encountered incidentally in the environment. These are generalist predators that are not reliant on a single species of mosquito for their food source. Indoors, household spiders may have the most likely opportunity to feed on *Aedes aegypti* adult mosquitoes. Studies looking at the efficacy of *Aedes aegypti* predators for pest control have noted a number of household spiders which eat *Aedes aegypti*. In Malaysia spiders of the genera Araneus and Neoscona tested positive for gut content containing *Aedes aegypti*, indicating that they were natural predators of *Aedes aegypti.* Further reports from Thailand and India show that the spider

Crossopriza lyoni is also a predator of *Aedes aegypti*. These spiders all have a broad distribution worldwide. However, the newly produced proteins in OX513A have not been demonstrated to have toxic or allergenic properties, making it unlikely that OX513A would adversely affect predatory invertebrates like spiders.

Aedes aegypti larvae are known to form part of the diet of carnivorous mosquito species such as *Toxorhynchites* which have been evaluated for biological control of mosquito larvae.
These mosquitoes are reported to inhabit most of the tropical regions of the world are most commonly found in tree holes, bromeliads and other ephemeral containers. The oral exposure of the predatory invertebrate *Toxorhynchites spp.* to OX513A has been evaluated experimentally and there was no significant impact on the development, fecundity and longevity of two predatory T*oxorhynchites* species (*Tx. amboinensis and splendes*) that were fed exclusively with OX513A larvae, compared to a diet consisting of non-modified wild type larvae. Therefore it is unlikely that OX513A will adversely affect predatory invertebrates.

Fish
Certain fish species are recognized as predators of mosquitoes when sharing mosquito breeding habitats, and have been evaluated as a potential mosquito control agent. *Poecilia reticulate* is used on Saba as part of the vector control program for *Aedes aegypti.* To determine the effects of ingestion of larvae and pupae of OX513A on the guppy fish *Poecilia reticulate* a 14-day feeding study was performed. No significant difference was observed in mortality, fish length, weight, appearance and behavior in fish fed with OX513A and the non-modified wild type control.

Parasitoids of Aedes aegypti
No specific parasitoids are known to be associated with *Aedes aegypti*. The nematodes *Romanomermis culicivorax* and *Strelkovimermis spiculatus* from the family Mermithidae are generalist parasitoids infecting a number of mosquito species. Although these species are known to infect *Aedes aegypti* in the laboratory, they have not been found infecting natural populations.

Aedes aegypti's role in pollination
Although female *Aedes aegypti* mosquitoes take blood meals from humans in order to obtain protein for ovary development, mosquitoes of both sexes require plant juices as an energy source. Floral nectars are the best-known sources, but mosquitoes are also known to obtain sugars from extra-floral nectaries, damaged fruits, damaged and intact vegetative tissues, and honeydew. However, there are no reports that *Aedes aegypti* is a pollinator for any plant species.

Data indicate that abundance or species composition of representatives of ecological functions on Saba are unlikely to be affected by OX513A-induced suppression of local *Aedes aegypti* populations

Ad 2) Effects on biodiversity, concerning species of conservation value (rare or threatened species), or of cultural value (aesthetic value) and food chain effects

An analysis of protected, charismatic and valued species that may occur in the release areas on Saba has been conducted using detailed online searches from sources such as the International Union for Conservation of Nature (IUCN) Red List. Other sources were the biological inventory reports from the Caribbean Research and Management of Biodiversity foundation, and the Dutch Caribbean Nature Alliance.
The snake red-bellied racer (*Alsophis rufiventris*) is listed as endangered on Saba, however there is negligible potential for exposure to OX513A due to lack of habitat overlap, and its non-insectivorous feeding habits.
For the Saban anole *(Anolis sabanus)* the potential for exposure due to habitat overlap may be considered moderate.
For other *Anoles spp.* it was found that ants primarily comprise the diet by number, but lepidopteran larvae, and orthopterans comprise the main food items by bulk. It is therefore unlikely that the Saban anole could be adversely affected by OX513A.
Data indicate that adverse effects of OX513A on protected, charismatic and valued species of Saba are unlikely

Ad 3) Effects on other ecosystem services including *Aedes aegypti* as a decomposer and as a resource for decomposers, nutrient cycling, water regulation and purification

Aedes aegypti as a decomposer
Aedes aegypti larval development is in an aquatic environment, predominantly in artificial breeding sites that frequently contain detritus metabolized by microbial communities.
Although there is limited research in this area, it is thought that *Aedes aegypti* survives on the micro-organisms that break down the detritus, and it is the nitrogen, phosphorus and carbon availability that influences relative abundance of *Aedes aegypti* in breeding sites
Although *Aedes aegypti* occupies man-made or artificial containers where plant and animal detritus is broken down, it is unlikely to be the mosquito itself contributing to direct decomposition of the material. It is more likely that the mosquito mainly acts as a consumer of the elements from the breakdown of detritus by other organisms, rather than as a decomposer.

Aedes aegypti as a resource for decomposers
Fungi such as *Metarhizium anisopliae*, a well-known entomopathogenic fungus which is found in the soil, is capable of infecting *Aedes aegypti* eggs. The fungus *Beauveria bassiana* has also recently been evaluated as a potential biological control agent for *Aedes aegypti.* However, *Aedes aegypti* is not known as a specific resource for decomposers on Saba.
Data indicate that adverse effects on decomposition as a consequence of the OX513A releases on Saba are unlikely

Ad 4) Effects on abundance or species composition of host plants or host animals and the ecosystem services they provide
Given the data that are described above, it is unlikely that abundance or species composition of host plants or host animals and the ecosystem services they provide are affected by OX513A.

Ad 5) Effects of toxins or allergens associated with the GM insect on insectivorous vertebrates
Given the above describe data and the fact that the newly produced proteins in OX513A are not toxic or allergenic, no adverse effects on insectivorous vertebrates are expected.

Conclusions
The GMO Office confirms conclusions from Oxitec in that OX513A, in comparison to the non-modified *Aedes aegypti*, is unlikely to adversely affect non-target organisms in semi-natural and natural areas, also taking into account current vector control.
This based on the following information:

1. As an invasive species, *Aedes aegypti* is not considered a keystone species on Saba
2. OX513A is unlikely to adversely affect natural enemies/predators, based on data that indicate that:
   - there is lack of habitat overlap or overlap in activity between *Aedes aegypti*/OX513A and predators
   - *Aedes aegypti* only forms a small or negligible part of the diet of predators
   - predator(s) is (are) only generalist
   - toxicity studies with OX513A as a sole diet demonstrate no adverse effects on predators
   - the newly expressed proteins in OX513A are not toxic
3. OX513A is unlikely to adversely affect other ecosystem services since *Aedes aegypti* does not play a role in decomposition, nutrient cycling or pollination
4. OX513A is unlikely to adversely affect protected and charismatic species due to lack of habitat overlap and not being a part of the diet of these species on Saba

2.6 Environmental impacts of the specific techniques used for management

The hypothesis tested is that the specific management practices used with respect to OX513A do not have adverse environmental impacts on the receiving environment.

The key considerations based on EFSA (2013)[2] guidance are:

1. Changes in (current) vector management practices associated with the production and subsequent release of OX513A;
2. Potential adverse effects to the environment resulting from these changes in management practices;
3. The overall risks associated with changes in management of the production and release of OX513A and their environmental consequences.

Ad 1) Changes in (current) vector management practices associated with the production and subsequent release of OX513A
The current vector control measures, the use of biological control agents, will remain applicable when OX513A is released. OX513A is seen as an additional tool to control *Aedes aegypti.* A site visit of Oxitec to Saba occurred early in 2016, and discussion with vector control

managers confirmed that the principle means of control are with thebiological control agents larvivorous guppy fish (*Poecilia reticulata)* and *Bacillus thuringiensis* var. *israelensis* SH 14 (Bti). Also pesticides are applied as vector control. If vector control methods were to change on Saba, it would be through the adjustment of pesticide applications so as to avoid adulticide applications the same day as OX513A releases are taking place.

Risk management strategies for rearing and release of OX513A
SOPs are in place for emergency procedures that cover circumstances which have the potential to cause inadvertent release through damage to the integrity of the rearing facility. These include, but are not limited to natural events such as adverse weather, long term power outages, fire and vandalism. Additionally, SOPs including cleaning and waste disposal procedures and entry and exit are in place to prevent the inadvertent release of OX513A. SOPS are also in place to prevent any inadvertent release as a consequence of egg production, transportation, adult production, transport, release, ovitrap processing and analysis.

Potential release of tetracycline into the environment
During the rearing of OX513A in the mobile rearing unit, OX513A is grown in the presence of tetracycline. Waste water will be disposed through residential waste disposal systems. The maximum amount of tetracycline to be discarded as a result of the rearing practices for OX513A males has been estimated as 1.7 grams/week. The amount of tetracycline generated by the rearing facility is negligible as compared to the amount of tetracycline generated by human medical applications. Disposal of waste water from the rearing unit in a manner consistent with that of the residential waste disposal systems would thus represent a negligible contribution to tetracycline in the environment on Saba.

Potential for tetracycline resistant bacteria to be released into the environment
Due to the rearing of OX513A in the presence of tetracycline, the potential for insect gut bacteria to acquire tetracycline resistance genes and to spread those genes to other organisms in the environment upon their release was considered. The potential for this to occur was considered to be negligible as gut bacteria are expelled during mosquito metamorphosis from larvae to adults. Pupae and adults are not subsequently treated with tetracycline during the rearing process and pupae are washed in fresh water several times during the sorting process.

Conclusions
Conventional vector control measures at the release site are not anticipated to be modified as a result of the OX513A release program Therefore no adverse effects on human health and the environment are foreseen as a consequence of a change in management, as compared to effects of the vector control program in place.
The GMO Office confirms the above conclusion

2.7 Impact on human and animal health

The hypothesis tested is that the environmental release of OX513A will not have an adverse impact on human and animal health in comparison to the non-GM comparator.

The key considerations based on EFSA (2013)[2] are:

1. Potential toxic effects of the new compound(s), their derived metabolic products and/or the GM insects to humans and animals, e.g. qualitative or quantitative change in the production of toxins by the GM insects when compared with their non-GM comparators;
2. Potential allergenic effects of the new compound(s), their derived metabolic by-products and /or the GM insects to humans;
3. Loss of immunity in the human population and reliance on continued long-term positive effects of vector suppression or replacement strategy.

The interaction between humans and the released male OX513A are expected to be limited to accidental dermal or ocular contact or by accidental inhalation. The most plausible route of human exposure would be through biting of OX513A females.

Ad 1) Potential toxic effects of the new compound(s), their derived metabolic products and/or the GM insects to humans and animals, e.g. qualitative or quantitative change in the production of toxins by the GM insects when compared with their non-GM comparators
Ad 2) Potential allergenic effects of the new compound(s), their derived metabolic by- products and /or the GM insects to humans

Exposure characterization.
OX513A has been reared and used in contained use and in regulated environmental releases since 2002 (over 115 generations) with no reported adverse effects on the staff working with the strain.

Exposure through OX513A saliva: the bite of an adult female
A small amount of OX513A females will be introduced in the environment as a consequence of the sorting process and of the incomplete penetrance of the trait in offspring of OX513A and wild type females. These females could bite and thereby expose humans to tTAV and DsRed2 proteins that are potentially present in saliva. tTAV and DsRed2 proteins were not detected in the saliva of OX513A females, therefore humans will not be exposed to these proteins through biting.

Toxicity and allergenicity of the OX513A traits
Independent bioinformatic searches performed with tTAV or DsRed2 proteins did not uncover any concerns related to potential allergenicity, allergenic cross-reactivity or potential toxicity. Updated (2017) bioinformatics analysis confirmed these results (additional information January 2017). Also scientific literature searches in the PubMed (NCBI) database for tTAV and DsRed2 or other genetic elements in the OX513 construct did not indicate any safety issues.

This is consistent with findings of both the USDA and FDA in independent reviews on the safety of the tTAV or DsRed2 traits in other genetically modified organisms (plants, insects) and are confirmed by expert opinions.

Potential for exposure of humans and animals to tetracycline resistance genes.
No tetracycline resistance genes have been used in the construction of OX513A. However tetracycline resistant bacteria could develop within the controlled conditions of the rearing process within the MRU. This is considered to be unlikely since gut bacteria are expelled during mosquito metamorphosis from larvae to adults. Also antimicrobial resistance arising in bacteria in the rearing water and the subsequent transfer of this trait to other bacteria that could cause food or water-borne diseases would be highly unlikely due to the short duration of the mosquito life cycle.

Ad 3) Loss of immunity in the human population and reliance on continued long-term positive effects of vector suppression or replacement strategy
It has been suggested that, paradoxically, vector control could lead to increased dengue transmission through reduced immunity in the human population as less people are exposed. The impact of a reduction in vector population density on the transmission threshold of dengue has been estimated in relation to pupae per person (as a proxy for adult mosquito population), ambient temperature and herd immunity. It was estimated that the average pupae per person decreased in the treated area from 0.7 pre-treatment to 0.04 post-treatment, which would be sufficient to prevent epidemic transmission under these conditions, or indeed under the most adverse conditions modelled for a naive human population with 0 % sero-prevalence. This indicates that vector control by OX513A would not lead to increased dengue transmission.

Conclusions

1. Potential toxic effects of the new compound(s), their derived metabolic products and/or the GM insects to humans and animals have not been identified
2. Potential allergenic effects of the new compound(s), their derived metabolic by- products and /or the GM insects to humans have not been identified
3. Loss of immunity in the human population and reliance on continued long-term positive effects of vector suppression or replacement strategy do not pose any additional risk, relative to existing or intended interventions

The GMO Office confirms the above conclusions.

3 Overall risk evaluation and conclusions

3.1 Uncertainty in the Environmental Risk Assessment

Uncertainty in the ERA may arise from various sources such as variability in parameters measured, assumptions and extrapolations made, and from limitations of the current scientific literature. The ERA for the deliberate environmental release of OX513A on Saba is

qualitative in nature, while often reliant on quantitative measurement endpoints to formulate conclusions. In qualitative risk assessments, expert judgment in the field is the basis of informing the degree of uncertainty.
The key areas of uncertainty which have been identified through this ERA are addressed below:

● What is the likelihood of dispersal of OX513A and their progeny beyond Saba?
There is uncertainty on the likelihood of dispersal of OX513A and their progeny beyond Saba, but this level of uncertainty is low since OX513A released males are demonstrated to have limited dispersal due to the geographic isolation of Saba. This is based on results from previous trials of OX513A in other countries, information from published literature and the location and features of Saba.
There is a higher level of uncertainty about passive dispersal by transport of OX513A by airplanes or boats. However, even if passive dispersal will take place there is sufficient information from previous field releases that the OX513A males live only for a short time. If these OX513A males are able to mate with wild type females after their dispersal, their offspring will die as a consequence of the presence of the lethality trait.

● What is the likelihood of establishment of OX513A on Saba?
There is a low level of uncertainty about the likelihood of establishment of OX513A on Saba. This is based on the information from previous field releases of OX513A, indicating that the lifespan of the released insects was approximately 1-3 days and that more than 95% of progeny die before reaching adulthood. This is also based on evidence from the scientific literature on potential sources of tetracycline.

Ongoing monitoring of the local mosquito population is integral to the OX513A program and is fundamental to vector control activities generally. Post suppression phase monitoring will allow an ongoing assessment of the lack of establishment of OX513A on Saba.

● What is the likelihood of inadvertent release of OX513A?
Uncertainty related to the likelihood of inadvertent release of OX513A is decreased based on the containment measures at the MRU and risk management strategies in place, as referenced throughout the ERA. Rearing would be conducted consistent with ACL2 containment levels. Staff working at the MRU would be under the supervision of an Oxitec site manager with a high degree of experience in handling OX513A and other GM insects in contained conditions. Locally recruited staff working in the MRU would be trained in the Standard Operating Procedures (SOPs) for the OX513A program as referenced throughout the ERA.

Some uncertainty with respect to inadvertent release exists in case of adverse weather conditions. This uncertainty is minimized by a Hurricane Preparedness Policy being in place, where adult and larval insect life stages would be killed within an appropriate time frame in advance of an adverse weather warning of a defined magnitude. Even if some OX513A were to escape containment, the intended effect of the self-limiting trait would prevent establishment in the environment.

3.2 *Conclusions*

This Environmental Risk Assessment has been conducted following the methodology and guidelines described in the EFSA Guidance on the environmental risk assessment of genetically modified animals (EFSA, 2013). Seven areas of concern have been evaluated according to the six steps recommended in this Guidance as an interpretation of Directive 2001/18/EC.

The risk assessment was carried out considering one of the following comparators:

a) Wild type *Aedes aegypti* (unmodified laboratory strains)
b) Wild *Aedes aegypti* (native wild local populations)
c) Existing control measures for *Aedes aegypti*

A comparative safety assessment has been conducted using a weight-of-evidence approach which considers the molecular characterization of OX513A together with its phenotypic and behavioral characteristics. The results of this comparative safety assessment demonstrated that the only differences of biological relevance are the expression of the two introduced traits; the self-limiting trait (tTAV) and the fluorescent marker trait (DsRed2). A significant focus of the environmental risk assessment was thus to characterize tTAV and DsRed2 with respect to potential adverse effects on human health and the environment.

For all areas of concern there was adequate information to conclude that potential adverse effects on human health and the environment as a consequence of the intended release of genetically modified OX513A on the island of Saba are considered to be negligible as compared to effects of non-modified *Aedes aegypti* and in the context of standard vector control. Measures have been described regarding physical containment and procedural controls which are intended to prevent the unintended environmental release of OX513A as a matter of good practice and consistent with common conditions for regulatory compliance. While the measures described may be characterized as risk management measures, they are not in response to any identified risk in this ERA for OX513A on Saba.
In all regulated environmental releases to date OX513A has performed consistently with respect to parameters identified in risk assessment submissions, and no unanticipated results nor unintended effects have been observed, nor required regulatory reporting.

The GMO Office concludes that potential adverse effects on human health and the environment as a consequence of the potential release of genetically modified OX513A on the island of Saba, under the conditions as described in the documentation of Oxitec and in the context of standard vector control, are considered to be negligible as compared to effects of non-modified *Aedes aegypti*. This is in line with recent related environmental risk assessments such as from Brazil3,4 and the United States Food and Drug Administration5.
The GMO Office recommends post-release monitoring by an independent party, as advised by the WHO6, on a monthly basis until populations of OX513A are below the level of detection.

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