

# Appendix 1

Ref. No.	A	S	I	P			Office use
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CAYMAN ISLANDS GOVERNMENT



Cayman Islands, Environmental Centre . 580 North Sound Road . PO BOX 10202GT . George Town . KY1-1106

CAYMAN ISLANDS

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## PERMISSION TO IMPORT & RELEASE ALIEN OR GENETICALLY ALTERED SPECIES

Once date-stamped by the Department of Environment, this permission constitutes an agreement between the following Parties:

### Department of Environment

(on behalf of the National Conservation Council)

and

### The Applicant

The Applicant undertakes to import alien species as follows:

<b>APPLICANT NAME</b>	Dr. William Petrie
<b>POSITION</b>	Director
<b>INSTITUTION</b>	Mosquito Research and Control Unit
<b>ADDRESS</b>	Marco Giglioli Centre 99 Red Gate Road P.O. Box 486 Grand Cayman KY1-1106 Cayman Islands
<b>PHONE</b>	949 2557
<b>EMAIL</b>	William.Petrie@gov.ky
<b>Intended Date Of Importation</b>	June 2016

All particulars in the attached documents make up part of this Permit.

Importation requires a separate Permission by the Department of Agriculture.

Under the National Conservation Law and the Animals and Plants Laws the import and release of alien or genetically altered species without permission of the Department of Agriculture and the National Conservation Council respectively is illegal.

This Permit serves as

- 1) Advice to the Department of Agriculture on the application to import alien or genetically altered species;
- 2) Permission or denial to release alien or genetically altered species.

1. SPECIES & QUANTITIES TO BE IMPORTED			
row	Species Common Name	Species Scientific Name	Quantity
1	Yellow fever mosquito	<i>Aedes aegypti</i> (OX513A)	1.650 Kg (eggs)
Add rows as necessary, or attach list separately.			

For those species which are IUCN listed certification of captive breeding or sustainable harvest should be provided along with application to import.

2. PURPOSE OF IMPORT			
	Pet		Breeding
	Display		Farming or other consumption
	Human Interaction		Retail
X	Other: Mosquito population control		
Tick as appropriate. Fill in if other purposes.			

3. LOCATION WHERE SPECIES WILL BE KEPT
<p>The eggs would be sent from Oxitec Limited in UK approximately once a month and then hatched and processed in a dedicated ACL-2 production facility situated in MRCU compounds, 99 Red Gate Road, George Town, Grand Cayman. Up to 600,000 Oxitec males (strain OX513A) would be produced every week for up to a nine month period.</p> <p><b>Rearing and Sorting</b></p> <p>Eggs would be vacuum hatched and larvae distributed into trays. Over the following 7 to 9 days, the larvae would be fed fish food until pupation. After pupation, larvae and pupae would be separated using special mechanical and/or chemical processes. Pupae would then be separated by sex on the basis of size using specialised equipment (males are smaller than females) and aliquoted in release devices. Male pupae (a sub-sample) are therefore manually screened for the presence of females and re-sorted if the female threshold criteria is not met (see section 5). Male adults would be provided with sugar solution until they reach sexual maturity and then released within the following seven days.</p> <p>A general description of the activities being proposed/requested/permited.</p>

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#### 4. BIOSECURITY MEASURES

Biosafety Standard Operating Procedures (SOP) are in place and staff will be trained to ensure conformance with these standards.

The production facility is built to Arthropod Containment Level 2 (ACL-2) standards to minimise unintentional escapees from the unit. A double entry door will secure access, a sieve on the sink drains will prevent any biological material from escaping. Mosquito life stages not used for the release or other procedures are frozen at -15°C or colder for more than 12 hours and disposed of.

The inserted trait is self-limiting in the environment, whereby released mosquitoes will die in the environment and the transgene will rapidly disappear following cessation of releases (26). *Aedes aegypti* are not native to the Cayman Islands and have previously been eradicated on the islands. The strain is fully susceptible to insecticides used in mosquito control programs.

Indicate whether biosecurity measures (anti-escape & species/people security) are in place or intended.

#### 5. HEALTH & SAFETY CONSIDERATIONS / MEASURES TAKEN

##### **Tetracyclines in the environment:**

Tetracyclines are an antidote to the self-limiting technology, whereby if transgenic larvae are being reared in the presence of tetracycline they will survive to adulthood. An estimated threshold level of 0.01 to 0.1 µg/ml of tetracyclines is required to see an effect on survival of the mosquitoes in the laboratory. The literature suggests that there is an average concentration of 0.00066 µg/ml in the environment, well below the threshold required for survival of OX513A mosquitoes.

Further reading on Tetracyclines in the environment:

Curtis Z, Matzen K, Neira Oviedo M, Nimmo D, Gray P, Winskill P, Locatelli MAF, Jardim WF, Warner S, Alphey L, Beech C. 2015. Assessment of the impact of potential tetracycline exposure on the phenotype of *Aedes aegypti* OX513A: implications for field use. *PLOS Neglected Tropical Diseases*. 9(8): e0003999

Duff, B. (2005). Presence of tetracycline antibiotics in surface water. A study of the presence/absence of tetracycline in the Racoon river watershed, Des Moines water works laboratory.

Gulkowska A, He Y, So MK, Yeung LW, Leung HW, Giesy JP, et al. The occurrence of selected antibiotics in Hong Kong coastal waters. *Mar Pollut Bull*. 2007; 54(8):1287–93. doi: 10.1016/j.marpolbul.2007.04.008 PMID: 17553528.

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Le-Minh N, Khan SJ, Drewes JE, Stuetz RM. Fate of antibiotics during municipal water recycling treatment processes. *Water research*. 2010; 44(15):4295–323. Epub 2010/07/14. doi: 10.1016/j.watres. 2010.06.020 PMID: 20619433.

Locatelli MA, Sodre FF, Jardim WF. Determination of antibiotics in Brazilian surface waters using liquid chromatography-electrospray tandem mass spectrometry. *Archives of environmental contamination and toxicology*. 2011; 60(3):385–93. Epub 2010/06/11. doi: 10.1007/s00244-010-9550-1 PMID: 20535610.

McQuillan D, Hopkins S, H., Champman T, Sherrell K, Mills D. Drug Residues in Ambient Water: Initial Surveillance in New Mexico, USA. 7th Annual New Mexico Environmental Health Conference; October 28–30 2002; Albuquerque, New Mexico 2002.

#### **Effect on Human Health:**

In order to present a risk to human health, the expressed proteins tTAV and DsRed2 would have to (a) be expressed in salivary glands, (b) be secreted into the saliva, and (c) be toxic or otherwise hazardous to humans if injected in relevant quantities. Of these, (a) and (b) relate to potential exposure, while (c) relates to the hazard incurred by the gene products. Addressing (c) first, Oxitec has commissioned extensive analysis on the safety of the expressed gene products using internationally recognized criteria, and it has been determined that there are no toxicological or allergenicity concerns from the gene products (tTAV and DsRed2) expressed in the OX513A *Ae. aegypti*. In addition tTAV expression is reported in a wide variety of organisms [2, 3] and a feeding study using OX513A [4] showed no adverse effects. Should a person be bitten by a OX513A female, the risk to human health is thus minimal as bites from Oxitec females are no different from wild females apart from the fact that inadvertently released females will be disease free.

Male mosquitoes do not bite or transmit diseases. Though the aim is to only release males which do not bite, there is a small chance that females will be released which come through the mechanical sorting process. The sorting is performed using a proprietary piece of equipment known as a wire sorter. This equipment sorts male and female pupae by size, males are smaller and can pass through the wire grid whereas females are larger and cannot. Quality assurance procedures are in place during the sorting and in practice, efficiencies of sorting result in very low numbers of females (0.07% in Cayman (1) and 0.02% in Brazil (27). Therefore although there is the potential for females to be released, these are unlikely to be fit and survive well in the environment and are unlikely to survive long enough in the environment to acquire and transmit diseases.

In the laboratory, under optimal conditions, the survival of functional adults is 5% or less. Evidence suggests that survival is much lower than this in the field; monitoring of ovitraps in Cayman in 2010

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(1) failed to detect any evidence of the transgene in eggs from the field two weeks after releases had stopped, and similar results were seen in Panama (26). Additional studies investigating the characteristics of these low numbers of heterozygous survivors have also been carried out. When reared through immature stages at temperatures other than the ideal 27°C, the number of surviving adults is further reduced.

Vectorial competence is the intrinsic ability for the mosquito to become infected and subsequently transmit the pathogen [5]. Laboratory studies detected no significant differences in infection rates between OX513A strain and the comparative wild type strain for dengue serotypes 1-3 and chikungunya. The only significant difference detected was that the comparator strain females had a higher infection rate for dengue 4 than OX513A. Overall the impact of the transgene insertion had no effect on dengue or chikungunya susceptibility of the OX513A strain in this study.

## 6. LOCATION WHERE SPECIES WILL BE RELEASED

The releases are planned to be conducted in the South part of West Bay. The area comprises about 1,800 inhabitants over 300 acres. West Bay is one of the hotspots for *Aedes aegypti* and transmission of mosquito borne viruses and thus a suitable place to evaluate OX513A mosquitoes.

### Releases

3 to 7 times a week, the release devices would be placed in large containers and brought to the treated area in West Bay where they would be released following a predetermined path enabling an even distribution of the Oxitec males in the area.

### Monitoring

- Ovitrap (which mimic natural oviposition sites in which females lay eggs)
- BG- Sentinel adult traps (Biogents) chemically attract all mosquito species towards them and the mosquitoes are subsequently trapped.
- Both of these recapture methods will be used in and around the release area; and an equivalent untreated control site. This is specialised monitoring which is conducted alongside routine island-wide MRCU monitoring and control activities for all mosquito species.
- Ovitrap will be serviced weekly, BG Sentinels weekly or daily at different stages of the experiment and the mosquitoes collected. Eggs from the ovitrap will be hatched and the resulting larvae analysed for paternity and/or species. The GM mosquitoes have an internal fluorescent marker (DsRed2) and the recaptured mosquitoes are examined by microscopy-allowing the MRCU to track gene flow through the wild population, and the GM mosquito dispersal throughout the study site and beyond.

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**Fluorescent marker**

The introduced DsRed2 protein is a fluorescent marker that allows the OX513A to have a fluorescent phenotype when excited by illuminations of a specific wavelength under a special microscope. Expression of this non-toxic protein will facilitate the detection of the OX513A monitoring

**Other measures**

Existing mosquito controls will continue to be used if necessary in both treatment and control sites, although will need co-ordination with release dates.

**Dispersal**

The dispersal of OX513A adults by spontaneous flight, has been shown to be less than 100 meters [11] and the average distance travelled by wild adults is well-known to be less than 200 meters [11-25]. Therefore the risk of dispersal of adults into other countries is extremely low, especially from an island. Adults could move by passive transport further and so can eggs (in cars, plant pots, plastic containers etc.); however any offspring that inherit the OX513A gene would have a large selective disadvantage (a maximum of 5% survive) and would be rapidly lost from the recipient population. In addition, control of the *Aedes aegypti* population where the OX513A males are released (previously shown to take about 6 months to get over 80% reductions) would significantly reduce the risk of egg dispersal as far less eggs would be produced.

A general description of the activities being proposed/requested/permitted.

**7. OTHER RELEVANT INFORMATION TO BE CONSIDERED**

The mosquito-borne diseases dengue, chikungunya and Zika are transmitted primarily by the *Aedes aegypti* mosquitoes. These diseases generally cause symptoms such as fever, headache, vomiting, diarrhoea, rash and joint pain; in severe and complicated cases chronic arthritis, haemorrhagic fever, dengue shock syndrome, Guillain-barré syndrome and microcephaly.

It is not possible to prevent the introduction of such diseases to the Cayman Islands; hence MRCU currently conducts adulticiding and larviciding of all mosquito species across the islands to prevent transmission. However, insecticide resistance is increasing. It is conventionally accepted that novel strategies will have to be employed alongside traditional methods to control mosquito-borne diseases. Scientists at Oxitec ([www.oxitec.com](http://www.oxitec.com)), a UK based biotechnology firm, have inserted a self-limiting gene into a strain of *Aedes aegypti* (OX513A). The technology works by releasing males, as they do not bite or transmit diseases, in the environment. These genetically modified males compete with wild

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males for females. When the wild females are inseminated by OX513A males (females typically mate once in a lifetime) their progeny receive the self-limiting gene and die before reaching adulthood. With sustained release of the OX513A mosquitoes over a period of time, the population of *Aedes aegypti* in the area is anticipated to be significantly suppressed. In studies in Brazil (27), Cayman Islands (1) and Panama (26) local populations of *Aedes aegypti* have been suppressed by over 90%. Suppression of *Aedes aegypti* to this extent could greatly reduce the risk of transmission of dengue, chikungunya and Zika transmission in the Cayman Islands.

*Aedes aegypti* is not native to the Cayman Islands, it is an introduced peri-domestic species 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 as well as ephemeral containers, such as puddles. Once eclosed the adult *Ae. aegypti* mosquitoes live in and around houses where females have easy access to the blood meal necessary for egg development. As such suppression of the local *Ae. aegypti* population is thus not expected to alter population dynamics of non-target organisms as it is not a keystone species in the local food chain, nor does it perform services like pollinating flowers. Reducing the *Ae. aegypti* population is thus unlikely to have a negative impact on the environment.

The inserted trait is self-limiting in the environment, whereby released mosquitoes will die in the environment and the transgene will rapidly disappear following cessation of releases (26).

Animals that eat self-limiting *Aedes aegypti* will be exposed to nutritional elements- protein, fat, sugar and others- as they would be from eating any mosquito: they cannot take up genes from this route, and the introduced genes are non-toxic and non-allergenic and thus do not impact organisms which eat them. The effect of the self-limiting gene on the offspring of OX513A is through causing subtle adjustments to their cells in a way that is non-toxic to organisms that eat those cells.

Vertical gene transfer refers to the ability of genes to move via sexual reproduction, to a closely related species or wild relative. *Ae. aegypti* mating is extremely species-specific. There have been reports of mating with the related species *Ae. albopictus* in both the laboratory and the field [6, 7], but even these matings, if successful, appear to fail to produce embryos capable of developing to adulthood. Forced laboratory matings have been conducted with OX513A and *Ae. albopictus* [8]; none of the eggs produced were fertile. Consequently vertical gene transfer to closely related mosquito species is both theoretically and practically possible but is likely to be an extremely rare event, and when these matings occur naturally or are forced to occur, as in close contact in a laboratory

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situation, fertile offspring are not produced. The strain OX513A contains no toxic proteins or DNA that would confer a selective advantage to an organism; in fact the contrary is true as they confer a strong selective disadvantage. Consequently any hypothetically transferred genetic material would be rapidly lost from the recipient population. Reviews of the fate of DNA from transgenic organisms ingested by animals conclude there are no safety concerns that could be identified [9, 10]. Studies have been conducted on non-target organisms consuming OX513A at 100% of diet and no adverse effects were noted.

#### References:

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2. Gill G, Ptashke M: Negative effect of the transcriptional activator GAL4. *Nature* 1988, 334(6184):721-724.
3. Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC, Condon GC, Morrison NI, Kelly OW, Dafa'al la T et al: A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat Biotechnol* 2005, 23(4):453-456.
4. Nordin O, Donald W, Ming WH, Ney TG, Mohamed KA, Halim NAA, Winskill P, Hadi AA, Lacroix R, Scaife S et al: Oral ingestion of transgenic RIDL *Ae. aegypti* larvae has no negative effect on two predator *Toxorhynchites* species. *PLoS One* 2013, 8(3) e58805.
5. Kramer L, Ebel G: Dynamics of flavivirus infection in mosquitoes. *Adv Virus Res* 2003, 60: 187-232.).
6. Nasci RS, Hare CG, Willis FS: Interspecific mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and the laboratory. *Journal of the American Mosquito Control Association* 1989, 5:416-421.
7. Tripet F, Lounibos LP, Robbins D, Moran J, Nishimura N, Blosser EM: Competitive Reduction by Satyrization? Evidence for Interspecific Mating in Nature and Asymmetric Reproductive Competition between Invasive Mosquito Vectors. *Am J Trop Med Hyg* 2011, 85(2): 265-270.
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9. Alexander T, Reuter T, Aulrich K, Sharma R, Okine E, Dixon W, McAllister TA: A review of the detection and fate of novel plant molecules derived from biotechnology in livestock production. *Anim Feed Sci Tech* 2007, 133:31-62.

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10. Flachowsky G, Schafft H, Meyer U: *Animal Feeding Studies for nutritional and safety assessments offeds from genetically modified plants: A review. J Verbr Lebensm* 2012, 7: 179-194.
11. Lacroix R, McKemey A, Raduan N, Lim K-W, Ming WH, Ney TG, Siti Rahidah AA, Salman S, Subramaniam S, Nordin O et al: *Open Field Release of Genetically Engineered Sterile Male Aedes aegypti in Malaysia. PLoS One* 2012, accepted.
12. Getis A, Morrison AC, Gray K, Scott TW: *Characteristics of the spatial pattern of the dengue vector, Aedes aegypti, in Iquitos, Peru. Am J Trap Med Hyg* 2003, 69(5):494-505.
13. MacDonald PT: *Population characteristics of domestic Aedes aegypti (Diptera: Culicidae) in villages on the Kenya coast. II. Dispersal within and between villages. Journal of Medical Entomology* 1977, 14:49-53.
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21. Reiter P, Amador MA, Anderson RA, Clark GG: *Short report: dispersal of Aedes aegypti in an urban area after blood feeding as demonstrated by rubidium-marked eggs. Am J Trap Med Hyg* 1995, 52(2):177-179.
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27. Carvalho, D. O., A. R. McKemey, et al. (2015). "Suppression of a Field Population of *Aedes aegypti* in Brazil by Sustained Release of Transgenic Male Mosquitoes." *PLoS Negl Trop Dis* 9(7): e0003864.

By signing my initials to each line, I, <b>The Applicant</b> , confirm that I have read, understand, and agree to the following:		<b>Applicant Initials</b>
1.	I will conduct my actions as described in this document, and will abide by the terms set forth in this agreement. Imported species will be used only for the reasons listed above. Any change of use of these species should be reviewed by the Department of Environment on behalf of the National Conservation Council, and the Department of Agriculture.	WP
2.	I will get the permission of and abide by all requirements of the Department of Agriculture before importing any alien species.	WP
3.	I will follow all procedures required by the Cayman Islands Government Department of Environment, as set out in this permission.	WP
4.	There will be no unpermitted release of live organisms or their offspring (if incidental reproduction occurs) or viable material, e.g., seeds, cuttings, etc., into any uncontrolled environment, either natural or artificial, of the Cayman Islands.	WP
5.	To avert possible introduction of exotic parasites or diseases, as well as offspring, there will be no discharge of effluents, disposal of waste, transport or housing media or other materials associated with the alien species or disposal of diseased or dead organisms or parts thereof except via lawful municipal waste discharge or disposal methods, i.e., sewage or garbage disposal.	WP
6.	These conditions follow the specimens imported and applicant should inform recipients of specimens (offspring, retail sale, etc.) of the above stipulations.	WP
7.	I understand that previous import permission for a listed or related species does not mean continuing permission to import nor does it mean that future permission to import will necessarily be granted.	WP



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I, **The Applicant**, undersigned, confirm that I have read, understand, and agree to abide by the agreement outlined in this document.

<i>William D. Petri</i>	<i>9 June, 2016</i>
<b>SIGNATURE OF APPLICANT</b>	<b>DATE</b>

**FOR OFFICIAL USE ONLY:**

<b>PERMISSION GRANTED</b>	<b>YES / <del>NO</del></b>
<b>PROCEDURES REQUIRED / RESTRICTIONS ON IMPORTATION / REASON FOR DENIAL</b>	
<ul style="list-style-type: none"><li>i) No more than 22 million adult male <i>A. aegypti</i> may be released in the course of the project.</li><li>ii) Un-used mosquitoes (eggs, larvae, adults) must be destroyed. (Frozen at -15 degrees Celsius or colder for more than 12 hours.)</li><li>iii) Importation and release shall be as in this document and shall conclude by June 30 2017.</li><li>iv) A copy of the Oxitec trade and business licence and a copy of the Certificate of Occupancy issued by the Department of Planning for the ACL-2 egg rearing are required and shall be supplied to the National Conservation Council to be retained with a copy of this Permit by the Department of Environment.</li><li>v) MRCU continue public outreach throughout the study period.</li><li>vi) MRCU report to the Council of progress as at June 30, 2016 and at the conclusion of the project (for the Council Annual Report).</li></ul>	
<b>NAME OF PERMIT ISSUER</b> John Bothwell	<b>DATE</b> 8 June 2016
<b>SIGNATURE OF PERMIT ISSUER</b> 	<b>DATE STAMP</b> 

**SPECIES RECOMMENDATIONS**

row	Species Scientific Name	Quantity	Importation & Release
1	<i>Aedes aegypti</i> (OX513A)	1.650 Kg (eggs)	Approved

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