GeneWatch UK comments on docket identification (ID) number EPA-HQ-OPP-2019-0274-0001: New Active ingredient for Oxitec OX5034 *Aedes aegypti* mosquitoes.

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This document contains GeneWatch UK's comments on Oxitec's application for a permit for experimental releases of its genetically engineered (GE) *Aedes aegypti* OX5034 mosquitoes expressing tTAV–OX5034 protein in the states of Florida and Texas.[1] Oxitec plans to make open releases of GE mosquitoes on up to 6600 total acres at a maximum rate of 20,000 male OX5034 mosquitoes, per acre per week.[1] The GE mosquitoes which Oxitec plans to release are of the *Aedes aegypti* species, which transmits diseases including dengue fever, zika and chikungunya.

The summary of the application states that female offspring of the OX5034 mosquitoes in the environment are expected to die before they mature into adults and therefore exposure to biting female mosquitoes is not anticipated: however, no evidence has been provided to support these claims. Further, numerous other issues of relevance to the protection of human and animal health and the environment have not been properly considered and the information provided is inadequate to make any meaningful assessment of these risks. These problems are compounded by the applicant's long history of making incorrect and unsubstantiated assertions about the efficacy and potential risks of its GE mosquito products.

Oxitec previously made an application to release the OX513A strain of the *Aedes aegypti* mosquito, which is genetically engineered to contain a red fluorescent marker and Oxitec's RIDL 'conditional lethality' trait.[2] GeneWatch UK objected to this application, which has since been withdrawn, and to an earlier application to the FDA.[3,4] Oxitec's OX513A GE mosquitoes are genetically engineered to (mostly) die at the larval stage in the absence of the antibiotic tetracycline, which acts as a chemical switch to allow breeding in the laboratory. Oxitec states that this GE mosquito has now been superseded by a new GE mosquito, OX5034, and thus the previous applications to release OX513A have been withdrawn. Very limited information regarding the newer OX5034 strain has been provided by the applicant in a published letter to the EPA.[5] The main substantive difference, compared to the earlier OX513A strain, is that the genetically engineered killing mechanism in OX5034 is intended to kill the female GE mosquitoes only, with GE males surviving for multiple generations. Although there are some important differences between the OX513A strain and the 2nd generation OX5034 strain, many of the issues raised regarding the 1st generation releases remain of concern and have not been addressed. In addition, because the OX5034 strain is female-killing only, GE males are expected to survive for multiple generations and this will considerably increase the spread of genes from the introduced strain into the wild population. In an online presentation, Oxitec presents this as a benefit because it argues that the released laboratory-derived strain will spread insecticide susceptibility genes into the wild mosquito population[6]: however, there is no guarantee that only beneficial and no harmful traits will be spread in this way.

Although Oxitec frequently described its OX513A GE mosquitoes as "sterile", this is not the case. The released GE males mate and produce offspring which inherit the genetically engineered late-lethality trait. This means that most (but not all) of the GE mosquitoes' offspring die at the late larval stage, in the water where the female mosquitoes lay their eggs. GeneWatch UK has repeatedly warned (including in its previous regulatory submissions cited above) that this partial survival rate, even if low (a reported 3 to 4% in laboratory conditions), would lead to the establishment of hybrid mosquitoes in the environment, which might possess altered properties, including the potential for enhanced disease transmission or resistance to insecticides. A recent paper, reporting monitoring of

[1] Genetically engineered (GE) organisms are also known as genetically modified (GM) organisms, or as living modified organisms (LMOs).

wild mosquito populations following some of Oxitec's experiments in Brazil, has confirmed that such hybrid mosquitoes have indeed spread into the area surrounding the release sites.[7]

An important lesson from this research is that the EPA cannot adequately protect human and animal health and the environment by focusing the assessment of risks solely on the active ingredient tTAV–OX5034 (which provides the genetically engineered killing mechanism for the mosquitoes). This is because other introduced traits, which are present due to the use of a non-native strain of mosquito (such as altered disease transmission properties), may also pose serious risks to human and animal health and the environment. As noted above, Oxitec's male OX5034 GE mosquitoes are 'female-killing' only: they are intended to mate with wild females and produce female offspring which die as larvae, whilst GE male mosquitoes from each generation continue to survive and reproduce. Thus, due to the survival of GE males for multiple generations, the OX5034 strain is expected to increase, rather than reduce, the spread of genes from the released GE non-native strain into the wild *Aedes aegypti* mosquito population, compared to the OX513A strain.

It is also notable that no public information has been provided in the Docket or elsewhere relating to the survival rates of GE females to adulthood, in the presence or absence of sources of tetracycline: this makes it impossible to assess Oxitec's claim that no biting GE females will be released or survive to adulthood.

Commercial use of GE mosquitoes has yet to be approved anywhere in the world, but this would entail repeated releases of many billions of GE males, vastly outnumbering the wild male mosquito population, with the intention of reducing the total adult population of mosquitoes over time. Contrary to Oxitec's claims, the release of its first-generation OX513A GE mosquitoes has not been successful, as GeneWatch UK has documented extensively: the company has no evidence of any impact on disease transmission and has made repeated, exaggerated claims about the impact of its experimental releases on wild mosquito populations.[8,9] As a result of this poor performance, trials of OX513A have ceased worldwide, with a single trial of OX5034 GE mosquitoes being undertaken solely in Brazil. The summary of the application in the Docket states that the proposed experiments are to evaluate the efficacy of Oxitec's alternative 2nd generation OX5034 GE mosquitoes as a tool for suppression of wild *Aedes aegypti* mosquito populations. However, Oxitec's claim[10] that "effective mosquito control, with built-in biosafety" has been demonstrated in field trials of its 2nd generation OX5034 GE mosquitoes in Brazil is not supported by any published evidence.

The documents provided by the Environmental Protection Agency (EPA) include no details of Oxitec's proposed experimental program, and no environmental assessment (EA) or environmental impact statement (EIS) has been provided.

This consultation follows Guidance clarifying the regulatory roles of the EPA and FDA in relation to proposed releases of GE mosquitoes[11] and prior consultation by the FDA on similar proposed releases of Oxitec's OX513A GE mosquitoes in Key Haven, Monroe County, Florida.

We conclude that:

(1) As a first step, the EPA should clarify the legal basis under which it proposes that Oxitec should be released from the contained use requirements of its import permit, in order to allow its GE insects to be deliberately released into the environment.
(2) A full EIS should be prepared under the National Environmental Policy Act (NEPA), and this should be subject to further consultation. The EIS should include consideration of the EPA's responsibilities under other environmental legislation, including the Endangered Species Act.
(3) Although further demonstration of efficacy would be necessary before Oxitec could submit an application to register a pesticide under section 136a of the Federal Insecticide, Fungicide

and Rodenticide Act (FIFRA), more laboratory and caged trials are first essential to establish that use of the pesticide under the permit, and its method of delivery via living genetically engineered (GE) pest organisms, does not cause unreasonable adverse effects on the environment.

1. Deliberate release of disease vectors into the environment

Regulatory actions under the Insecticide, Fungicide and Rodenticide Act (FIFRA) focus largely on the active ingredient (intended to act as a pesticide by killing pests), namely the tetracycline Trans-Activator Variant (tTAV) protein that Oxitec's GE mosquitoes have been genetically engineered to express. However, in this case, Oxitec is not releasing an inert ingredient but a living organism. Thus, not only the active ingredient, but also its method of delivery must be carefully considered.

The *Aedes aegypti* mosquito that Oxitec proposes to release is itself categorised as a pest, under 7 U.S.C. § 136(t) and § 136w(c)(1), because this mosquito species may be injurious to health or the environment. Mosquitoes are listed as Pests of Significant Public Health Importance.[12] Further, the *Aedes Aegypti* mosquito is a disease vector, as defined in 42 CFR §71.54: "*Any animals (vertebrate or invertebrate) including arthropods or any noninfectious self-replicating system (e.g., plasmids or other molecular vector) or animal products (e.g., a mount, rug, or other display item composed of the hide, hair, skull, teeth, bones, or claws of an animal) that are known to transfer or are capable of transferring an infectious biological agent to a human*". The movement of human disease vectors requires permits from the Centers for Disease Control (CDC). Import permits are granted under import regulations for infectious biological agents, infectious substances, and vectors (42 CFR §71.54) and the importer is required to remain in compliance with all of the permit requirements and conditions that are outlined in the permit issued by the CDC, which would not normally allow any open release of such disease vectors into the environment. A permit issued under this part is not required under certain circumstances[2], but these do not include the issuing of a licence for experimental use, or full product approval, of a pesticide under FIFRA (although an FDA licence as a New Animal Drug – the previous regulatory process - does allow exemption from a CDC permit). It is therefore unclear whether open release of such organisms can be lawfully permitted through the proposed mechanism of granting an experimental use permit under FIFRA. Particular concerns arise in this regard because of the potential release of biting females (see below) and the use of a non-native imported strain of the *Aedes aegypti* mosquito (see below), which is expected to lead to non-native hybrid mosquito strains becoming established in the environment.

As a first step, the EPA should therefore clarify the legal basis under which it proposes that Oxitec should be released from the contained use requirements of its import permit, in order to allow its GE mosquitoes to be deliberately released into the environment.

2. National Environmental Policy Act (NEPA)

The National Environmental Policy Act (NEPA), 42 U.S.C. §4321 et seq., as implemented by the Council on Environmental Quality (CEQ) Regulations (40 CFR Parts 1500 through 1508), requires that Federal agencies include in their decision-making processes appropriate and careful consideration of

[2] Including if: *(5) It is a product that is cleared, approved, licensed, or otherwise authorized under any of the following laws:*
(i) The Federal Food, Drug, and Cosmetic Act (21 U.S.C. 301 et seq.), or
(ii) Section 351 of the Public Health Service Act pertaining to biological products (42 U.S.C. 262), or
(iii) The Virus-Serum-Toxin Act (21 U.S.C. 151-159).
(6) It is an animal or animal product listed in 42 CFR Part 71 and its importation has been authorized in accordance with 42 CFR 71.52, 71.53, or 71.56.

all environmental effects of proposed actions, analyze potential environmental effects of proposed actions and their alternatives for public understanding and scrutiny, avoid or minimize adverse effects of proposed actions, and restore and enhance environmental quality to the extent practicable (40 CFR §6.100). The EPA shall integrate these NEPA requirements as early in the Agency planning processes as possible. The environmental review process shall be the focal point to ensure NEPA considerations are taken into account. This is the process used to comply with section 102(2) of NEPA or the CEQ Regulations including development, supplementation, adoption, and revision of NEPA documents.

As part of these requirements, the EPA must undertake an environmental review and prepare either an Environmental Assessment (EA) and Finding of No Significant Impact (FONSI) or an Environmental Impact Statement (EIS) and record of decision (ROD) for the proposed action. Consistent with 40 CFR 1500.5(g) and 1502.25, the Responsible Official must determine the applicability of other environmental laws and executive orders, to the fullest extent possible (40 CFR §6.201). This is likely to include, for example, the Endangered Species Act, so that the risks to threatened and endangered species (for example, through consumption of the GE mosquitoes) can be assessed. Public participation requirements are outlined in 40 CFR §6.203, including requirements for public consultation.

Previous proposals by Oxitec for experimental releases of its OX513A GE mosquitoes in Key Haven, resulted in the publication of a final Environmental Assessment by the FDA, following a period of public consultation.[13] The EA was prepared under the FDA's environmental impact considerations regulations (21 CFR part 25), consistent with NEPA. Now that the EPA is the lead agency on proposed releases of GE mosquitoes into the environment, it too must prepare either an EA and FONSI, or an EIS, for public consultation, under its own environmental impact considerations regulations (40 CFR Part 6). GeneWatch UK submits that the EPA should prepare an EIS, as the potential impacts of the proposed action are complex and significant.

Further, the EPA must also fulfil its obligations under the Endangered Species Act and other relevant environmental laws and executive orders, by including relevant assessments in the EIS.

Actions under FIFRA have traditionally been exempt from NEPA, but this depends on whether the assessment under FIFRA is functionally equivalent to the assessment under NEPA, ensuring full and adequate consideration of environmental issues. It is not a broad exemption but a "*narrow exemption from the literal requirements for those actions which are undertaken pursuant to sufficient safeguards so that the purpose and policies behind NEPA will necessarily be fulfilled*". [14] Although this exemption may apply for traditional applications of chemical and biochemical pesticides, there are many issues associated with the release of GE mosquitoes into the environment which may not be adequately captured by assessment under FIFRA (discussed further below). Therefore an assessment under NEPA is also required.

The issues covered by the EA or EIS are likely to be broader than those considered under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), because regulatory actions under FIFRA focus largely on the active ingredient (intended to act as a pesticide by killing pests), namely the tetracycline Trans-Activator Variant (tTAV) protein that Oxitec's GE mosquitoes have been genetically engineered to express. However, in this case, Oxitec is not releasing an inert ingredient but a living organism. This organism is a pest and human disease vector (as discussed above), and a genetically engineered (GE) organism (regulated as a plant pest under 7 CFR part 340). It is also an organism which may introduce or disseminate a contagious or infectious disease of animals (regulated under 9 CFR part 122): relevant diseases include dog heartworm, *Dirofilaria immitis*[15]; lumpy skin disease virus[16]; myxoma virus[17]; fibroma virus[18]; and Rift Valley Fever[19], as well as human

diseases such as dengue which may also infect primates and perhaps dogs[20]. Thus, not only the active ingredient, but also its method of delivery, and the impact on the environment and human and animal health of associated complex changes in ecology, must be carefully assessed in a manner which ensures compliance with all relevant regulations and protects human and animal health and the environment. This method of delivery of the active ingredient introduces additional concerns and potential adverse impacts on the environment and human health (discussed further below).

As part of the assessment, Oxitec's compliance with all other relevant laws must be considered, including those covering the deliberate release of disease vectors. As noted above, it is unclear whether open release of *Aedes aegypti* mosquitoes can be lawfully permitted through the proposed mechanism of granting an experimental use permit under FIFRA. Concerns regarding this process are exacerbated if biting females are included in the release (discussed in more detail below), and because Oxitec is using a non-native strain of the *Aedes aegpti* mosquito (see further below). Further, the new OX5034 strain of Oxitec's GE mosquitoes is female-killing only and male GE mosquitoes are therefore expected to survive for multiple generations, spreading the non-native genes of the introduced mosquito strain more widely into the wild mosquito population. At the very least, the deliberate release of disease vectors into the environment merits very serious consideration, including a full EIS and comparison with alternatives that do not involve such a risk.

Some examples of relevant issues are outlined below. However, we anticipate that more in-depth consideration would be needed in a full EIS issued for further consultation.

2.1 Potential release of biting female GE *Aedes aegypti* mosquitoes

Oxitec aims to release only male GE mosquitoes, however in practice large numbers of female GE mosquitoes – which may bite and transmit disease - have been released during past experiments with Oxitec's OX513A GE mosquitoes.

Oxitec used a mechanical method to sort its OX513A GE mosquitoes by size, with the aim of releasing mainly male mosquitoes, which do not bite. In 2014, Oxitec published a number of figures on the number of biting female GE mosquitoes that are inadvertently released.[21,22] In practice, these criteria were often exceeded. For example, checks by the Mosquito Research and Control Unit (MRCU) in the Cayman Islands on one production batch on May 12th 2017 revealed 9 females in one release pot of 500 (1.8%), nine times the agreed level.[23] The Cayman Islands' report also shows significant increases (spikes) in adult female mosquito numbers (green line in Figure 1B) in the release area 5 to 7 weeks after the releases begin, and again 7 to 8 weeks after the releases are increased.[24] These spikes in the adult female population exceed 150% of the comparator population, but their true extent is not shown as the peaks are cut off on the graph. These female GE mosquitoes pose a risk to the public because they can bite and transmit disease. Emails released as a result of a Freedom of Information (FoI) request in the Cayman Islands highlight "*a significant increase in the number of female mosquitoes collected in the treatment area*", rather than a decrease, which is thought to be due to the accidental release of GE female mosquitoes.[25] The emails reveal a high level of concern about the inadvertent release of GE female mosquitoes, from the Mosquito Research and Control Unit (MRCU) scientist with access to the data.[26]

Whilst continuing to fail to acknowledge this serious mistake, in its letter to the EPA, Oxitec states that its new OX5034 strain will avoid this problem because it provides "*genetic separation to 100% males*". [27] However, Oxitec has provided no evidence that the female-killing mechanism engineered into the OX5034 strain is 100% effective. It is essential that such evidence is published and made available for independent scrutiny and consultation in order to assess the risk of release of female GE mosquitoes in the proposed experiments.

Steps are also required to ensure that the GE mosquito line is not contaminated with potentially surviving females, or that other unexpected events do not occur. This has already been a major problem with during caged experiments using Oxitec's flightless female GE mosquitoes in Mexico. Quartz reports[28]: "*However, during an experiment, one of the research partners found that some of the GM mosquitoes only had one copy of the gene rather than the two needed to pass on the trait consistently—meaning half of their female offspring could fly, and mate. The GM mosquito line was likely contaminated during an earlier experiment in Colorado; at some point, a wild mosquito probably sneaked into the GM mosquito insectary. The line returned to Oxitec in the UK before shipping to Mexico, said Luca Facchinelli, a medical entomologist at the University of Perugia, who managed the field site*". The GE mosquitoes to be released under the proposed permit are different: however, open release trials are premature in the absence of a full, published investigation into this incident, to establish whether or not contamination was the cause, and protocols to prevent further errors of this kind.

As noted above, the movement of insects, mites and ticks that affect man or vector human diseases require permits from the CDC, and it is unclear whether open release of such organisms, especially if biting females are included and/or non-native strains are used, can be permitted. It is unclear why taking this risk would be justified, in comparison with alternatives, and at the very least a full assessment of this risk must be made by publishing a full EIS for consultation under NEPA, before the proposed experimental releases of GE mosquitoes are undertaken. The EIS must include data to quantify the effectiveness of the female-killing mechanism engineered into the OX5034 strain, rather than relying on Oxitec's claim that it is 100% effective.

2.2 Potential release of infected mosquitoes

Biting females may transmit disease even if they are disease-free on release (or at the time of birth in the environment), since they may encounter one of the diseases for which the *Aedes aegpti* mosquito is a vector (e.g. dengue, zika, chikungunya, yellow fever) by biting an infected person or animal, and spread that disease by subsequently biting an uninfected person or animal.

However, the possibility that the released GE mosquitoes are already infected with diseases also needs to be considered. Oxitec's draft Environmental Assessment for its OX513A strain, as submitted to the FDA (page 31), stated that the horse blood it uses to feed the GE mosquitoes at its UK production facility is screened for equine infectious anemia (EIA) and equine viral arteritis (EVA) among other pathogens, to minimize the potential for contamination of the blood by virus, bacteria, or other pathogenic agents.[29] It also notes that the host range of *Aedes aegypti* and *Aedes albopictus* does not extend to the UK, so the risk of transmission of arbovirus such as dengue and chikungunya to these horses is negligible. However, the range of *Aedes albopictus* has been expanding in Europe and there have been warnings that this vector could reach the UK in future.[30,31] The UK has several endemic mosquito species (mainly *Culex* species) that could potentially act as vectors for West Nile Virus in the future. It is also unclear what feed source Oxitec intends to use in its US rearing facilities. To reduce the risk that infected mosquitoes (potentially including some biting females) are released, a protocol for testing the GE mosquitoes for pathogenic agents should be introduced at the proposed rearing facilities. Up-to-date information regarding the feeding of the OX5034 strain also needs to be provided.

2.3 Survival and spread of GE mosquitoes

Oxitec's OX5034 GE mosquitoes are genetically engineered with the aim of killing all female offspring carrying the genetic trait at the late larval stage. However, there are several mechanisms which

could allow many more of the mosquitoes to survive to adulthood. Eggs may survive for several months when dried out on the inner walls of containers and may be transported elsewhere.[32] Any assessment therefore needs to consider the potential global transport of such eggs, and not be limited to considering the lifespan of adults and dispersal through adult flying.

In its 2004 report, the National Research Council's (NRC's) Committee on the Biological Confinement of Genetically Engineered Organisms (GEOs) states that biological confinement (bioconfinement) includes the use of biological barriers, such as induced sterilization, that prevent GEOs or transgenes from surviving or reproducing in the natural environment (page 15).[33] The report emphasises the importance of considering the large scale at which bioconfined organisms could be released and the possibility that even carefully planned, integrated bioconfinement methods could fail. It concludes that research is needed to characterize potential ecological consequences of bioconfinement methods and to develop methods and protocols for assessing environmental effects should confinement fail (page 12).

Oxitec's approach to reducing the reproductive capacity of its GE mosquitoes has a number of major weaknesses. Firstly, the killing trait may not be fully penetrant (meaning not all the GE insects will die) and is late-acting (meaning the insects are not sterile, but mostly die at the late larval stage). In the case of its OX513A strain, Oxitec published evidence that 3 to 4% of these GE mosquitoes unintentionally survived to adulthood[34]: however, no information has been provided on the penetrance of the female-killing trait in OX5034. This means it is impossible to assess how many GE female mosquitoes might survive to adulthood. Secondly, the lethality trait is conditional: the company uses the common antibiotic tetracycline as a chemical switch to turn off the killing mechanism, allowing the insects to be bred in the laboratory. This mechanism can therefore fail if the GE mosquitoes encounter high enough levels of tetracycline in the environment. Thirdly, resistance to the killing mechanism could evolve in the GE mosquito factory or in the environment.

When OX513A GE mosquitoes were fed cat food containing industrially farmed chicken, which contains the antibiotic tetracycline, the survival rate increased to 15-18%. Oxitec originally hid this information[35] but later admitted to an 18% survival rate of larvae fed on cat food in a published paper.[36] In the case of the OX5034 strain, no information has been provided whatsoever on the impacts of tetracycline on the likely survival rates of GE female mosquitoes.

In the case of the OX513A strain, Oxitec claimed that an increased survival rate due to tetracycline contamination would not happen in the wild because the GE larvae would breed only in clean water. However, a number of studies have found that *Aedes aegypti* mosquitoes can breed in septic tanks where there can be high levels of contamination with antibiotics such as tetracycline. [37,38,39,40,41,42] A 2004 study found that sewage treatment plants, septic tanks, and cesspits were larval development sites for *Aedes aegypti* in the Florida Keys.[43] In 2004, there were more than 36,000 septic systems and 5,000 to 10,000 cesspits in Florida.[44] *Ae. aegypti* also commonly live in areas where discarded takeaways are likely to contain meat contaminated with tetracycline. Oxitec uses a diet supplemented with 30 µg/ml of the tetracycline to breed its OX513A mosquitoes in the lab: again, figures are not available for the OX5034 strain. The tetracycline derivatives oxytetracycline (OTC) and doxycycline (DOX, used to prevent malaria) could also allow the GE mosquitoes to breed. Oxytetracycline can be found at concentrations above 500 µg/g in animal manure and doxycycline at up to 78516.1 µg/kg dry weight in broiler manure, which may be sufficient to inactivate the killing mechanism.[45,46]

The percentage of surviving GE mosquitoes could also increase if resistance to the genetic killing mechanism evolves over time.[47] In comparison, the traditional Sterile Insect Technique (SIT), used to control some pests, results in multiple chromosome breaks when the insects are exposed to

radiation, severely limiting any potential for resistance to evolve during the production process. In contrast, any genetic or molecular event that allows the GE mosquitoes to survive and breed successfully could be rapidly selected for during mass production.[48] Increased survival rates would reduce the effectiveness of any population suppression effect over time, increase the number of biting GE females, and potentially allow the GE mosquitoes to establish in the wild.

In a conventional SIT programme in Japan, wild females appeared that were unreceptive to mating with irradiated males.[49] Therefore, adaptive behaviour in wild females to increase survival of their offspring, including avoiding GE males or seeking out tetracycline-contaminated sites to lay their eggs, must also be considered.

These risks must therefore be assessed as part of a full EIS. To enable a proper assessment of the risks, relevant information on survival rates to adulthood, with and without tetracycline, must be provided.

2.4 Use of antibiotics to feed the GE mosquitoes

Oxitec feeds its GE mosquitoes on the antibiotic tetracycline, as this acts as a chemical switch to turn off the genetic killing mechanism. The use of tetracycline to breed the GE mosquitoes in the lab carries the risk of spreading antibiotic resistance, which could pose a major risk to human and animal health. Insect guts are reservoirs for antibiotic resistance genes with potential for dissemination.[50,51] Insect production in factories exposed to antibiotics could lead to drug resistance in their microbiota so that the insects disseminate antibiotic resistance when released into the environment.[52,53] Disposal of waste water, containing tetracyclines and/or tetracycline-resistant bacteria, may also spread antiobiotic resistance. A postgraduate student working with Oxitec's GE *Aedes aegypti* mosquitoes has conducted relevant experiments which found that "*Colonies grew on plates supplemented with 50 µg ml^{-1} of chlortetracycline, indicating that the larvae bore chlortetracycline-resistant bacteria*".[54]

Oxitec's letter to the EPA states that released male OX5034 *Aedes aegypti* will be reared in the absence of tetracycline. This is not possible for the OX513A strain, but is possible for OX5034, because the latter strain is female-killing only (so male larvae do not need to be fed the antibiotic in order to survive). However, the OX5034 strain will require tetracycline at the egg production stage as the female parent mosquitoes of the males that are released need the antibiotic in order to survive to adulthood to lay their eggs. This means there will likely be tetracycline-resistant bacteria in the egg stage of the GE males, which may persist until their release on adulthood. There is also potential for intergenerational transfer of antibiotic resistant bacteria, although we are not aware of any studies of this in *Aedes aegypti*. Considerably more information is needed to be able to confirm or rule out the presence of such antibiotic resistant bacteria in the GE mosquitoes intended for release. Antiobiotic resistant bacteria could pose a major risk to health if spread into the environment.

Protocols released under the Freedom of Information Act (FOIA) raise further questions about the use of antibiotics by Oxitec. The documents reveal that the company feeds its adult OX513A *Aedes aegypti* mosquitoes on sugar solution containing the antibiotics penicillin and streptomycin, during egg production (Section 1.2 of the Quality Control Protocol for the Assessment of Mating Competitiveness, page 88 of the pdf; and Section 1.2 of the Quality Control Protocol for Colony Genotyping, page 101 of the pdf).[55] This raises further concerns about the development of antibiotic resistant bacteria in the insects or the water in which they breed, and whether this enters the environment during waste disposal. It is unclear from the information provided, whether penicillin and streptomycin are fed to adult GE mosquitoes only during specific experiments, or also during

mass production, prior to open release into the environment. This would raise additional concerns because:

- The scale of the disposal problem would increase if these antibiotics are used during mass production;
- It could lead to the spread of antibiotic resistant bacteria by the GE mosquitoes on release;
- There is some evidence that antibiotics may increase the transmission of dengue fever by *Aedes aegypti* mosquitoes.[56]

The potential spread of antibiotic resistance could pose a serious risk to human and animal health. It is therefore essential to consider whether Oxitec's use of antibiotics is lawful under the Veterinary Feed Directive (21 U.S.C. §354) and any other relevant legislation or executive orders, and to assess the risks to human health and the environment in a full EIS.

2.5 Use of a non-native strain of the *Aedes aegypti* mosquito

Oxitec's GE mosquitoes have been developed from a non-native strain (the Rockefeller laboratory strain,[57] originally from Cuba[58]). In the Cayman Islands, this was backcrossed into a Mexico-derived genetic background[59] and it appears that this same strain was then used in Brazil and probably also in Panama. As described in Oxitec's draft Environmental Assessment for OX513A, originally submitted to the FDA, (pages 21 and 22), the GE strain OX513A was produced in 2002 by microinjection into individual embryos of *Aedes aegypti* from a Rockefeller strain background. [60] The strain was made homozygous by repeated back-crossing and then the insert was introgressed into an *Ae.aegypti* Latin strain background from Instituto Nacional de Salud Publica (INSP), Mexico. The Rockefeller strain is a common laboratory strain of *Aedes aegypti*, which appears to have been derived from a strain established in Havana, Cuba, by Carlos J. Finlay in 1881, used in the original experiments which established that *Aedes aegypti* mosquitoes are a vector for Yellow Fever.[61,62]

As GeneWatch noted in its previous submission regarding proposed releases of Oxitec's OX513A GE mosquitoes, when Oxitec's GE mosquitoes breed with wild mosquitoes some of their other genetic characteristics will be passed on to the local wild mosquito population. A recent paper, reporting monitoring of wild mosquito populations following some of Oxitec's experiments in Brazil, has confirmed that such hybrid mosquitoes have indeed spread into the area surrounding the release sites.[63] Because the OX5034 strain is female-killing only, GE males are expected to survive for multiple generations and this will considerably increase the spread of genes from the introduced strain into the wild population. In an online presentation, Oxitec presents this as a benefit because it argues that the released laboratory-derived strain will spread insecticide susceptibility genes into the wild mosquito population.[64] Consistent with this presentation, Oxitec has demonstrated the effects of rapid introgression of insecticide-susceptible traits in its own research and modelling of its GE agricultural pests.[65,66] However, there is no guarantee that only beneficial and no harmful traits will be spread in this way. In particular, the use of a non-native strain risks spreading altered disease transmission properties into the wild mosquito population and/or creating strains which exhibit "hybrid vigour" (for example, becoming more fertile, as has been demonstrated for hybrid strains of other mosquito species[67]).

Different strains of the same species are found in different places and some strains are more resistant to insecticides than others or better transmitters of disease (the four serotypes of the dengue virus and/or other viruses, such as chikungunya, zika and Yellow Fever). *Aedes aegypti* may transmit zika, chikungunya, yellow fever and four different serotypes of dengue, yet strains may vary significantly in their ability to transmit these tropical diseases.[68,69,70,71,72,73,74,75] In the case of zika, little is known about vector strain variation and its consequences. The possible introduction of such traits needs to be considered very seriously. Harm to people's health can be increased if some

serotypes or viruses can be transmitted more easily by the introduced strain than they were by the wild species already in the area, or if the strain is resistant to insecticides.

The proposed releases could lead to the introduction of undesirable disease transmission traits (or an insecticide-resistant trait) into the wild *Aedes aegypti* population at the site and/or create hybrid mosquitoes that display other undesirable traits (such as increased fertility) due to "hybrid vigour". This risk therefore needs to be considered in a full EIS.

For comparison, in the UK, Oxitec has been prevented from releasing a GE diamondback moth (an agricultural pest) because of concerns about the use of a North American background strain, which is subject to controls under plant pest control regulations.[76] As noted above, the movement of insects that affect man or vector human diseases require permits from the CDC, and it is unclear whether open release of such organisms, especially if derived from non-native organisms, can be permitted merely by issuing an experimental use licence under FIFRA (see Section 1).

2.6 Mosquito population responses

Releases of Oxitec's GE *Aedes aegypti* mosquitoes are intended to suppress the wild population of *Aedes aegypti*. Unlike removing breeding sites or using larvicides, this is a single-species approach which does not reduce populations of non-target species. If population suppression of *Aedes aegypti* is successful, one important question for the risk assessment is whether *Aedes albopictus* (Asian Tiger) mosquitoes, which also transmit dengue and other viruses (including chikungunya), will increase in numbers and perhaps establish in new areas as a result of competitive displacement of one species by another. *Aedes albopictus* is widespread in the USA, including in Texas and Florida.[77]

In a draft risk assessment for its OX513A strain from 2011, Oxitec states (page 25): "*It is not clear to what extent Ae. albopictus could or would expand its range into areas currently dominated by Ae. aegypti but it is reasonable to expect a degree of such expansion if no countervailing activities are undertake*n".[78] Oxitec has also published a paper which uses computer modelling to show how *Aedes aegypti* and *Aedes albopictus* may interact.[79] The authors acknowledge that this could have important consequences for the persistence of disease. In its application to the Cayman Islands, Oxitec states: "*Should Aedes albopictus begin to occupy the Aedes aegypti niche upon reduction in their numbers, a concurrent operation will begin to reduce the numbers of Aedes albopictus*".[80] However, no such operation has ever taken place, so there is no evidence it would be effective or cost-effective. More recently, Oxitec's former Chief Scientific Officer, Luke Alphey stated, "*Since* Aedes aegypti *and* Aedes albopictus *are known to compete … it is possible that the successful implementation of* …[GE mosquito] *gene drives could lead an existing* Ae. aegypti *population to be displaced by* Ae. albopictus *where it would not otherwise have been. This would likely hamper efforts to eliminate viruses such as dengue since* Ae. albopictus *are also competent vectors*...".[81]

Both species can spread extremely rapidly and can interact with and displace one another: for example, *Aedes albopictus* has replaced *Aedes aegypti* in much of Florida and in Bermuda.[82,83] The results of a 2013 study show that Florida *Aedes aegypti* and *Aedes albopictus* mosquitoes are both competent vectors of the DENV-1 strain of dengue isolated from Key West in 2010.[84]

Aedes albopictus has been responsible for epidemics of dengue and chikungunya elsewhere in the world[85,86] and for the re-emergence of dengue in southern China.[87] The role of *Ae. albopictus* may have been underrated and this species is likely to play an important role in the maintenance and transmission of the virus.[88,89] Oxitec frequently cites a review by Lambrechts et al. (2010) to support its claim that *Ae. albopictus* is a less effective vector of dengue than *Ae. aegypti*.[90] However this paper also warns that it is not possible to predict the epidemiological outcome of competitive

displacement of *Ae. aegypti* by *Ae. albopictus* and warns that vector status is a dynamic process that in the future could change in epidemiologically important ways.

Grard et al. (2014) have identified the presence of ZIKV (Zika virus) in *Aedes albopictus* in Gabon.[91]

In the case of zika, some scientists have argued that common *Culex* species of mosquitoes may also play an important role in transmission of disease.[92,93,94] Although the evidence is not definitive (and some scientists have found that *Culex* species do not appear to transmit zika in some regions[95,96,97]) the southern house mosquito, *Culex quinquefasciatus*, also known as the common mosquito, may be a vector for zika in certain environments.[98] If this is the case, attempting to reduce zika transmission by targeting *Aedes aegypti* may be the wrong approach.

Another issue is whether or not releases of GE mosquitoes could cause an increase in the numbers of mosquitoes in surrounding areas. This effect is predicted by some models for the release of sterile insects.[99] There is evidence from Oxitec's experiments with its OX513A strain that numbers in neighbouring control areas may increase as the population is suppressed in the target area. For example, in its 2009 Cayman Islands experiments, the number of wild *Aedes aegypti* mosquito eggs, measured using egg traps (ovitraps), was observed to increase in the neighbouring control area as the population in the release area decreased (Figure 2c).[100] The same effect can be seen in Oxitec's experiments in Itaberaba (Brazil), which compare ovitrap data from the control area with data from adult male traps in the release area (Figure 2D).[101] Thus, there appears to be a real possibility that some of the wild mosquitoes, when swamped by very high releases of GE males, simply migrate to mate in the surrounding area, potentially increasing health risks for the people there. More information is needed to either confirm or rule out this possibility.

Further, any assessment of the potential impact on the environment of the proposed releases must consider more than the desired reduction in the *Aedes aegypti* population in the release area on wild animals that may feed on them. In reality, there will be a very large increase (several orders of magnitude) in *Aedes aegpti* numbers (largely GE adult males, but perhaps also spikes in adult females, as discussed above) in the target area during the releases, and potential increases in surrounding areas (possibly including large numbers of wild males if they migrate from the release site to avoid competition with the GE males that are released). This may be followed by a fall in wild numbers at the release site if the experiment is successful in achieving population suppression, and perhaps a subsequent rebound if the mosquitoes evolve resistance or begin to breed in tetracycline-contaminated sites, or if continued releases become technically difficult or uneconomic. Consideration of the impacts requires consideration of a dynamic ecosystem that may respond in complex ways. For example, species that feed on mosquitoes may initially be attracted to the site, but lose access to the new food supply as the numbers of the target species at the site reduce. Oxitec's treatment of this issue to date has been inadequate because it does not consider the complex and dynamic nature of the ecosystem.

In addition, species which feed on adult *Aedes aegpti* are likely to have an increased proportion of this species in their diets, due to the need to swamp wild males by several orders of magnitude during the releases (the issue of ingestion of the GE mosquitoes is considered further in Section 3 below).

Increases in non-target mosquito species as a result of the proposed releases could pose risks to human and animal health, as could increases in the target species in areas neighbouring the releases. Complex ecosystem responses could result from altered mosquito population dynamics and wildlife may be affected by ingestion of the GE mosquitoes. These risks must be assessed in a full EIS, including potential risks to relevant species under the Endangered Species Act.

3. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)

Under section 5 of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), 7 U.S.C. 136c, the EPA can allow manufacturers to field test pesticides under development. When any experimental use permit is issued for a pesticide containing any chemical or combination of chemicals which has not been included in any previously registered pesticide, the EPA may, under 136c(d), specify that studies be conducted to detect whether the use of the pesticide under the permit may cause unreasonable adverse effects on the environment.

According to the summary of the application, the proposed experiments are to evaluate the efficacy of OX5034 mosquitoes as a tool for suppression of wild *Aedes aegypti* mosquito populations. The proposal does not mention any investigations of potential adverse effects on the environment. However, many more such studies (in contained use, and by monitoring and modelling the behaviour of wild mosquito populations and their ecosystems) would be required before an adequate risk assessment could be undertaken.

3.1 Poor efficacy

To date, Oxitec has not established the efficacy of its technique for reducing *Aedes aegpti* populations, or the impact on relevant diseases (which may continue to be transmitted even by relatively small numbers of mosquitoes, including other species). Existing data from experiments elsewhere suggests the efficacy of this approach is poor (discussed further below) and there is no efficacy data for the United States. Further efficacy data would therefore certainly be needed before Oxitec could register its GE mosquitoes as a pesticide under 7 U.S.C. 136a. However, GeneWatch UK opposes the granting of the experimental use permit, as further studies are first essential to establish that the proposed experimental use will not cause unreasonable adverse effects on the environment (see Section 3.2).

Oxitec has conducted experimental open releases of its OX513A GE mosquitoes in the Cayman Islands, Malaysia, Brazil and Panama. In 2018, the Environmental Health Minister in the Cayman Islands confirmed that trials of Oxitec's GE mosquitoes there did not work and would be abandoned.[102] Oxitec's releases of GE mosquitoes in Panama and Malaysia ceased earlier, due to concerns about costs, effectiveness and risks. In Malaysia, trials were abandoned following a small open release experiment to measure flying distances and survival rates.[103] The health ministry concluded that "*the method was not practical besides involving high costs*".[104] In Panama, open release trials of Oxitec's GE mosquitoes were conducted in 2012 and then ceased, reportedly due to the high costs.[105] Proposed trials in other countries never actually took place. Oxitec notes that its former subsidiaries in Singapore, Mexico, Australia and Costa Rica are all now dormant.[106] Since its Cayman Island operations have now closed,[107] only the company's Brazilian office remains active. In Brazil, Oxitec released GE mosquitoes in Jacobina and Juazeiro in the state of Bahia, from 2011 to 2013. In 2016, Oxitec began larger-scale trials of its GE mosquitoes in Piracicaba, a city located in the state of São Paulo.[108] However, in 2018, Oxitec Brazil decided to close its GE mosquito factory in Piracicaba.[109] According to the company, the reason was the transition to the newer OX5034 version of its GE mosquitoes, which began to be released in a pilot project in Indaiatuba in the Campinas region, in mid-2018. In November 2018, Oxitec announced that in future it would only conduct trials with this new generation of GE insects.[110]

Oxitec has repeatedly claimed that its experiments have been successful. In a brochure published in 2016, the company stated, "*Oxitec has developed a paradigm shift in mosquito control leading to unparalleled levels in the suppression of* Aedes aegypti*, the main vector for several of the world's most damaging viruses including zika, dengue and chikungunya*" and, "*In five separate efficacy trials across three different countries, releases of Oxitec OX513A mosquitoes led to a greater than 90%*

reduction in the local Aedes aegypti *populations*".[111] However, these claims are not supported by the evidence.[112] For example, emails released as a result of Freedom of Information requests to the Cayman Islands' Mosquito Research and Control Unit (MRCU) reveal comments from scientists there with access to the data, which state, "*Whilst Oxitec and MRCU are making public statements proclaiming major reductions in the* Aedes aegypti *population in the treatment area the data I have seen does not support this.*"[113] and "*To date all the measures recorded have shown no significant reduction in the abundance of* Aedes aegypti *in the release area.*"[114]

Oxitec's decision to stop releasing its OX513A mosquito and begin trials with a new female-killing version effectively confirms that its trials to date have all been a failure. In Brazil, commercial releases have never been approved by the Brazilian health authority ANVISA, which wants to see evidence of benefits to health before giving its approval, in line with recommendations from the World Health Organisation (WHO).[115,116,117] There is no commercial approval for releases because the company lacks any evidence of efficacy in tackling dengue or other diseases spread by this mosquito.

Further, GE mosquito production is extremely costly and there have been production problems. In 2014, the release of 300,000 GE mosquitoes in Panama was reported to have cost $620,000 (more than $2 per mosquito).[118] In the Cayman Islands, production issues included the release of a high percentage of female GE mosquitoes, high adult and larval mortality, and mould in the rearing unit.[119]

Oxitec's letter to the EPA claims that effective mosquito control has been demonstrated with OX513A, in complete contradiction to the evidence outlined above. Further, it claims that effective mosquito control has also been demonstrated for OX5034 in a trial in Brazil: however, there is no published evidence that this has been the case.

The role of Oxitec's GE mosquitoes in Integrated Pest Management (IPM) is also highly questionable. Continuing to use traditional control methods for mosquitoes (adulticides and larvicides) could further limit the effectiveness of Oxitec's technology by killing the GE males before they mate with the wild female mosquitoes, or the larvae before they survive to reproduce the trait and spread it through the wild population. Moreover, since there is little data regarding the effectiveness of existing measures, it is hard to see how the claimed benefits of adding GE mosquito releases to existing measures will be evaluated. On the other hand, failure to use existing control methods (if and when they are effective) in order to allow GE mosquito releases to take place, may put people at unnecessary risk of dengue or other diseases, or simply add to the nuisance of mosquito bites, perhaps with negative impacts on tourism or quality of life.

We note that, were an experimental use licence to be granted, the requirements of EPA's human studies rule (40 CFR Part 26) should be followed, due to the exposure of human subjects (including children) to the proposed open releases of GE mosquitoes, and the potential limitations on the use of other methods of mosquito control that may need to be applied during the experiments.

3.2 Prior assessment of adverse effects on the environment

Potential adverse effects include those discussed under Section 2 above, plus the negative effects on IPM strategies (Section 3.1) and any direct negative effects of the tTAV or DsRed2 proteins on humans, animals and wildlife (discussed below).

Because the female GE mosquitoes mostly die at the larval stage, there will be large numbers of dead GE larvae in the water where the female mosquitoes lay their eggs, and these might be ingested by humans, animals or wildlife. Humans, animals and wildlife will also swallow adult GE mosquitoes. Journalists have reported that in Brazil, during experiments with Oxitec's OX513A GE mosquitoes, "*…it's impossible to talk during the liberation sessions without accidentally swallowing a*

few…" due to the very large numbers of GE mosquitoes being released to try to swamp the wild population.[120] In addition, people and animals may be bitten by female GE mosquitoes, if any survive or are inadvertently released.

In its application to release GE moths in New York State (since withdrawn but later resubmitted, although a brief open-release trial has now ceased), Oxitec provides a commercial reference for toxicity testing of the red fluorescent marker, DsRed2, by Pioneer DuPont.[121] Oxitec also cites a 26-day feeding study in rats, using GE oil seed rape (canola) genetically modified to express green (not red) fluorescent protein (GFP), which concludes: "*These data indicate that GFP is a low allergenicity risk and provide preliminary indications that GFP is not likely to represent a health risk*".[122] However, other than a bioinformatics report (desk study), Oxitec has to date provided limited evidence regarding the safety of the RIDL genetic mechanism and the high level expression of tTAV that kills the insects at the larval stage. The mechanism of action of this killing mechanism is not fully understood and very limited safety data is available. The tetracycline transactivator (tTAV) protein is created by fusing one protein, TetR (tetracycline repressor), found in *Escherichia coli* bacteria, with the activation domain of another protein, VP16, found in the Herpes Simplex Virus. Researchers commonly use this mechanism to switch on and off different genetic traits, for example in transgenic (GE) mice used in medical research, but it is not normally present in the human food chain. Oxitec has published one feeding study, in which OX513A GE *Ae. aegypti* mosquito larvae were fed to two different species of a type of mosquito that eats other mosquitoes (known as *Toxorhynchites*).[123] More recently, Oxitec published a feeding study on the impact of GE olive flies on one parasitoid (a wasp) and two predators (a spider and a beetle), reporting no adverse effects.[124] A report on a feeding trial with Guppy fish (*Poecilia reticulate*) was included in Oxitec's draft Environmental assessment to the FDA (Appendix H, page 184 of the pdf) for OX513A. [125] As far as we are aware, no feeding trials have been published which study potential impacts on birds, mammals, reptiles or amphibians, such as lizards or frogs. Further, no independent studies have been published. In addition, it is unclear whether or not tTAV-OX5034 is the identical to the protein in the OX513A strain, and no studies specific to the OX5034 strain have been provided.

In the scientific literature, there is some evidence that enhanced tTAV expression can have adverse effects (loss of neurons affecting cognitive behaviour) in transgenic (GE) mice.[126] Other mice studies have detected adverse effects on the lung.[127,128] These studies should act as warning signs that further evidence is needed.

For biopesticides, the EPA typically requires Tier I testing done on the following non-target organisms: birds (oral and inhalation), mammals, freshwater fish and invertebrates, estuarine/marine fish and invertebrates, plants, insects, and honeybees. Tier II, III, and IV testing is triggered only when unacceptable effects are seen at the Tier I testing level.

Considerably more data, based on specific feeding trials in relevant species, is therefore needed to establish that consumption of OX5034 GE mosquito adults or larvae is not harmful to humans, farm animals, pets or wildlife. As noted above, wildlife consuming the GE mosquitoes may also include threatened or endangered species, and this risk also needs to be assessed.

European Union (EU) standards are relevant here because Oxitec is required by EU law to provide a risk assessment which meets EU standards to the importer, before exporting its GE mosquitoes.[129] EU Guidance on risk assessment of GE insects (known as GM insects in Europe) published by the European Food Safety Authority (EFSA) requires applicants to assess the effects of toxins or allergens associated with the GE insect in animals such as birds, mammals, reptiles and amphibians.[130] It also states (page 8): "*…applicants should also assess the likelihood of oral exposure of humans to GM animals or their products which are not intended for food or feed uses. If such exposure is likely and*

ingestion or intake will occur at levels which could potentially place humans at risk, then applicants should apply the assessment procedures described in the EFSA Guidance Document on the risk assessment of food and feed from GM animals and on animal health and welfare aspects". To meet the requirements of the cited Guidance on risk assessment of food and feed, it is likely that repeated dose toxicity studies using laboratory animals would be required.[131]

No GE insects have been released to date in the EU, although Oxitec has applied to do so. Oxitec's application to release GE olive flies in Spain was withdrawn in 2013, following a request for further information from the regulator, including toxicity testing using feeding trials in relevant species.[132,133] Oxitec re-applied to release GE olive flies in Spain in 2015, without providing the necessary safety information.[134] This application was rejected.[135]

In summary, considerable further evidence is needed to assess whether the use of the pesticide under the proposed permit (including its method of delivery) may cause unreasonable adverse effects on the environment. As well as considering the legal obligations highlighted in Sections 1 and 2, the EPA should therefore specify that further studies be conducted before publishing a full EIS for public consultation. It is notable that information supplied to the various review processes for OX513A GE mosquitoes and other insects (to the FDA, APHIS and the EPA, as well as overseas agencies) is almost entirely lacking in the current process for the proposed release of the new generation of OX5034 GE mosquitoes. In addition, there are no published peer-reviewed papers for Oxitec's GE *Aedes aegypti* OX5034 mosquitoes. All studies should be independently replicated. The necessary studies include, for example:

- Laboratory safety tests, including feeding trials for relevant wild species and laboratory rats to better establish the claim of no harmful effects of ingestion and/or biting.
- Independent verification that the new OX5034 strain provides Oxitec's claimed "*genetic separation to 100% males*": plus estimates of the numbers of GE biting female mosquitoes that may be released during the proposed experiments, or that may survive from subsequent generations, taking into account the potential to encounter tetracycline in the environment.
- Studies of the potential of the GE mosquitoes to evolve resistance to the killing mechanism during mass breeding or following release, plus studies of the potential for wild females to evolve behavioural resistance.
- A protocol for testing the GE mosquitoes for pathogenic agents prior to release.
- Identification of relevant septic tanks and cess pits where mosquitoes may breed and testing of tetracycline levels in them.
- Identification of potential sites where GE mosquitoes could encounter industrially farmed meat (e.g. discarded takeaways, pet food) and testing of tetracycline levels at these sites.
- Laboratory studies of the potential for antibiotic resistant bacteria to be spread into the environment via adult mosquito releases or disposal of larval rearing water or other wastes from the mosquito production facility.
- Information about which existing control methods will continue to be applied during the proposed releases.
- Published criteria for assessing the impact of existing control measures and the proposed releases on the target pest and the risks of all the relevant diseases.
- Full independent testing of the non-native strain proposed for release, for disease transmission traits for all relevant diseases and insecticide resistance for all relevant insecticides, plus contained studies addressing concerns about the potential 'hybrid vigour' of any hybrid strains.
- More in-depth consideration of the risk of increasing other mosquito vectors, including: laboratory and caged trials on the impacts of interspecies competition; thorough baseline studies of mosquito populations; studies on the disease transmission properties of other

vectors for all relevant diseases; and consideration of the possibility that viruses will evolve in response to ecosystem changes.

- Confirmation that *Aedes aegypti* is the main vector of zika and that other species do not also play a role.
- Further consideration of the dynamic changes in local ecosystems as a result of the proposed releases, including the impacts of a large (potentially several orders of magnitude) increase in the number of adult mosquitoes in the target area during the releases.
- Publication of laboratory studies, including studies of proteins in mosquito saliva, feeding trials with mosquito predators, and larval survival rates in the presence and absence of tetracycline contamination.
- A full, published investigation into the reported survival of hybrid GE mosquitoes in Brazil, including a specific investigation of the recent open release trials of OX5034 GE mosquitoes. This study should include detailed analysis of any hybrid mosquitoes for disease transmission properties. Published confirmation and independent verification of Oxitec's claim that its trial of OX5034 in Brazil has been successful is also missing.
- A full, published investigation into the unexpected survival of female mosquitoes in Oxitec's experiments in Mexico.
- The GPS coordinates and other relevant details of the proposed release sites and the scientific protocols for the proposed trials.
- A proposal for comprehensive post-release monitoring of the proposed releases and their potential impacts on the environment.

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