GeneWatch UK comments on FDA Docket FDA-2014-N-2235: Draft Environmental Assessment and Preliminary Finding of No Significant Impact Concerning Investigational Use of Oxitec OX513A Mosquitoes

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It is premature to issue a Finding of No Significant Impact (FONSI) in the absence of further information regarding a number of important areas of risk.

Potential impacts on human or animal health

Direct risk of ingestion and biting

Risk of ingestion of genetically modified (GM) mosquitoes has been entirely omitted from the risk assessment, and there have been no feeding tests in mammals, amphibians, reptiles, crustacea or birds. Tests that have been conducted are inadequate to demonstrate the safety of the GM mosquitoes for humans, animals or birds that may inadvertently swallow them. Neither the GM construct as a whole, nor the two expressed proteins DsRed and tTAV, have been adequately tested.

Journalists have reported that in Brazil "…*it's impossible to talk during the liberation sessions without accidentally swallowing a few*…" due to the very large numbers of GM mosquitoes released to try to swamp the wild population (at ratios of up to 54 to 1[1]).[2] In the Cayman Islands, Oxitec reported: "*The only consistent project-related criticism from the community related to nuisance from the large numbers of males in each individual release in the first part of Period 3. In response we promptly reduced these numbers and moved the release points further from habitations. We also partly substituted with pupal releases, from which adults emerge over a period of time*".[3] Therefore, it is clear that there is the potential for humans or animals, including pets, to swallow adult GM mosquitoes when they are released: indeed the likelihood of this is high. In addition, GM mosquito larvae will hatch in breeding sites which may include water available for consumption by birds or pets, so GM larvae may also be ingested by animals. In some species, exposures may be high because GM mosquitoes (largely adult males) will be released in very large numbers (several orders of magnitude higher than the numbers of wild males) in order to seek to suppress the wild population.

Signs of toxicity[4] and neurotoxicity[5] have been reported in mice expressing the tTAV protein, yet this evidence of risk has been dismissed by Oxitec in favour of reliance on a single bioinformatics report produced by an industry consultant. Feeding studies are reported for predator mosquitoes (known as *Toxorhynchites*)[6] and one species of fish (the guppy, *Poecilia reticulate*, Appendix E of Oxitec's draft Environmental Assessment), yet no feeding studies have been reported in mammals, amphibians, reptiles, crustacea or birds, which might consume the GM mosquitoes when they are released into the environment. In addition to trials with relevant wild species, feeding trials should also have been conducted with the mosquitofish, *Gambusia affinis*, which is used in the Florida Keys as a larvicide in permanent water bodies such as cisterns, abandoned pools, and ornamental ponds. Importantly, human safety seems to have been given a very low priority, as feeding studies in laboratory rats, normally part of a risk assessment for human health, have also been omitted.

Regarding the potential for direct harm from GM mosquito bites, Oxitec's draft Environmental Assessment includes a study (Appendix K) which the company states shows that the introduced proteins, tTAV and DsRed2 were not detected in OX513A *Aedes aegypti* saliva at and above the limit of detection (summarised on pages 89-90). However, results from a further preliminary study by the NIH (referred to on page 89) are not included. It would

increase public confidence in Oxitec's findings if independent research were published to confirm their claims. Independent replication is a cornerstone of the scientific method.

Potential spread of diseases by biting females

Oxitec's Draft Environmental Assessment acknowledges that it is inevitable that some biting female GM mosquitoes will be released. In the Cayman Islands, mechanical sorting led to about 5,000 biting female mosquitoes in every million males (additional sorting was then performed by hand before release).[7] In Brazil, Oxitec report that female contamination was on average 0.02% i.e. about 200 biting female GM mosquitoes were released in every million males.[8] Oxitec's draft Environmental Assessment states (page 34) states that if more than 0.2% of the sorted population is female the batch is re-sorted prior to release to ensure meeting the 0.2% criterion. However, Oxitec provides no public information on likely numbers for release: this should be corrected and a maximum number included for the licence, since larger release numbers will clearly increase the numbers of biting females that are inadvertently released. In its experiments in Panama, Oxitec reports that the equivalent of less than one female GM mosquito per person per year was released.[9] However, given the small numbers of *Aedes aegypti* that may be needed for disease transmission, these biting females could still play a role in the transmission of dengue, chikungunya or zika from an infected person to an uninfected person.

As well as being up to 0.2% of the released GM mosquito population, some next-generation GM females will hatch and survive to adulthood. In the absence of tetracycline, Oxitec's draft Environmental Assessment reports a survival rate from the larval stage to flying adults of 3.4% (Table 2, page 53). Survival rates may increase if GM mosquitoes breed in areas contaminated with sufficiently high doses of tetracycline (discussed further below). The percentage of surviving GM insects, including biting females, could also increase if resistance to the genetic killing mechanism evolves over time: for example, genetic mutations in the insects which allow the GM insects to survive and breed successfully could be rapidly selected for during mass production.[10,11] Surviving next-generation GM mosquitoes would be expected to include 50% males and 50% females, which will bite and feed on human blood.

Released or surviving biting females may spread tropical diseases (dengue, chikungunya, zika) if these diseases are present in the Keys, by biting an infected person and then a number of uninfected persons.

The possibility that the released GM mosquitoes are already infected with diseases also needs to be considered. Oxitec's draft Environmental Assessment (page 31) states that the horse blood it uses to feed the GM 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. 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.[12,13] The UK has several endemic mosquito species (mainly *Culex* species) that could potentially act as vectors for West Nile Virus in the future. To reduce the risk that infected mosquitoes (including some biting females) are released, a protocol for testing the GM mosquitoes for pathogenic agents should be introduced at the proposed Hatching and Rearing Unit (HRU) at the Florida Keys Mosquito Control District (FKMCD) site in Marathon.

Steps are also required to ensure that the GM 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 GM

mosquitoes in Mexico. Quartz reports[14]: "*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*". 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.

Potential spread of antibiotic resistant bacteria

The use of tetracycline to breed the GM mosquitoes in the lab or in factories for large-scale production carries the risk of spreading antibiotic resistance, which could pose a major risk to human and animal health.[15] Insect guts are reservoirs for antibiotic resistance genes with potential for dissemination.[16,17] 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.[18,19] For example, swallowing or being bitten by GM mosquitoes might transfer antibiotic resistance from bacteria in the insect's gut or salivary glands into bacteria in human or animal guts or bloodstreams which cause disease. If these bacteria become resistant to tetracycline as a result, some human or animal diseases may become difficult to treat.

This issue has been dismissed by Oxitec on page 76 of the draft Environmental Assessment, where the company states: "*A potential impact could be from insect gut bacteria acquiring antibiotic resistance genes as they are fed on antibiotics in the laboratory and could spread those genes in the environment. There is no causal pathway for this to occur as gut bacteria are lost during mosquito metamorphosis from larvae to adults (DeMaio et al., 1996; Moll et al., 2001). Larvae are treated with tetracycline, but as described above the gut bacteria are lost during the pupal stage (e.g., stay in the rearing water), and pupae and adults are not subsequently treated with tetracycline during the rearing*".

However, although Moll et al. (2001) do identify a process through which many midgut bacteria are lost during metamorphosis, it is not correct to say that there is no mechanism for transfer of antibiotic resistant bacteria from larvae to adults. Oxitec's claim considerably overstates what is known about the transfer of bacteria from larvae to adults and ignores some published evidence. For example, in one study, *Serrattia odorifera* was the only microbe commonly associated in the midguts of all pupae and adults studied, suggesting it remains with the *Aedes aegypti* mosquito from the larval stage: contradicting Oxitec's claim.[20] If antibiotic resistant bacteria are transferred from the larval stage to adulthood, they may be released with the adult (largely male) GM mosquito releases and pose a risk to human and animal health.

In general, very sparse information is available on the nature of the microbial community associated with *Aedes aegypti* mosquito larvae in domestic water containers in and around human dwellings. A study of bacteria associated with the larvae of *Aedes Aegypti* was published for the first time in 2014.[21] Hence many uncertainties remain and more studies are needed to quantify the risk of spread of antibiotic resistant bacteria into the environment during releases of Oxitec's GM mosquitoes.

In relation to the risks of disposal of tetracycline from the mosquito production facility, Oxitec presents information in its draft environmental assessment regarding the degradation of tetracycline (page 53). However, antibiotic resistant bacteria may persist long after degradation of the antibiotic which caused the resistance to develop. As cited above, the

draft Environmental Assessment (page 76) appears to accept that tetracycline resistant bacteria could stay in the rearing water due to their loss during metamorphosis. However, no information is provided about the disposal process for the rearing water which is likely to contain these bacteria, including the expected quantities to be produced. This is important because it could lead to the dispersal of such antibiotic resistant bacteria into the environment, posing a risk to human or animal health.

Risks of releasing a non-native strain

Oxitec's GM mosquitoes have been developed from a non-native strain of the invasive species *Aedes aegypti*. Oxitec's OX513A strain was developed using strains of *Aedes aegypti* which originated in Mexico and Cuba, rather than in Florida. As described in Oxitec's draft Environmental Assessment (pages 21 and 22), the GM strain OX513A was produced in 2002 by microinjection into individual embryos of *Aedes aegypti* from a Rockefeller strain background. 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.[22,23]

Different strains of *Aedes aegypti* will have different properties including differences in resistance to insecticides and in vector competence (the ability to transmit tropical diseases). These properties can be transferred to the wild mosquito population through mating and may persist in the wild population, even if the GM mosquitoes themselves die out. It is therefore risky to a release a non-native strain and very important to know its properties, especially its disease transmission properties and resistance to insecticides.

Oxitec reports some testing of its OX523A strain for insecticide resistance (Appendix E) against temephos (tested on 4th instar larvae) and permethrin, deltamethrin, bendiocarb and malathion (all tested on adults), and for two mutations associated with pyrethroid and DDT resistance. However, the company dismisses the finding of resistance to bendiocarb, on the grounds that this also appears in the control. In addition, the native wildtype *Aedes aegypti* from Florida was not used as a control in Oxitec's experiments, so no information is available regarding whether native *Aedes aegypti* are already resistant to bendicarb. Florida Keys Mosquito Control district currently uses *Bacillus thuringiensis israelensis* (Bti), applied from the ground or by air to larval habitats, and Spinosad (*Saccharopolyspora spinose*) as a larvicide for container breeding mosquitoes.[24] Susceptibility of the strain to these larvicides was not tested, which is a serious omission given the current reliance of mosquito control on these larvicides. The District uses permethrin, a pyrethroid (synthetic form of pyrethrum), to control adult mosquitoes when dispensed by truck-mounted sprayers and sprays the insecticide naled from aircraft to control adult mosquitoes. Although Appendix E includes susceptibility tests for permethrin, it does not include naled, which is another important omission from these tests. Other insecticides used for mosquito control in Florida include other artificially created pyrethroids, resmethrin and sumethrin; insect growth regulators such as methoprene and diflubenzuron; and *Bacillus sphaericus*.[25] Resistance to all these insecticides should have been included in the tests.

The experiment will introduce some biting female *Aedes aegypti* at the release site, and more may survive to future generations (as noted above). Due to the large number of males released and the aim to ensure that at least 50% of these mate with wild mosquitoes, there is a high probability of introgression of traits found in the released GM mosquito population into the wild population, even if the original and any surviving GM mosquitoes do die out. For example, in 1974 about 800 sex-linked translocation heterozygote males of *A. aegypti* were released daily for 10 weeks in a Kenyan village. Two genetic markers that were carried by

released males but unknown to the region persisted in the population during the entire period of observation of nearly a year after the experiments, even though few translocation-bearing progeny of released males survived.[26] Consistent with this real world evidence, Oxitec has demonstrated the effects of rapid introgression of insecticide-susceptible traits in its own research and modelling of its GM agricultural pests.[27,28] In the case of the proposed releases of GM mosquitoes in the Florida Keys, the opposite could occur and the proposed releases could lead to the introduction of an insecticide resistant trait into the wild *Aedes aegypti* population at the site.

Perhaps even more importantly, no testing for disease transmission traits in the GM *Aedes aegypti* proposed for release has been included in Oxitec's draft Environmental Assessment. This is critically important because *Aedes aegypti* may transmit zika, chikungunya, yellow fever and four different serotypes of dengue, yet strains may vary in their ability to transmit these tropical diseases.[29,30,31,32,33,34,35] Different strains of *Aedes aegypti* exhibit wide variation in vector competence to transmit dengue.[36] In the case of zika, little is known about vector strain variation and its consequences. Due to the likely introgression of traits into the wild species, as described above, there is a risk that wild type *Aedes aegypti* at the site become better vectors for one or more of these tropical diseases as a direct consequence of introgression of non-native disease transmission traits from the proposed releases of Oxitec's GM mosquitoes. This could have serious negative implications for human health. Preferably, non-native pest species should not be released at all: at minimum extensive testing of disease transmission properties should be required.

Risk of wild type Aedes aegypti mosquitoes migrating to surrounding areas in response to the releases

In Oxitec's experiments in both the Cayman Islands and Brazil, control areas were next to the release areas, making it impossible to assess whether wild *Aedes aegypti* simply move to neighbouring areas when the release ratio of GM males to wild males is high. In both the Cayman Islands (Figure 2(c))[37] and Juazeiro, Brazil (Figure 2(D))[38], the reported ovitrap index in the neighbouring area increases as the index decreases in the target area, suggesting more wild type *Aedes aegypti* mosquito eggs are being laid there. Although this is not conclusive proof of cause and effect, the risk that the releases increase wild mosquito populations in surrounding areas should have been included in the Environmental Assessment as this is a possibility that is consistent with this data.

Although the relationship between *Aedes aegpti* mosquito density and disease risk is not straightforward, the possibility that disease risk (for dengue, chikungunya and/or zika) increases in neighbouring areas needs to be considered.

Risk of increasing other mosquito vectors

Other species could increase in numbers due to the single-species population suppression of *Aedes aegypti*, perhaps due to reduced competition for breeding sites. If they become better vectors, or are more difficult to eradicate, this could lead to harm to human or animal health. This risk is considered further below, as it occurs via changes to the ecosystem.

Potential impacts on the ecosystem

Impacts of population suppression of Aedes aegypti on other disease vectors

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.[39] In the case of zika, the assumption in Brazil has been that *Aedes aegypti* has been the vector responsible for the current outbreak: however this has not been

demonstrated scientifically, and other species (including *Aedes albopictus*, and perhaps more common *Culex* species) may be responsible.[40]

There is inadequate consideration in the draft Environmental Assessment and FONSI of whether temporary population suppression of *Aedes aegypti* (if achieved) could enable an increase or expansion in territory occupied by other vectors, including the competitor species *Aedes albopictus*, an important vector for dengue and chikungunya in many countries which may be harder to eradicate than *Aedes aegypti*. [41,42,43,44] The consideration of possible zika transmission by *Aedes albopictus*, or by other more common *Culex* species, is also omitted from Oxitec's draft Environmental Assessment. Oxitec's draft Environmental Assessment merely mentions that *Aedes aegypti* is a vector for zika, but says nothing about the potential impacts of the proposed releases on this disease.

Impact of the proposed releases on other mosquito species is an important consideration because this is a single species approach, which targets only one vector, unlike most other vector control approaches which e.g. remove breeding sites within a given area, or limit biting by all species e.g. by using repellents. Other species could increase in numbers due to the single-species population suppression of *Aedes aegypti*, perhaps due to reduced competition for breeding sites. If they become better vectors, cause more serious diseases, or are more difficult to eradicate, this could lead to harm to human health.

Aedes albopictus has been responsible for concurrent epidemics of dengue and chikungunya in some countries and its presence can also extend the dengue season and perhaps introduce new viruses.[45,46,47,48,49,50]

In a draft risk assessment submitted to regulators in the USA 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".[51] Oxitec has also published a paper which uses computer modelling to show how *Aedes aegypti* and *Aedes albopictus* may interact.[52] The authors acknowledge that this could have important consequences for the persistence of disease. 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.[53,54] An added complication is that the effects of larval interactions on mosquito populations are different in different contexts, because they may be altered by ecological conditions.[55]

Benedict et al. (2007) report that *Ae. albopictus* (a native of Asia that has spread around the world) was established in Panama in 2002.[56] Researchers at Panama University have described *Aedes albopictus* as more dangerous than *Aedes aegypti* and regard it as a more invasive species which may be very difficult to tackle if it moves into an area.[57] In Brazil, both *Ae. aegypti* and Ae. *albopictus* play a role in transmission of the chikungunya virus.[58] The two species have overlapping habitats and sometimes co-exist.[59] *Aedes albopictus* has been responsible for concurrent epidemics of dengue and chikungunya in Gabon,[60] for an outbreak of dengue fever and dengue haemorrhagic fever in Dhaka, Bangladesh,[61] and for the re-emergence of dengue in southern China.[62]

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*. 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. Brazilian experts have warned that dengue may mutate so that *Aedes albopictus* becomes a more important dengue vector in such circumstances.[63] The potentially devastating effect of a single adaptive mutation in the virus has already been observed with chikungunya. The

mutation lead to improved virus replication and transmission efficiency in *Aedes albopictus*, causing to disease outbreaks as a result of adaption to this new vector.[64,65] Simultaneous dissemination of both dengue and chikungunya can occur via *Ae. aegypti* and *Ae. albopictus*.[66] Information on zika transmission remains sparse.

Competition among larvae may also affect the probability of virus transmission, which may have important consequences for dengue. [67] For *Aedes albopictus*, but not *Aedes aegypti*, competition increases the probability of acquiring disseminated infections of arboviruses. If invasion by *Aedes albopictus* results in competitive replacement of *Aedes aegypti*, so that the two species can coexist, this competitive effect could increase the vectorial capabilities of *Aedes albopictus* compared with that of *Aedes aegypti*. Thus, *Aedes albopictus* may assume a greater role in dengue transmission, because not only the numbers of *Aedes albopictus* but also its ability to transmit the virus could increase.

In the Philippines, Duncombe et al. (2013)[68] suggest that increased numbers of *Ae. albopictus* mosquitoes in vegetative areas later in the wet season may extend spatial and temporal opportunities for dengue fever transmission, which would not be possible if *Ae. aegypti* were the sole vector. They also note that increasing co-circulation of dengue fever virus serotypes in human populations with specific herd immunity may increase the incidence of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are more severe forms of dengue fever resulting from secondary infection with a different serotype. In Sri Lanka, Sirisena and Nordeen (2014) find that the role of *Ae. albopictus* has been underrated and this species is likely to play an important role in the maintenance and transmission of the virus. [69] The greater susceptibility of *Ae. albopictus* to infection is believed to have led to greater dengue virus adaptation, thus Sri Lanka as a whole may be at serious risk of multiple dengue fever/DHF outbreaks in the future with the evolution of new virus strains.

In 2014, Grard *et al.* identified the presence of Zika virus in the invasive mosquito *Aedes albopictus* in Gabon and raised the possibility of a new emerging threat to human health.[70] At the current time the efficacy of *Aedes albopictus* to transmit zika is not well established: however it could be another major vector, in addition to *Aedes aegypti*.[71] Preliminary research indicates that common *Culex* mosquito species may also transmit zika (further research into this possibility is ongoing).[72,73] There is particular concern about zika transmission at the current time due to a probable link with microcephaly cases in babies and with Guillain-Barré syndrome.[74]

In a 2004 study, *Aedes albopictus* was found to have infested Big Pine Key.[75] *Culex quinquefasciatus* (which might turn out to be a vector for the zika virus) is a common species in the Keys. However, *Cx*. *nigripalpus* is also a species of great interest because it is the dominant *Culex* in Florida during the summer and fall, occurs in wastewater systems varying widely in nutrient loads, and is the primary vector of St. Louis encephalitis virus (SLEV) and West Nile virus (WNV).[76] Following the detection of West Nile virus in New York State during the fall of 1999, Florida experienced extensive morbidity and mortality in humans and equines beginning in 2001. Therefore, potential impacts of the proposed releases on *Cx*. *Nigripalpus* populations also need to be considered.

In Panama, Oxitec has studied the response of the co-existing *Aedes albopictus* population to releases of about 4.25 million GM adults in 10 hectare area over a six month period.[77] Although the data shows increases in *Aedes albopictus* at the release site during the releases (Figure 3), Oxitec argues that the increases in the treated area are not significantly greater than at the control sites. However, the authors of this study accept that longer-term studies are needed. In addition, the local environment in Key Haven is different from the environment at the release site in Panama, and other species, in addition to *Aedes albopictus*, also need to be considered.

It is therefore critically important that more consideration is given to the potential expansion or establishment of other vectors if the population of *Aedes aegypti* is suppressed using the proposed single species approach. Relevant studies on species competition could be conducted in the laboratory or as caged trials. Further monitoring is also required at the proposed release site and in the Keys more widely, prior to the approval of any open release experiments, to establish the extent to which *Aedes albopictus* is already a problem, or is in the process of invading, the area. In addition, it is important to conduct studies, or await the availability of ongoing studies, on the role of other vectors, including both *Aedes* and *Culex* species, in transmission of all relevant diseases, including chikungunya and zika. In the case of zika, it remains possible to *Aedes aegypti* is the wrong species to target and therefore any intervention should await confirmation that this species is the main vector causing the outbreak in Brazil.

Impacts of ingestion on wild species and impacts of mass releases on ecosystems
As noted above, the risk of ingestion has been neglected and inadequate safety testing has been conducted (lack of feeding trials) to assess the impacts on wild species which may eat the released mosquitoes. No feeding studies have been conducted in mammals, amphibians, reptiles, crustacea or birds. More feeding trials are needed to assess the risk of ingestion to relevant wild species.

Further, Oxitec's draft Environmental Assessment considers only the potential impact of 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 GM adult males) 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 GM males that are released). This may be followed by a temporary fall in wild numbers at the release site if the experiment is successful in achieving population suppression. 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 is 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. Feeding studies need to take this higher than usual exposure into account (as discussed above).

Potential impacts associated with the failure of the introduced trait in OX513A mosquitoes

Failure of killing mechanism due to tetracycline in the environment and/or evolution of resistance

There are a number of mechanisms through which Oxitec's GM mosquitoes can survive and spread, including by feeding in areas contaminated with the antibiotic tetracycline, which is widely used in medicine and agriculture. In the laboratory, 3% of the offspring of Oxitec's GM mosquitoes survive to adulthood, even in the absence of the antidote tetracycline.[78] When Oxitec's GM 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[79] but later admitted to an 18% survival rate of larvae fed on cat food in a published paper.[80] This survival rate was presumed to be due to contamination of industrially farmed chicken in the cat food with tetracycline.

Oxitec's draft Environmental Assessment (page 52) claims that full survival occurs at tetracycline concentrations above 1 µg/mL and that the no observable effect level (NOEL) was determined to be 1ng/ml. Table 2 (page 53) nevertheless indicates that 4.3% of GM mosquito larvae survive to become flying adults at this 1ng/mL concentration (range 3.2%-5.4%), compared to 3.4% (range 2.4%-4.3%) with no added tetracycline. Numbers of survivors may be large, even if the percentage surviving is small and half the surviving offspring will be biting females. A clear definition of "non-viable adults" is also needed, as there may be potential for some of these females to bite too. It would increase public confidence in Oxitec's findings if independent research were published to confirm their claims regarding the no observable effect level. Independent replication is a cornerstone of the scientific method.

More detail is provided in Oxitec's published paper on this issue which finds that "*concentrations at or below 3 ng mL^{-1} tetracycline,1 ng mL^{-1} chlortetracycline, 10 ng mL^{-1} oxytetracycline and 0.1 ng mL^{-1} doxycycline gave no significant increase in the survivorship of OX513A larvae, i.e. did not increase the proportion of functional adults*". The figure give in this paper for doxycycline is particularly important because it is an order of magnitude lower than the NOEL reported in Oxitec's draft Environmental Assessment.[81] Further, this paper notes: "*In surveying the literature we found a few instances of reported environmental concentrations of doxycycline above the concentration which would allow a greater than the nominal fraction of OX513A larvae to develop to functional adults*". Oxitec argues that these concentrations do not occur in typical breeding sites. However, this claim is likely to be incorrect.

Ae. aegypti commonly live in areas where discarded takeaways are likely to contain meat contaminated with tetracycline. Pet food contaminated with tetracycline may also be available in containers which are accessible as breeding sites. Levels of tetracycline in takeaways or pet food are likely to be similar to those which Oxitec admits led to an 18% survival rate in the laboratory. No survey has been provided of potential sources of tetracycline-contaminated meat in or near the proposed release area. However, in the USA, tolerances are established for the sum of tetracycline residues in tissues of beef cattle, non-lactating dairy cows, calves, swine, sheep, chickens, turkeys, and ducks, of 2 parts per million (ppm) in muscle, 6 ppm in liver, and 12 ppm in fat and kidney and a tolerance is established for residues of chlortetracycline in eggs of 0.4 ppm.[82] In water, 1 ppm is 1 µg/mL (Oxitec's claimed NOEL), so the allowed concentrations in meat are sufficient to at least partially inactivate the killing mechanism in the GM mosquitoes, as the reported findings with cat food suggest. This suggests that Oxitec's claims that increased survival rates will not happen on encountering tetracycline in the wild because the doses available in the environment will be too low are incorrect.

Oxitec's draft Environmental Assessment includes a literature review which reports concentrations of tetracyclines from field sites around the world, however this review does not include any measurements of high risk areas such as septic tanks where mosquitoes may directly encounter human waste. A number of studies have found that *Aedes aegypti* mosquitoes can breed in septic tanks where there are likely to be higher levels of contamination with antibiotics such as tetracycline.[83,84,85,86,87] A 2004 study found that sewage treatment plants, septic tanks, and cesspits were larval development sites for *Aedes aegypti* in the Florida Keys.[88] In 2004, there were more than 36,000 septic systems and 5,000 to 10,000 cesspits in Florida.[89] Although Oxitec's Draft Environmental Assessment states that 99.9% of septic tanks have been eliminated in Key West, the company has not specifically identified any remaining septic tanks or cesspits, nor has any testing been conducted of tetracycline levels in such tanks.

In a conventional SIT programme in Japan, wild females appeared that were unreceptive to mating with irradiated males.[90] Therefore, adaptive behaviour in wild females to increase survival of their offspring, such as seeking out tetracycline-contaminated sites to lay their eggs, must be considered.

A key difference between the Sterile Insect Technique (SIT) using irradiated insects and the release of genetically modified (GM) insects is that radiation-induced sterility involves multiple chromosome breaks, whereas Oxitec's RIDL system relies on a specific genetic modification. Radiation-induced sterility therefore has built-in redundancy that is not provided by molecular genetic approaches.[91] A number of authors have therefore speculated that any genetic or molecular event that allows the GM mosquitoes to survive and breed successfully could therefore be rapidly selected for during mass production.[92] If this happens, the conditional lethality effect could rapidly disappear as resistance develops in production facilities or in the field. Oxitec has published some computer modelling of how resistance to RIDL might develop: whether or not resistance will develop in practice depends on a complex combination of factors.[93] Oxitec accepts that resistance may occur but argues that it could be monitored and detected before a significant loss of efficacy occurred, and a new RIDL line could be substituted. It is not clear how realistic this claim is likely to be in practice and no relevant information is provided in Oxitec's draft Environmental Assessment.

Failure of the killing mechanism could lead to wider spread of mosquitoes inheriting the genetic trait, or other traits acquired through the use of a non-native strain. This is relevant to the following risks:

- Risk of spreading mosquitoes with the intended genetic traits further into the environment, when these traits have not been properly safety tested for adverse health effects on humans or animals, particularly via ingestion (as described above);
- Introduction and reproduction of increased numbers of biting female *Aedes aegypti* that may spread diseases;
- Enhanced spread of traits from the introduced non-native strain that have not been adequately tested (potential for introduction of enhanced disease transmission traits or insecticide resistance).

Efficacy would also be reduced, as higher survival rates would compromise any population suppression effect (poor efficacy is discussed further below in the context of the No Action Alternative).

Surviving mosquitoes containing the GM traits and/or other traits may be widely dispersed via human movement e.g. of tyres, as acknowledged in Oxitec's draft Environmental Assessment. In particular eggs may survive for several months when dried out on the inner walls of containers and may be transported elsewhere.[94] It is therefore misleading to focus only on the lifespan of adults and dispersal through adult flying.

Lack of information about other control measures and the protocol for assessing the outcome of the experiments

Continuing to use traditional control methods for mosquitoes (adulticides and larvicides) could further limit the effectiveness of Oxitec's technology by killing the GM males before they mate with the wild female mosquitoes. Moreover, since there is no data regarding the effectiveness of existing measures, it is hard to see how the claimed benefits of adding GM mosquito releases to the existing measures will be evaluated. On the other hand, failure to use existing control methods – if and when they are effective - 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.

Oxitec's Draft Environmental Assessment states that control of *Ae.aegypti* is currently at best 50% effective, citing a personal communication from Florida Keys Mosquito Control District (FKMCD) (page 17), however no data is provided to confirm this and no information is given about current levels of control of other species. This is important information that should have been included.

The experiment is premature in the absence of any criteria for assessing the impact of existing or new control measures on the incidence of all the relevant diseases, and the absence of any information about how existing control methods will be combined with the proposed releases.

No Action Alternative

Oxitec's draft Environmental Assessment states correctly that the "No Action" alternative in this case would be for Oxitec not to carry out the field trial in Key Haven, Florida (page 16). However the company goes on to claim that the plausible outcomes of this decision are that Oxitec could continue development and commercialization of the product at locations outside of the United States with no intent to market the product in the United States, or select another location in the United States to conduct the field trials.

This assumes that there are likely customers or partners abroad or elsewhere in the United States willing to collaborate in the development and commercialisation of the product. However, this currently seems unlikely. Malaysia has abandoned trials of Oxitec's GM mosquitoes and plans to use the new dengue vaccine.[95,96,97,98] The Cayman Islands have not undertaken any further trials since 2010 and Panama has not undertaken further trials since 2014. Brazil's risk assessment for commercial releases includes a dissenting opinion and questions directly linked to dengue control are left to the Brazilian health authority, ANVISA, which has yet to approve the technology.[99] Oxitec was awarded a UK research grant in 2014 to conduct releases in India[100], however, this award has not been finalised due to lack of approval for these releases. A number of other countries have stated they do not intend to use Oxitec's technology, including Vietnam[101], Paraguay[102], El Salvador[103] and Dominica[104].

Further, Oxitec's claim that its product would likely be tested somewhere else is based on the company's claims that:

(i) Oxitec's GM mosquitoes have generally proved successful at suppressing the population of *Aedes aegypti* mosquitoes where they have been tested to date;
(ii) The expected population suppression effect has the potential to reduce the incidence of relevant tropical diseases.

However, there are several problems with Oxitec's claimed successes to date, which mean that these assumptions are not substantiated by the published evidence. As noted above, in the Cayman Islands and Brazil, control areas were adjacent to release areas and claimed population reductions in the release are could (partially or wholly) reflect migration of wild mosquitoes to surrounding areas. In Panama, the control areas are not adjacent to the release are but figures are reported as relative changes (Figure 6), so important information is missing about the actual impact of the releases on numbers of eggs and adults.[105] A further problem is Oxitec's reluctance to report the release ratios of GM males to wild males, which provide a measure of the effectiveness of the technology, or estimates of adult female population numbers. Unpublished data from Brazil shows release ratios of up to 54 to 1.[106] In both the Cayman Islands and Brazil, increased numbers of males were targeted on smaller release areas as the experiments progressed (for example, reducing the target area from 11 hectares to 5.5 hectares in Juazeiro, Brazil[107]), and in the Cayman Islands pupal releases were added, as the number of adults caused complaints about nuisance from the local population[108]. Modelling of the results from the Cayman Islands suggest this technology is very ineffective at reducing wild mosquito population numbers, requiring 2.8 million GM adult

male mosquitoes to be released per week to suppress a wild population of only 20,000 mosquitoes (10,000 males).[109]

Further, the releases are also unlikely to make a long-term impact on *Aedes aegypti* population density, and thus any potential beneficial impact is likely to be low, even if it were possible to scale the releases up to a commercial scale in future and if population reduction actually led to a reduction in disease risk. The US Centers for Disease Control and Prevention (CDC) suggests that even if all larvae, pupae, and adult *Aedes aegypti* were to be eliminated at once from a site, its population could recover two weeks later as a result of egg hatching following rainfall or the addition of water to containers harbouring eggs.[110]

In addition, the most important potential outcome of vector control is the impact on the future incidence or risk of dengue, chikungunya or zika, not on *Aedes aegypti* populations. Dengue transmission can sometimes continue even with very small numbers of mosquitoes and disease transmission thresholds are unknown.[111,112] The house index (HI, percentage of houses positive for larvae) and the Breteau index (BI, number of positive containers per 100 houses) have become the most widely used indices to study the success of measures to reduce *Aedes aegypti* populations, but their critical threshold has generally not been determined for dengue fever transmission: in addition, care should be taken when extrapolating findings to communities with different herd immunity levels or different environmental conditions.[113,114] Even less information is available regarding transmission thresholds for the chikungunya and zika viruses. Yet these are critical to assessing the success or failure of the proposed experiment. In its papers on its experimental trials in Brazil and Panama, Oxitec uses dengue disease transmission thresholds based on a model developed in 2000 using data from Thailand. These calculations are unlikely to be relevant to the Florida Keys where dengue is not endemic: thresholds are likely to be lower where there is no immunity, if the concept of a threshold is applicable at all. In addition, few details are reported: for example, data from one of the control areas is omitted in the Panama paper (Figure 10)[115]; and disease transmission for chikungunya and zika is not discussed. Oxitec should be required to provide a plausible mechanism through which its proposed releases might reduce the risk of such diseases in the Keys: otherwise the proposed experiment is at best pointless.

As Oxitec's draft Environmental Assessment notes, local transmission of dengue fever was reported in the Florida Keys in 2009 and 2010, with 22 people diagnosed in 2009 and a further 66 people in 2010 (page 15). The reasons for the outbreak are unknown but action taken in response (including prompt diagnosis, increased disease surveillance, increased control of larval and adult mosquito populations and an intense door-to-door campaign to find and eliminate mosquito breeding sites) appears to have been successful.[116] The 2010 cases appeared to be a continuation of the 2009 outbreak, suggesting local transmission for a period of one or two years.[117] However, further local transmission has not been reported since.

Countries or regions with endemic disease (including the US territory of Puerto Rico) would need to consider additional risks due to potential impacts of partial or temporary population suppression on human immunity. In areas of high mosquito abundance, where dengue is endemic, reducing the frequency of biting can increase the incidence of the more serious form and often fatal of the disease, dengue haemorrhagic fever (DHF), by reducing cross-immunity to the four different serotypes of the dengue virus, or increasing the incidence of dengue fever (DF) due to age-related effects (known as 'endemic stability').[118,119] The World Health Organisation has stated that full-scale programmatic deployment is not currently recommended for Oxitec's GM mosquitoes and that Randomized Controlled Trials (RCTs) with epidemiological outcomes should be carried out to build evidence for routine programmatic use of OX513A *Aedes* against *Aedes*-borne diseases.[120] Such trials would need to be conducted in dengue-endemic areas and thus would proceed or not proceed

independently of any trial in Florida, taking into account the additional risks associated with impacts on human immunity to the relevant diseases, and relevant local conditions (such as the role of other vectors in transmitting relevant tropical diseases).

However, vaccines are already emerging as important alternatives, in one case with proven impacts on disease. The first dengue vaccine, Dengvaxia (CYD-TDV) by Sanofi Pasteur, was first registered in Mexico in December, 2015. CYD-TDV is a live recombinant tetravalent dengue vaccine that has been evaluated as a 3-dose series on a 0/6/12 month schedule in Phase III clinical studies. It has been registered for use in individuals 9-45 years of age living in endemic areas.[121] The Philippines has just launched the world's first mass dengue vaccination programme using this vaccine[122]. There are approximately five additional vaccine candidates under evaluation in clinical trials, including other live-attenuated vaccines, as well as subunit, DNA and purified inactivated vaccine candidates. Additional technological approaches, such as virus-vectored and VLP-based vaccines, are under evaluation in preclinical studies.[123] An NIH-sponsored phase 2 clinical trial of chikungunya vaccine opened in late 2015, after promising results in a phase 1 trial.[124] Research on a zika vaccine is also being accelerated.

In Florida, a more likely consequence of refusing the trial (the "No Action Alternative") is that alternative approaches are developed and implemented instead, including the development and deployment of vaccines for travellers to countries where the relevant diseases are endemic.

NEPA Decision and Findings

The FONSI states: "*Most importantly, the status of the environment is restored when releases are stopped (i.e., the released mosquitoes all die, and the environment reverts to the pre-trial status)*". However, this statement is not correct. Potential changes to the environment include:

- Dispersal of non-native traits (potentially including insecticide resistance or enhanced disease transmission properties) via introgression into the native wild mosquito population and/or further afield e.g. via transport of dessicated eggs in containers such as tyres;
- Dispersal of the intended genetic traits via survival of some offspring to adulthood and thus to future generations: likely to be enhanced through encountering environmental tetracycline and/or the evolution of resistance;
- Ecosystem changes that may not be reversible e.g. increase in competitor mosquito species such as *Aedes albopictus*;
- Potential for dispersal of antibiotic resistant bacteria into the environment via the releases of adult mosquitoes or disposal of the rearing water;
- Direct adverse effects that may not be reversible (but which may occur due to lack of adequate safety testing) e.g. harm to humans or animals through ingestion.

In short, if the released mosquitoes all die, this doesn't necessarily mean that there are no ongoing effects on the environment.

Summary of concerns regarding the draft FONSI

In summary, it is premature to issue a Finding of No Significant Impact (FONSI) in the absence of further information, including:

- Further laboratory safety tests, including feeding trials for relevant wild species and laboratory rats to better establish the claim of no harmful effects of ingestion.
- Estimates of the numbers of GM 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 or evolve resistance to the killing mechanism during mass breeding.

- A protocol for testing the GM 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 GM 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 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 risk 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.
- 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 causing the outbreak of zika in Brazil 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 (several orders of magnitude) increase in the number of adult mosquitoes in the target area during the releases.
- Independent replication of Oxitec laboratory results, including studies of proteins in saliva and larval survival rates in the presence of tetracycline contamination.
- A full, published investigation into the unexpected survival of female mosquitoes in Oxitec's experiments in Mexico.

In addition, there is a need for further clarity over who will be liable if adverse impacts do arise and how such impacts would be identified.

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