UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON D.C., 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Review of Section G for an Experimental Use Permit 93167-EUP-E to Test OX5034 *Aedes aegypti* Mosquitoes
Decision #549240

FROM: Kara Welch, M.S., Biologist
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THRU: Eric Bohnenblust, Ph.D., Senior Biologist
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TO: Eric Bohnenblust, Ph.D., Senior Biologist
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ACTION REQUESTED

Review of the experimental protocol and acreage request for Oxitec, Ltd. to conduct field trials using genetically engineered male *Aedes aegypti* of the OX5034 strain carrying the female-specific self-limiting gene.

SUMMARY

Oxitec, Ltd. requests an experimental use permit to investigate the pesticidal efficacy of *Ae. aegypti* strain OX5034 for female larval mortality of wild mosquitoes to support a subsequent Section 3 submission. Oxitec, Ltd. plans to release male *Ae. aegypti* strain OX5034 and compare the survival rates to adulthood between treated female larval progeny (those fathered by OX5034 males) and untreated female larval progeny (those fathered by wild males), over-flooding ratio (i.e., the OX5034 male to wild male ratio), and proportion of treated individuals trapped (i.e., mating fraction). Additional metrics will examine OX5034 male dispersal capacity and persistence of the transgene post-release. The request is not to exceed a total of 6,600 acres for 2020, and if trials continue, into 2021. The site locations include Monroe County, Florida and Harris County, Texas (see Table 1).

In addition to summarizing the Section G protocol, herein EPA assesses the utility of the protocol for collecting data to be used for subsequent Section 3 commercial registration in the conclusions section. EPA requires a minimum of three testing locations for efficacy testing and discusses the mortality measure that will be used to assess product efficacy (i.e., larval mortality versus mating fraction).

CONCLUSIONS

BPPD has reviewed the experimental protocol (Section G) submitted with the application and determined the protocol to be **acceptable with revisions** pending the following changes:

- The rationale provided by Oxitec, Ltd. for only testing in Florida does not discuss how only testing in Florida will demonstrate efficacy against distinct populations of *Ae. aegypti* mosquitoes and satisfy EPA's requirement for at least three distinct testing locations. Justification for these points needs to be provided for testing only in Florida whether trial A only or trial A and trial B are conducted in Florida. In a letter dated February 14, 2020, EPA indicated that for large scale release, (i.e., trial B), data were necessary from at least three test locations. This was prior to EPA's understanding that only trial A might be conducted, EPA maintains that data must be collected from at least three distinct testing locations regardless of which trial or combinations is intended to support efficacy for a FIFRA section 3 registration application.
- In trial A, if only a single release is conducted then subsequent data are only valid for dispersal measurements. Multiple releases must be conducted to measure efficacy.
- For trial A, adult release for mosquito dispersal can only be used to support dispersal area subsequently used for trial B adult releases. To support dispersal distance in trial B for the mosquito rearing box method of deployment with eggs, dispersal distance must be measured through egg releases with the mosquito rearing box using the trial A protocol.
- In trial A, if OX5034 mosquitoes or their offspring are trapped at the outer perimeter of the study design (i.e., 400 m), then trials will need to be reconducted with, 1) a larger perimeter to determine maximum dispersal area, and 2) a greater than 500 m distance between control and treated trials. If trials need to be expanded, the number of replicate trials needs to be adjusted accordingly to not go over the maximum allowable acreage under the EUP.
- For trial B, the distance between trial locations (i.e., control and treated locations) will be greater than the maximum dispersal distance observed in trial A. If the maximum dispersal is less than 400 m, trial locations will be separated by at least 400 m.
- Oxitec, Ltd. must reference or submit the qPCR protocol that will be used to verify OX5034 mosquitoes and their offspring.
- Persistence monitoring must continue until no OX5034 fluorescent larvae are found for at least two successive generations, which may exceed the eight-week timeframe outlined in the EUP.
- Oxitec, Ltd. must describe how to dispose of OX5034 or fluorescent mosquitoes that have been trapped.
- Oxitec, Ltd. must ensure to the extent possible that mosquito abatement activity will be the same in the treated and untreated areas of the EUP and will be disclosed to EPA with data in the final report at the time of application for a registration.

- Field testing sites cannot directly abut the open ocean or other waterbody if this limits dispersal distance measures.
- Oxitec, Ltd. should consider measuring the hatch rate of OX5034 from the mosquito rearing boxes

Oxitec, Ltd. has proposed to measure efficacy as the mortality of treated individuals (i.e., larval progeny of matings between male OX5034 and wild type females). However, EPA has previously indicated mating fraction (i.e., how many insects that are treated, proportion of fluorescent individuals trapped) needs to be used as a measure of population suppression to assess efficacy for this product. Herein, Oxitec, Ltd. has proposed to measure both mortality of treated individuals as well as mating fraction, thus Oxitec, Ltd. under the proposed section G experimental protocol is collecting the types of data that could support efficacy of the product using either way of measuring efficacy. Because Oxitec, Ltd. is collecting the types of data that could be used to support efficacy using either evaluation method, EPA will further consider outside of this Experimental Used Permit Application Oxitec's argument for their measurement of efficacy on the most valid measure of efficacy for OX5034 with respect to adequacy to support a FIFRA Section 3 commercial registration.

Additionally, Oxitec, Ltd. has proposed two testing locations, Florida and Texas, but indicated the possibility of measuring efficacy through trial A alone in Florida, in a single study with three replicates (control, low dose, and medium dose). Oxitec, Ltd. posits that a single test in a suitable climate zone (i.e., Florida, where pest population pressure is high) is sufficient to provide efficacy data, given that sufficient experimental replicates were included in that geographic location. At this time, Oxitec Ltd. has not adequately justified only testing in Florida or only conducting a single trial A, as noted in the 1st deficiency bullet above. EPA's decision about whether a single trial A in Florida might support a FIFRA Section 3 registration application will depend on additional rationale for justification submitted by Oxitec Ltd. and EPA's decision on which efficacy measure EPA determines to be valid for measuring efficacy of OX5034.

BACKGROUND

The target pest is the mosquito species *Aedes aegypti*, also known as the yellow fever mosquito. The applicant, Oxitec, Ltd., is seeking to investigate efficacy amongst other product performance related metrics of *Ae. aegypti* strain OX5034 through experimental field trials. Male OX5034 mosquitoes are homozygous for the female-specific self-limiting gene (i.e., tTAV-OX5034) that is lethal to female offspring when tetracycline is absent from larval diet and results in only hemizygous male offspring survivorship that pass on the transgene to subsequent generations. Importantly, male mosquitoes do not bite and therefore do not transmit diseases. While conversely, females, which as adults do have the capacity to bite, have been shown in laboratory testing shows to die as larvae 100% of the time if they have a single copy of the OX5034 transgene. In addition to the self-limiting trait, OX5034 express DsRed2-OX5034, a red fluorescent marker protein, which aids in identification of OX5034.

TEST LOCATIONS AND ACRES

Oxitec, Ltd. proposes to test male OX5034 *Ae. aegypti* mosquitoes in Monroe County, Florida and Harris County, Texas. In total, Oxitec, Ltd., requests 6,600 acres for testing between the

years 2020 and 2021. Of this acreage 4,800 will be treated and 1,200 will be control sites. The requested test locations and acres are listed in the applicant provided table (see Table 1).

JUSTIFICATION FOR ACREAGE AND TEST LOCATIONS

The stated purpose of this investigation is to determine the pesticidal efficacy of OX5034 *Ae. aegypti* for larval mortality. The primary justifications for the acreage and test locations are:

1. Oxitec, Ltd. is seeking to measure product dispersal/coverage. From a single release point, the expected mean dispersal of male *Ae. aegypti* mosquitoes is expected to be 50 meters (i.e., 1-2 acres), the maximum distance travelled may be as high as 500 meters (i.e., 200 acres). Thus, for a trial that includes three replicates of the untreated control, low dose, medium dose, and high dose scenarios, a minimum of 2,400 acres is needed per site location (i.e., 4,800 acres). Further studies will be conducted to assess the efficacy of multiple release points following determination of mean distance travelled. These studies will have three replicates each of a control, low, and medium dose option for a total of 900 acres per site location if mean distance travelled is less than 100 acres (i.e, 1,800 acres).
2. The test location in Florida was identified as a suitable high-pressure mosquito area because of the climate that is conducive to high pest pressure year-round which allows evaluation of the impact of immigration, timing and frequency of release, and evaluation of release ratio. Testing in Texas represents a different climatic zone.

EXPERIMENTAL DESIGN

Protocol. The purpose of the protocol is to generate efficacy data which will be used to support a FIFRA Section 3 registration. Including:

a. Female larval mortality as a measure of OX5034 product efficacy
b. Adult over-flooding ratio achieved (i.e., OX5034 males to wild males)
c. The proportion of treated (i.e., fluorescent) individuals trapped (i.e., mating fraction)
d. Dispersal of OX5034 males in the field
e. Persistence of the transgene post-release (i.e., distance the transgene disperses, duration/scale of residual activity)

Application Method. Oxitec, Ltd. provided a general protocol which outlined the experimental design that would be applied to all sites. Methods unique to the two trials, the single release and multi-release point studies, were discussed separately and will be paraphrased below in the "Study Designs" section. Treatment areas will be a maximum of 200 acres in size.

Two different deployment methods will be tested in the trials discussed below, egg and adult release modes. The trial will be conducted with eggs or adults only and the two deployment methods will not be mixed in one study. For the egg release, a known quantity of OX5034 eggs will be released in mosquito rearing boxes. In the case of adults, known quantities of contained adults will be acclimatized and released. Differences in the two release methods will be assessed for their impact on efficacy.

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Mosquito sampling will be done weekly using both BG Sentinel traps for the adult population and ovitraps for eggs in all trials. OX5034 mosquitoes and resulting offspring will be identified by fluorescent markers visible in all lifestages. Eggs from ovitraps will be reared in laboratory settings to adult emergence and fluorescence will be confirmed. Additional PCR analysis will be conducted on a minimum of 40 fluorescent and non-fluorescent individuals to confirm accurate identification. Ovitrap data will be used to assess larval mortality, trait persistence, and mating fraction. The adult over-flooding and male dispersal distances will be measured by adult counts in BG Sentinel traps.

The trapping density varies amongst the two separate trial protocols but will be the same within each comparative experimental site. The adult collection and ovitrap egg collection data from the experimental sites will be compared to the control sites to examine for the effect of the release of the product on female larval mortality. There will be a minimum of 400 meters between treatment and control areas.

For data analysis, efficacy will be assessed via Mulla's formula to account for female survival rates in untreated replicates versus treated trials to determine adjusted percent mortality. A general linear mixed-effects model (GLMM) will assess the difference in female survival between control and treatment sites to account for the random effect of site, a crossed random effect of trap distance, and the covariates life stage and release rate. Persistence of the transgene will use the Kaplan-Meier estimator to characterize the estimated mean/median values, 95% confidence intervals, interquartile ranges, and maxima of dispersal/residual activity.

Containment. Known quantities of OX5034 eggs and adults will be delivered to release sites in triple layered containment. Full rearing and quality control protocols are provided in Oxitec, Ltd. (2019, MRID 50889424). For egg release, the rearing box will be drained and disposed of with no parts left behind. Adult male mosquitoes will be transported to field sites, where they will be released after 10 minutes of acclimation. Any OX5034 mosquitoes not used in the program will be killed by freezing and disposed of in general waste. No disposal protocol was provided for the collected eggs/adult transgenic mosquitoes. Additionally, a storage and disposal protocol for individuals used in PCR must be provided.

Study Designs. Although the general protocol will be followed in each location for transport and release of mosquitoes will be followed in both trials, two unique study designs are proposed. The objectives of Trial A will be to quantify various parameters from a single release point and Trial B will be to quantify various parameters over multiple release points. The trials may take place simultaneously and Oxitec, Ltd. indicates it may be sufficient to conduct only trial A.

Trial A The objectives of trial A will be to quantify from a single release point: efficacy of the active ingredient (i.e., percent mortality of female progeny compared with untreated), the adult over-flooding ratio in BG traps, proportion of treated fluorescent individuals in ovi-traps (mating fraction), dissemination of the transgene (maximum travel distance of fluorescent individuals in ovi-traps), and duration/scale of residual activity (time until no adult or fluorescent larvae are found). The protocol may use eggs or adults in the trial, but not both at the same time. Each replicate will be a maximum size of 200 acres. The total acreage for this protocol is 2400 acres in both Texas and Florida for a total of 4800 acres.

Trial B The objectives of trial B will be to quantify from multiple release points: efficacy of the active ingredient (i.e., percent mortality of female progeny compared with untreated), the adult over-flooding ratio in BG traps, proportion of treated fluorescent individuals in ovi-traps, duration/scale of residual activity (time until no adult or fluorescent larvae are found), and presence of fluorescent larvae in natural breeding sites. The trial will release eggs only. The acreage requested per replicate for trial B is 100 acres and may be less. The acreage will be based on mean dispersal distance identified in trial A across multiple release points. The total acreage for this protocol is 900 acres in both Texas and Florida for a total of 1800 acres.

The primary difference between trial A and B is the single versus multi-release point deployment options. Additionally, the former measures dispersal distance and the latter additionally assess presence of larvae in natural breeding sites. Note, penetration of the transgene in cryptic breeding sites will not be submitted as an efficacy measure for commercial registration.

AMOUNT OF ACTIVE INGREDIENT

Oxitec, Ltd. projects no more than 24,360,000 males will be released per week across both trials over the 24-month test period. The amount of active ingredient (i.e., the transgene, tTAV-OX5034) has been submitted to EPA and is pending review. The Agency will report this figure in a subsequent addendum to this review following product characterization review.

Table 1. Number of sites, application rates, and replicates for Trials A and B, including the treated acreage and life-stages assessed.

Trial	Location*	Number of untreated areas (required)	Number of treated areas -low dose (required)	Number of treated areas - medium dose (optional)	Number of treated areas - high dose (optional)	Max acreage per trial site	Maximum total treated acreage	Life stage assessed
Trial A	Florida - Monroe Co.	3	3	3	3	200	2400	Eggs or adults (one life stage only)
	Texas – Harris Co.	3	3	3	3	200	2400	Eggs or adults (one life stage only)
Trial B	Florida - Monroe Co.	3	3	3	N/A	100	900	Eggs only
	Texas – Harris Co.	3	3	3	N/A	100	900	Eggs only

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*Note, both or only one location (FL or TX) may be used