Primary Reviewer: Cassandra Kirk, Ph.D., Biologist, OPP/BPPD **Date:** 3/10/20 Date: 2020.03.10 16:08:29 -04'00'

Secondary Reviewer: Chris A. Wozniak, Ph.D., Biotechnology Special Assistant, OPP/BPPD **Date:** 3/10/20 CHRISTOPHER R WOZNIAK Digitally signed by CHRISTOPHER WOZNIAK Date: 2020.03.10 17:04:28 -04'00'

DATA EVALUATION RECORD

REQUIREMENT: Non-Guideline

TEST MATERIAL: *Aedes aegypti* OX5034

SYNONYMS: OX5034

CITATION: Dose Response of Hemizygous *Aedes aegypti* OX5034 to Tetracyclines and Effects of Environmental Exposure to Tetracyclines.
MRID: 50889415

SPONSOR: Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX United Kingdom

AUTHOR: Oxitec Ltd.

TEST SITE: Not specified but assume Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX United Kingdom since Oxitec Ltd. is documented as the author.

COMPLIANCE: Good Laboratory Practice Standards, 40 CFR Part 160, are not applicable to this document.

EXECUTIVE SUMMARY:

The tTAV-OX5034 expression is made female-specific by inclusion of a splicing module, derived from the *Ae. aegypti Aeadsx* (doublesex) gene, that is naturally alternatively spliced between males and females as part of the sexual differentiation pathway (Salvemini et al., 2011). This has been linked to the tetracycline-off (tet-off) system. The tet-off system activates tTAV-OX5034 expression in females in the absence of a tetracycline analogue, therefore all females carrying a copy of the transgene die at an early larval stage due to the accumulation of tTAV-OX5034 protein produced by a positive feedback loop (Gossen & Bujard, 1992; Gong et al., 2005). Females die in the absence of tetracycline analogues whether they are homozygous or hemizygous for the OX5034 rDNA. However, if a suitable tetracycline analogue is added to the larval rearing medium in sufficient quantities, tTAV-OX5034 expression is repressed, allowing for normal development of females to adulthood. Of the tetracycline analogues available, doxycycline is used to maintain colonies of the OX5034 in the insectary. The OX5034 strain is therefore also referred to as "male-selecting".

The report evaluated the dose-response of OX5034 mosquitoes to a range of tetracycline analogues, and related the concentrations of each tetracycline analogue, required to rescue female OX5034 mosquitoes, to the levels of each tetracycline analogue typically found in the environment as a result of industrial or household tetracycline usage.

The response of OX5034 hemizygous larvae to different doses of tetracycline, oxytetracycline, chlortetracycline and doxycycline was evaluated under laboratory conditions to identify the lowest concentrations that allow for survival of hemizygous OX5034 female adults.

Key results are as follows:

- Female-specific larval death occurs in OX5034 individuals reared at low concentrations of tetracyclines, with survival to pupal stage occurring at different concentrations of each antibiotic. In general, doxycycline achieves rescue of OX5034 life stages at lower concentrations than the other three tetracyclines tested.
- Female pupae were observed at concentrations above 100 pg/mL (DOX), 3ng/mL (CTC), 10 ng/mL (OTC) and 100 ng/mL (TC), however, they proved non-viable, failing to develop into functional, adults capable of flight.
- Eclosion and survival of functional adult females starts at 10ng/mL (DOX), 1 µg/mL (TC, OTC) and above 1 µg/mL (CTC), with the proportion increasing as concentration increases.

Table 1. The EC_{50} (half maximal effective concentration; the concentration which induces a response halfway between the baseline and maximum) for OX5034 larvae reared in the presence of different tetracycline analogues. Results displayed are for functional (*i.e.* flying) females, modelled using a log-linear model.

Tetracycline analogue	Adult Female Rescue concentration	EC_{50}	95% Confidence interval		Pupal Rescue concentration	Non-functional female[1] Rescue concentrations
Doxycycline	10 ng/mL	0.24 µg/mL	0.13 – 0.34 µg/mL		100 pg/mL	300 pg/mL to 10 ng/mL
Chlortetracycline[2]	*> 1 µg/mL*	*> 1 µg/mL*	N/A		3 ng/mL	3 ng/mL to >1µg/mL
Oxytetracycline	1 µg/mL	1.42 µg/mL	1.15 – 1.69 µg/mL		10 ng/mL	30 ng/mL to 1 µg/mL
Tetracycline	1 µg/mL	1.37 µg/mL	1.35 to 1.38 µg/mL		100 ng/mL	100 ng/mL to 1µg/mL

[1]Non-viable adults are a composite of dead adults on the water of the eclosion container, dead adults on the floor of the cage and non-flying adults; all these classes are considered to have zero survivability as *Aedes aegypti* court and initiate mating on the wing, and flight ability is required in the field to avoid predators and to find mates, hosts and oviposition sites.
[2]For larvae reared in the presence of chlortetracycline, no concentration tested produced any functional females and therefore the EC_{50} will be in excess of 1 µg/mL.

CLASSIFICATION: SUPPLEMENTAL

I. MATERIALS AND METHODS:

A. GUIDELINE FOLLOWED: Not Applicable

B. MATERIALS:

1. Test Material:

Stock solutions of the tetracycline analogues were stored at -20 C°:

- Doxycycline hydrochloride (Sigma-Aldrich, UK)
- Chlortetracycline hydrochloride (Sigma-Aldrich, UK)
- Oxytetracycline hydrochloride (Sigma-Aldrich, UK)
- Tetracycline hydrochloride (Sigma-Aldrich, UK)

2. Control Substance:

- Deionized water

3. Test Organism:

Species (common and scientific names): OX5034 (*Aedes aegypti*)
Number of test individuals: 200 L1 (first instar) larvae per 3 replicates.

Strain/Source:
Hemizygous OX5034 eggs were obtained by outcrossing 100 OX5034 males to 200 Wild Type females. Eggs were obtained by providing mated females with defibrinated horse blood and allowing access to wet seed germination paper as an oviposition substrate. Homozygous OX5034 eggs were obtained from a laboratory colony, established for 27 generations.

All strains were reared under standard insectary conditions: 26 C° [± 2 C°], 70% [± 10%] relative humidity, 12h: 12h light: dark cycle.

4. Control Organism:

Species (common and scientific names): *Aedes aegypti* Latin Wild Type (LWT)
Number of test individuals: 200 L1(first instar) larvae per 3 replicates.

Strain/Source:
This non-transgenic strain was originally collected in the region of Chiapas (Mexico) and was transferred from Mexico's Institute of Public Health to Oxitec's UK laboratories in 2006 where a colony has been maintained continuously. All strains were reared under

standard insectary conditions: 26 C° [± 2 C°], 70% [± 10%] relative humidity, 12h: 12h light: dark cycle.

C PURPOSE OF THE STUDY:

The presence of tetracycline(s) in the environment may affect survivability and thus persistence of female OX5034 *Aedes aegypti* in the receiving environment. The objective of this study was to determine the lowest concentration of chlortetracycline hydrochloride, oxytetracycline hydrochloride and tetracycline hydrochloride, and doxycycline that allows survival of *Aedes aegypti* females, hemizygous for the OX5034 construct and then compare these concentrations to concentrations of exogenous tetracyclines that might be encountered in the environment.

D METHODS:

Eggs were hatched in new 16oz. deli pots under vacuum for two hours. For each strain, three cohorts of 200 L1 larvae were counted into new 16oz. deli pots using tetracycline-free methods and reared in 200 mL of deionized water. Additional cohorts of 200 L1's were counted into 16 oz. deli pots and reared in 200ml of dl water containing a range of tetracycline analogue concentrations as follows:

- *Doxycycline:*

Eleven concentrations were tested: 100 pg/mL, 300 pg/mL, 1 ng/mL, 3 ng/mL, 10 ng/mL, 30 ng/mL, 100 ng/mL, 300 ng/mL, 1 µg/mL, 3 µg/mL and 6 µg/mL. Experimental doxycycline solutions were prepared from 0.1g/L stock solution stored at -20°C.

- *Chlortetracycline hydrochloride:*

Seven concentrations were tested; 1 ng/mL, 3 ng/mL, 10 ng/mL, 30 ng/mL, 100 ng/mL, 300 ng/mL and 1 µg/mL. Experimental chlortetracycline solutions were made from a 0.2 mg/mL stock solution.

- *Oxytetracycline hydrochloride:*

Eight oxytetracycline hydrochloride concentrations were tested; 3ng/mL, 10ng/mL, 30ng/mL, 100ng/mL, 1 µg/mL, 3 µg/mL, 10 µg/mL and 30 µg/mL. Experimental oxytetracycline solutions were made from a 0.2 mg/mL stock solution.

- *Tetracycline hydrochloride:*

Five tetracycline hydrochloride concentrations were tested; 10 ng/mL, 100 ng/mL, 1 µg/mL, 3 µg/mL and 10 µg/mL. Experimental tetracycline solutions were made from a 0.2 mg/mL stock solution.

Upon pupation, dead larvae, dead pupae and live pupae were collected and counted daily. Pupae from each cohort were screened for DsRed2 fluorescence and male and female pupae transferred to individual cages for eclosion and adult survival assessment. Adults were provided with a 10% sucrose solution food source. Three days after the last pupa was added, cage contents were scored for eclosion rate based on the criteria in Table 2.

Table 2. OX5034 phenotypes scored in this study

Descriptor	Phenotype
Live pupae	Live, swimming pupae.
Dead pupae	Pupae that died in the eclosion container, including those partially eclosed.
Dead adult on water	Fully eclosed adults that have died on the surface of the water in the eclosion container.
Dead adult on cage floor	Fully eclosed adults that have left the eclosion container and subsequently died.
Non-functional adults	Fully eclosed, live adults that are unable to maintain flight.
Functional adults	Fully eclosed, live adults able to maintain flight.

Statistical analysis:
Dose response data were analyzed using the RStudio software package version 1.1.456 (RStudio, USA) running the Dose Response Curve (DRC) packages. Log-linear distributions were used to model the data. The models were used to calculate the EC_{50} (Effective Concentration50) with 95% confidence intervals. Graphs were designed in GraphPad Prism 7.05.

II RESULTS:

Dose-response of hemizygous OX5034 *Aedes aegypti* to tetracyclines
Female-specific larval death occurs in OX5034 individuals reared at doxycycline concentrations at or below 100 pg/mL dox (Table 3.). Between doxycycline concentrations of 300 pg/mL and 3 ng/mL the presence of female pupae was observed, however, they proved non-viable, failing to develop into functional, adults capable of flight. Eclosion and survival of functional adult females starts at 10 ng/mL with the proportion increasing as concentration increases. Doxycycline concentrations at or above 30 ng/mL prove to be significantly different from 0 µg/mL ($p=0.019$).

Table 3. OX5034 hemizygous female larvae reared at different doxycycline concentrations

Doxycycline concentration	Dead female pupae	Non-viable female adults	Flying female adult
6 µg/mL	1.3% (±0.7%)	2.0% (±1.1%)	62.0% (±19.0%)
3 µg/mL	4.1% (±3.2%)	1.3% (±1.7%)	66.1% (±14.6%)
1 µg/mL	2.3% (±1.3%)	10.0% (±3.9%)	64.3% (±21.3%)
300 ng/mL	2.4% (±0.7%)	30.7% (±2.8%)	31.0% (±5.5%)
100 ng/mL	4.4% (±5.6%)	41.0% (±9.3%)	25.8% (±8.7%)
30 ng/mL	3.4% (±2.4%)	62.7% (±17.3%)	4.0% (±5.9%)
10 ng/mL	4.0% (±1.2%)	48.0% (±14.4%)	1.00% (±1.1%)
3 ng/mL	23.2% (±13.6%)	39.3% (±5.8%)	0.0% (±0.0%)
1 ng/mL	20.3% (±10.5%)	12.7% (±4.7%)	0.0% (±0.0%)
300 pg/mL	7.7%	5.3%	0.0%
100 pg/mL	0.0% (±0.0%)	0.0% (±0.0%)	0.0% (±0.0%)
0 µg/mL	0.0% (±0.0%)	0.0% (±0.0%)	0.0% (±0.0%)

Percentages are means of L1 individuals reaching the specified stage based on counts of 200 L1 larvae per replicate (n=3) and assuming a 50:50 sex ratio (n=300), excluding any non-fluorescent pupae. 95% confidence intervals are displayed in parentheses. Non-viable adults include; dead adults on the water surface, dead adults in the cage and non-flying adults.

At all chlortetracycline concentrations tested (1 ng/mL to 1 µg/mL), no functional females were observed (Table 4.). Between concentrations of 3 ng/mL and 1 µg/mL the presence of female pupae was observed, however they proved non-viable, failing to develop into functional adults capable of flight. Eclosion and survival of functional adult females was not observed within this concentration range. Male survival was unaffected by chlortetracycline concentration, with an average of 88.3% (±5.3 CI) functional adults observed.

Table 4. OX5034 hemizygous female larvae reared at different chlortetracycline concentrations

Chlortetracycline concentration	Dead female pupae	Non-viable female adults	Flying female adult
1 µg/mL	6.0% (±4.9%)	56.7% (±3.3%)	0.0% (±0.0%)
300 ng/mL	28.3% (±1.7%)	13.3% (±10.2%)	0.0% (±0.0%)
100 ng/mL	6.3% (±2.4%)	0.0% (±0.0%)	0.0% (±0.0%)
30 ng/mL	5.0% (±4.5%)	0.7% (±1.3%)	0.0% (±0.0%)
10 ng/mL	1.0% (±1.1%)	0.3% (±0.7%)	0.0% (±0.0%)
3 ng/mL	0.7% (±1.3%)	0.3% (±0.7%)	0.0% (±0.0%)
1 ng/mL	0.0% (±0.0%)	0.0% (±0.0%)	0.0% (±0.0%)

Percentages are means of L1 individuals reaching the specified stage based on counts of 200 L1 larvae per replicate (n=3) and assuming a 50:50 sex ratio (n=300), excluding any non-fluorescent pupae. 95% confidence intervals are displayed in parentheses. Non-viable adults include; dead adults on the water surface, dead adults in the cage and non-flying adults.

Female-specific larval death occurs in OX5034 individuals reared at oxytetracycline concentrations at or below 10 ng/mL (Table 5.). At a concentration of 30 ng/mL the presence of female pupae was observed, however, they proved non-viable, failing to develop into functional adults capable of flight. Eclosion and survival of functional adult females starts at 1 µg/mL with the proportion increasing as oxytetracycline concentration increases. Male survival was unaffected by oxytetracycline concentration, with an average of 83% (±4.5%, 95% CI) functional adults observed.

Table 5. OX5034 hemizygous female larvae reared at different oxytetracycline concentrations.

Oxytetracycline concentration	Dead female pupae	Non-viable female adults	Flying female adult
30 µg/mL	1.7% (±0.7%)	1.7% (±1.7%)	65.7% (±16.2%)
10 µg/mL	1.3% (±0.7%)	5.3% (±2.8%)	52.7% (±16.1%)
3 µg/mL	1.0% (±0.0%)	30.7% (±3.3%)	52.7% (±14.6%)
1 µg/mL	2.0% (±2.3%)	57.7% (±0.7%)	19.7% (±6.2%)
100 ng/mL	15.8% (±4.6%)	29.2% (±14.1%)	0.0% (±0.0%)
30 ng/mL	4.0% (±1.1%)	0.3% (±0.7%)	0.0% (±0.0%)
10 ng/mL	0.0% (±0.0%)	0.0% (±0.0%)	0.0% (±0.0%)
3 ng/mL	0.0% (±0.0%)	0.0% (±0.0%)	0.0% (±0.0%)

Percentages are means of L1 individuals reaching the specified stage based on counts of 200 L1 larvae per replicate (n=3) and assuming a 50:50 sex ratio (n=300), excluding any non-fluorescent pupae. 95% confidence intervals are displayed in parentheses. Non-viable adults include; dead adults on the water surface, dead adults in the cage and non-flying adults.

Female-specific larval death occurs in OX5034 individuals reared at tetracycline concentrations at or below 10 ng/mL (Table 5.). At a concentration of 100 ng/mL the presence of female pupae was observed, however they proved non-viable, failing to develop into functional adults capable of flight. Eclosion and survival of functional adult females starts at 1 µg/mL with the proportion increasing as concentration increases. Male survival was unaffected by tetracycline concentration, with an average of 92.9% (±7.1 CI) functional adults observed.

Table 5. OX5034 hemizygous female larvae reared at different tetracycline concentrations.

Tetracycline concentration	Dead female pupae	Non-viable female adults	Flying female adult
10 µg/mL	2.0% (±2.3%)	5.3% (±3.6%)	50.0% (±5.2%)
3 µg/mL	1.7% (±1.7%)	27.0% (4.9±%)	44.3% (±7.4%)
1 µg/mL	2.3% (±0.7%)	66.0% (±8.5%)	15.7% (±1.7%)
100 ng/mL	5.3% (±2.4%)	53.0% (±7.1%)	0.0% (±0.0%)
10 ng/mL	0.0% (±0.0%)	0.0% (±0.0%)	0.0% (±0.0%)

Percentages are means of L1 individuals reaching the specified stage based on counts of 200 L1 larvae per replicate (n=3) and assuming a 50:50 sex ratio (n=300), excluding any non-fluorescent pupae. 95% confidence intervals are displayed in parentheses. Non-viable adults include; dead adults on the water surface, dead adults in the cage and non-flying adults.

The data demonstrated that doxycycline concentrations at and above 10ng/mL result in eclosion of functional female adults and the minimum concentration of tetracycline and oxytetracycline required to suppress expression of the female specific transgene, allowing development of functional adult female progeny, was 1µg/mL, which rescued 15.7% (±1.5%) and 19.7% (±5.5%) flying females respectively. No female pupae developed when reared in the absence of tetracycline analogues.

Effects of Environmental Exposure to Tetracyclines

Because the presence of tetracycline(s) in the environment may affect survivability and thus persistence of female OX5034 *Aedes aegypti* in the receiving environment, this effect was evaluated by studying the potential for exposure to exogenous tetracycline concentrations that might be encountered in the environment. To this end, a review of the potential exogenous tetracycline concentrations that could be encountered in the environment was conducted from the scientific literature. The reported concentrations of each tetracycline analogue in various environmental water bodies were compared to the OX5034 female rescue doses for each analogue. In all cases the minimum concentration for each analogue, required to rescue OX5034 females, is higher than the mean concentrations found in environmental water bodies for the studies reviewed (Table 6).

Table 6. Study Authors Results of literature search (February 2019) comprising [doxycycline AND waste water AND USA], [doxycycline AND effluent AND USA], [doxycycline AND ground water AND USA], [doxycycline AND surface water AND USA], and the same set of search terms for chlortetracycline, tetracycline and oxytetracycline antibiotics.

Tetracycline analogue	Reported value	Units	Location	Likely *Aedes aegypti* breeding environment?	Reference*
Tetracycline	0.053	ng/mL	Waste water treatment plant (downstream)	unknown	(Haggard & Bartsch, 2009)
	0.1	ng/mL	Surface water	unknown	(Yang, Cha & Carlson, 2004)
	0.11	ng/mL	Surface water	unknown	(Lindsey, Meyer & Thurman, 2001)
	0.2	ng/mL	Waste water treatment plant influent	no	(Yang, Cha & Carlson, 2005)
	0.56	ng/mL	Waste water treatment plant effluent	no	(Batt, Bruce & Aga, 2006)
	0.98	ng/mL	River downstream of agricultural land	unknown	(Yang & Carlson, 2003)
	1	ng/mL	Downstream of swine production facilities	unknown	(MacKie et al., 2006)
	1.1	ng/mL	Waste water treatment plant	no	(Batt, Kim & Aga, 2007)
	1.2	ng/mL	Waste water treatment plant	no	(Karthikeyan & Meyer, 2006)
	2	ng/mL	Farm spring	unknown	(Campagnolo et al., 2002)
	4	ng/mL	Waste water treatment plant influent	no	(Kim et al., 2005)
	188	ng/mL	Waste water treatment plant influent	no	(Kulkarni et al., 2017)
	410	ng/mL	Hog lagoons	no	(Campagnolo et al., 2002)
Chlortetracycline	0.06	ng/mL	Waste water treatment plant effluent	no	(Yang, Cha & Carlson, 2005)
	0.11	ng/mL	surface water	unknown	(Yang, Cha & Carlson, 2004)
	0.15	ng/mL	surface water	unknown	(Lindsey, Meyer & Thurman, 2001)
	0.27	ng/mL	waste water treatment plant influent	no	(Yang, Cha & Carlson, 2004)
	0.28	ng/mL	river downstream of agricultural land	unknown	(Yang, Cha & Carlson, 2003)
	0.5	ng/mL	runoff from manure-treated agricultural land	unknown	(Dolliver & Gupta, 2008a)
	0.5	ng/mL	Surface water	unknown	(Meyer et al., 2000)
	1.5	ng/mL	Field stream	unknown	(Campagnolo et al., 2002)
	2	ng/mL	Field tile	unknown	(Campagnolo et al., 2002)
	210	ng/mL	direct runoff from manure stockpiles (not standing water)	no	(Dolliver & Gupta, 2008b)

Tetracycline analogue	Reported value	Units	Location	Likely *Aedes aegypti* breeding environment?	Reference*
	870	ng/mL	Hog lagoons	no	(Meyer et al., 2000)
	1000	ng/mL	Hog lagoons	no	(Campagnolo et al., 2002)
Oxytetracycline	0.02	ng/mL	Waste water treatment plant (downstream)	unknown	(Haggard & Bartsch, 2009)
	0.15	ng/mL	surface water	unknown	(Yang, Cha & Carlson, 2004)
	0.66	ng/mL	river downstream of agricultural land	unknown	(Yang & Carlson, 2003)
	1.34	ng/mL	surface water	unknown	(Lindsey, Meyer & Thurman, 2001)
	2	ng/mL	farm spring	unknown	(Campagnolo et al., 2002)
	2	ng/mL	downstream of swine production	unknown	(MacKie et al., 2006)
	410	ng/mL	hog lagoons	no	(Campagnolo et al., 2002)
Doxycycline	0.07	ng/mL	waste water treatment plant effluent	no	(Yang, Cha & Carlson, 2005)
	0.08	ng/mL	surface water	unknown	(Yang, Cha & Carlson, 2004)
	0.25	ng/mL	waste water treatment plant influent	no	(Yang, Cha & Carlson, 2005)
	0.34	ng/mL	river downstream of agricultural land	unknown	(Yang & Carlson, 2003)

*Complete references for these citations can be found in **Appendix A.**

Effects of Tetracycline Use in the Facility

No tetracyclines will be used in facilities used either to produce OX5034 male adults for release, or to package and distribute OX5034 release devices for field deployment. This is because OX5034 male adults develop to adulthood without the presence of tetracyclines, while females die at early larval stages when no tetracyclines are present. This method is used to ensure male-only OX5034 for release (see MRID 50889424 for full details of manufacturing and rearing and release device).

Development of Antimicrobial Resistance with Tetracycline Exposure

The likelihood that the production and release of OX5034 *Aedes aegypti* would lead to the development of antimicrobial resistant prokaryotes and represent a risk to human health or the environment is negligible, as no tetracyclines are used to rear OX5034 males for release.

Citrus Applications Using Oxytetracycline

The availability of tetracyclines in the environment due to agricultural exposure from oxytetracycline applications in Florida to combat citrus greening will not impact the survival of OX5034 where released, as treated areas will be located greater than 400m from commercial citrus growing areas.

III CONCLUSIONS:

Study Authors Conclusion
The study authors conclude that the likelihood that environmental sources of tetracycline would cause any change in the survival of OX5034 due to the availability of tetracyclines in the environment is extremely low, as supported by tetracycline dose response studies (Appendix I, II of original study report MRID 50889415). Further, they conclude that there is no likelihood of adverse effects associated with the production of OX5034 mosquitoes, as no tetracyclines are used in the facility used either to produce OX5034 male adults for release, or to package and distribute OX5034 release devices for field deployment. The development and spread of antimicrobial resistant prokaryotes is negligible, as no tetracyclines are used to rear OX5034 males for release, or in the OX5034 release devices for field deployment.

Reviewers Comments
The reviewer agrees with the study author's conclusion that the development and spread of antimicrobial resistant prokaryotes is negligible because no tetracyclines are used to rear OX5034 males for release, or in the OX5034 release devices for field deployment.

The reviewer also agrees that the concentrations of tetracycline analogues found in the literature review appear to be below the levels required to fully rescue adult females capable of maintaining flight, however two main uncertainties are noted:

1) **Although a literature review of environmental concentrations across the US was performed, a survey was not conducted to determine the presence of potential sources and/or concentrations of environmental tetracyclines in the specific Experimental Use Permit (EUP) Treatment Areas (TAs), such as hospitals (sewage), wastewater treatment plants, livestock sewage lagoons, or aquaculture.**

Tetracyclines in the environment can come from human or animal drugs, or non-drug sources (such as in agriculture). A review of environmental antibiotic degradation indicated that, in general, the highest sources of environmental tetracyclines (in the μg/L range) were from hospitals and municipal wastewater, whereas surface waters, and sea and ground waters were in the ng/L range (Homem and Santos 2011). Tetracyclines are well known to degrade rapidly in sunlight (photolysis) in the presence of catalysts (iron and hydrogen peroxide, both of which can occur naturally in sunlit water) where degradation of tetracycline was complete after 1 minute (Bautitz and Nogueira 2007). The rate of degradation is dependent on the initial concentration and the pH of the water. It is also reported that in natural water samples the rate of photo-degradation is higher than in pure waters due to aquatic matrix effects (Lopez-Penalver et al. 2010).

Aquaculture facilities, farms, hospitals, or municipal sewage facilities are likely the only sources that theoretically could introduce sufficiently high levels of tetracycline into the environment to allow survival of OX5034 females. Given that the TAs are likely to be in relatively developed (urbanized) areas, the presence of livestock or aquaculture is not expected. In general, *Ae. aegypti* prefer man-made containers such as gutters, water containers, and tires that hold rainwater or clean still water for their breeding sites (Tun-Lin et al. 1995; Hribar et al. 2001). A

2004 study did find that sewage treatment plants, septic tanks, and cesspits were larval development sites for *Aedes aegypti* in the Florida Keys (Hribar et al; 2004). However, these are not preferred breeding sites and breeding in septic tanks can only occur where the lid is cracked or broken, providing the female access to a novel oviposition site (Burke et al. 2010). Furthermore, Key West and surrounding areas in Monroe County have eliminated the majority of septic tanks and use a public sewer line system as the major means of waste disposal. In 1999, with nearshore water quality deteriorating around the Florida Keys, the State of Florida mandated through legislation that the entire island chain install advanced wastewater treatment systems to eliminate the use of tens of thousands of septic tanks, illegal cesspits and ineffective small treatment units (Monroe County, 2020). Most of the County is now served by the Cudjoe Regional Wastewater System that includes a deep injection well, which disposes of treated effluent 3,200 feet below the surface, thus excluding exposure of *Aedes aegypti* to effluent. Each of the Florida Keys Aqueduct Authority Wastewater Districts[1] has its own municipal wastewater treatment facility, which consist of a series of open holding tanks, thus allowing access to mosquitoes. Whether or not these tanks contain high enough levels of tetracycline to rescue OX5034 females is unknown as sampling was not conducted. The likelihood of this actually occurring, however, is low due to the fact that *Aedes aegypti* prefers to oviposit in clean waters and tetracycline rapidly undergoes aqueous photolysis in the presence of sunlight. However, the Agency recommends that the perimeter of a TA should be at least 500m from any wastewater treatment facility in either Monroe or Harris County. In Harris County, reclaimed water from all of Houston wastewater plants is discharged directly into a surface waterway, usually one of the area bayous (City of Houston, 2020), however, bayous are not typical breeding sites for *Aedes aegypti*. The handling of sewage effluent from hospitals in the TAs is unknown, however these are likely to be closed systems connected to centralized wastewater systems. A study examining tetracycline levels in environmental samples, including wastewater treatment plants, would be helpful in limiting uncertainties regarding potential sources of tetracycline in the environment. This recommendation is made out of an abundance of caution, despite the fact that the reviewer does not have reason to believe that environmental tetracyclines levels would be higher in the EUP TAs than what was found in the literature survey.

2) **The degradate of tetracycline, 5a,6-anhydrotetracycline is much more effective at inactivating tTA (Gossen and Bujard, 1993; Werten et al., 2014), and thus lower levels are likely required to rescue "functional" adult females as compared to tetracycline, however no degradates were tested in the rescue assay. The current study did not consider the rescue capacity of any of the tetracycline metabolites, thus it is uncertain whether levels of degradates present in the environment may allow of the development of adult females capable of maintaining flight.**

The abiotic degradation products or reversible epimers may be formed through hydrolysis or photolysis, including epi-tetracyclines and anhydrotetracyclines (Brain et al., 2005). Under photochemical degradation, tetracyclines will form a number of degradates, many of which are highly soluble and have been shown to be the more stable form in receiving waters (Halling Sørensen et al., 2002). Degradation products can be as active and/or toxic as their parent (Watkinson et al. 2007). One metabolite of tetracycline, 5a,6-anhydrotetracycline, binds to the tet repressor protein (tTA) approximately 35-fold more strongly as compared to tetracycline

[1] Navy, Key Haven, Big Coppit, Bay Point, Cudjoe Regional, Duck Key, and Layton Long Key.

(Degenkolb et al. 1991). Gossen and Bujard (1993) demonstrated 5a,6-anhydrotetracycline as an alternative effector for tetracycline-controlled gene expression in higher eukaryotic systems. They found that 5a,6-anhydrotetracycline is much more effective than the parent tetracycline in inactivating tTA, and that it completely abolishes tTA mediated luciferase activity at concentrations as low as 3 ng/ml. Data regarding environmental concentrations of 5a,6-anhydrotetracycline could not be found for the US, but a study in Turkey measured 5a,6-anhydrotetracycline downstream from a wastewater treatment plant at 3.57±0.17 ng/mL (Topal and Arslan-Topal, 2015) The concentration of tetracycline found in this same study was 4.12±0.20.

Reviewer's Conclusion and Recommendations:
Because a survey for sources of environmental tetracycline was not performed and degradates were not assessed, this study is classified as scientifically sound but **SUPPLEMENTAL** for use in risk assessment.

For the current requested action (i.e. the EUP) the Agency recommends:

- The perimeter of any TA should be not less than 500m from a wastewater treatment plant for the current experimental use permit without data from the relevant wastewater treatment plant regarding tetracycline concentrations. This measure would also mitigate potential exposure of OX5034 mosquitoes to tetracycline degradates.

For future actions, the Agency recommends:

- Studies examining the capacity for tetracycline degradates to rescue OX5034 females should be conducted prior to commercial registration.
- Future environmental sampling for tetracyclines in a proposed use area should include an examination of degradate levels.

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APPENDIX A:
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