Additional information requested in RIVM communication of January 19th 2017

Molecular characterization OX513A

Q1. The experimental data contained in the submission support the conclusion that backbone sequences of vector pOX53A are absent in genetically modified *Aedes aegypti* 0X513A, but do not exclude this possibility completely. Therefore data are requested that demonstrate the absence of the *bla* gene in 0X513A Aedes aegypti by using PCR targeting the *bla* gene, by Southern analysis or by sequencing.

Standard Operation Procedures

Q2. Is it correct that within the rearing unit (male) mosquitoes are always reared and kept in their primary containers and that no activities with mosquitoes are performed in the rearing unit outside of the primary containers? If activities with mosquitoes outside of the primary container are performed in the rearing unit, these should be addressed in the SOPs.

Q3. The SOPs and ASL-2 mention that records should be kept on inadvertent escape within rearing units. Please indicate whether it can be concluded from these records that containment within the rearing units is adequate.

Q4. What extra measures are taken to prevent access to the rearing unit by unauthorized persons or to prevent vandalism, such as a fence?

Q5. Which measures are taken to safeguard the stability of the mobile rearing unit, for example in case of hurricanes?

Q6. In several SOPs there is reference to inactivation of biological material by freezing or chemical decontaminants before material is discarded. Please indicate if efficacy of decontamination and inactivation has been validated.

Q7. Please indicate whether dead insects remaining after cleaning of cages are frozen before they are treated as waste, like is done for all other biological material.

Q8. It is noted that not all SOPs have yet been adjusted for use in Saba, for example who will be contacted in case of an accident or injury (the Saba Department of Agriculture, Hygiene & Vector Control?), how room/area decontamination will take place, whether an air curtain or an inner door will be used etc. Please indicate if and when these SOPs will be adjusted.

Q9. It is mentioned that in some mobile rearing units an air curtain is fitted above the entrance door to the rearing room. Is the air curtain instead of an inner door in agreement with ACL-2?

Q10. Which measures are taken to prevent spillage of tetracycline stocks from the rearing unit into the environment?

Q11. In part B 1.4 it is stated that conditions for transit via St Maarten will need to be explored with local regulatory officials of Saba and St Maarten, and has been identified with Saba legal affairs officials. This may require support from RIVM for attestation of adequate containment of 0X513A in transit. Which support of RIVM is meant?

Q1.

The experimental data contained in the submission support the conclusion that backbone sequences of vector pOX53A are absent in genetically modified *Aedes aegypti* 0X513A, but do not exclude this possibility completely. Therefore data are requested that demonstrate the absence of the *bla* gene in 0X513A Aedes aegypti by using PCR targeting the *bla* gene, by Southern analysis or by sequencing.

See study report SR-00043 Ed1a CONFIRMATION OF ABSENCE OF THE *BLA AMP[R]* GENE IN OX513A *AEDES AEGYPTI* Effective Date: 13 March 2017.

CONFIRMATION OF ABSENCE OF THE *BLA AMP[R]* GENE IN OX513A *AEDES AEGYPTI*

SR-00043 Ed1a

THIS DOCUMENT CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

THIS DOCUMENT CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

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1 SUMMARY

This study was designed to detect the presence or absence of the *bla amp[R]* gene in the OX513A strain of *Aedes aegypti* using Southern blot analysis and complements the previous molecular characterisation of *Aedes aegypti* OX513A whereby the absence of the complete ~4 kb plasmid backbone containing the *bla amp[R]* gene was confirmed by PCR using primers located near the 3' and 5' ITR sequences of the fragment.

The *bla amp[R]* gene was not detected in the OX513A strain and wild-type *Ae. aegypti,* but was readily detected in the positive control samples, both the plasmid from which OX513A was derived and a mosquito genomic sample known to contain *bla amp[R]*. The tTAV gene was detected in all but the wild-type samples as expected, demonstrating the integrity of the DNA used in the Southern blot analysis.

2 ASSOCIATED PERSONNEL

Name	Affiliation	Role/Tasks
Mirel Puinean	Oxitec Ltd.	Senior Scientist
Caroline Philips	Oxitec Ltd.	Research Scientist

3 INTRODUCTION

The *piggyBac*-mediated transformation system is routinely used to insert genetic constructs into insect genomes. The genes to be inserted are part of a larger plasmid DNA construct which, in the case of OX513, includes an ampicillin resistance gene (*bla amp[R]*) and a bacterial origin of replication (pUC ori) to allow the plasmid to be grown under selective conditions in *E. coli* cultures. Previously we have shown that the complete plasmid backbone fragment could not be detected in the genomic DNA of the OX513A strain of *Ae. aegypti.* This study was developed to specifically detect the *bla amp[R]* gene in genomic DNA samples using Southern blot analysis.

In brief, genomic DNA was extracted from OX513A, a wild-type *Aedes aegypti* (negative control), and an archival sample of a discarded development strain no longer maintained, but known to contain the plasmid backbone as previously confirmed by PCR (positive control). Similar amounts (gene copies) of pOX513 plasmid DNA used to transform the insects was also run alongside the gDNA samples. The probes used in this assay were designed to detect the *bla amp[R]* gene as well as the *tTAV* gene (known to be present in OX513A) as a positive control to assess gDNA integrity. Southern blot analysis utilised DIG hybridisation probes, and the presence of the genes was assessed using the DIG detection system.

4 STANDARD OPERATING PROCEDURES.

SOP	Function
TD_SOP_00066	Genomic DNA extraction using phenol-chloroform
TD_SOP_00302	Measurement of nucleic acids concentration using Qubit fluorimeter
TD_SOP_00215	Procedure for Southern blotting

5 ADDITIONAL METHODS

Genomic DNA was isolated using phenol-chloroform from homozygous OX513A mosquitoes and from wild-type *Aedes aegypti* (TD_SOP_00066). Included in the study was also gDNA extracted from archival samples of a development strain of *Ae. aegypti* (no longer in culture), which is known to contain the complete pOX513

backbone comprising an ampicillin resistance gene (*bla amp[R]*) and a bacterial origin of replication (pUC ori) inserted in the genome (positive control strain). The genomic DNA and pOX513 plasmid DNA were digested with restriction enzymes and quantified on a Qubit (Thermo, TD_SOP_00302).

About 5 µg gDNA and 42 pg pOX513 plasmid DNA which both correspond to similar gene copies number (3.25x 10^6) were run in duplicate on a 0.7% agarose gel at 20-60V for ~ 6 hours, and then transferred to a positively charged nylon membrane. The probes for detecting the sequence bla amp[R] (702 bp) and tTAV (645 bp) as a control were amplified by PCR and labelled using the PCR DIG Probe Synthesis Kit (Roche, Germany). Hybridisation was performed at 40°C for 16 hours, with one probe per batch of samples and then the probes were visualised using the labelling and detection kit "DIG High Prime DNA Labelling and Detection Starter II Kit" (Roche, Germany). The enzyme chosen to cut the DNA do not cut the areas recognised by the probes and their position and size of the expected products detailed in section 7.

6 SAMPLES

Sample ID	Description
pOX513 plasmid DNA	pOX513 plasmid DNA construct
Control sample	Genomic DNA extracted from a discontinued *Ae. aegypti* development strain with confirmed plasmid backbone (bla amp[R] gene and pUC ori inserted in the genome) and two copies of the tTAV gene.
OX513A	Genomic DNA extracted from OX513A pupae
WT	Genomic DNA extracted from wild-type *Ae. aegypti* pupae

7 CONSTRUCT MAP AND SCHEMATIC

Below there is a schematic map of the pOX513 construct used to obtain the OX513A strain indicting the features of the construct, the positions of the restriction sites used to digest the DNA and the relative size and position of the Southern blot hybridization probes (figure 1). Also, figure 2 indicates the construct inserted into the *Ae. aegypti* genome with the size of the fragments expected on the Southern blot after digestion. The fragments and their sizes are also summarised in the table below.

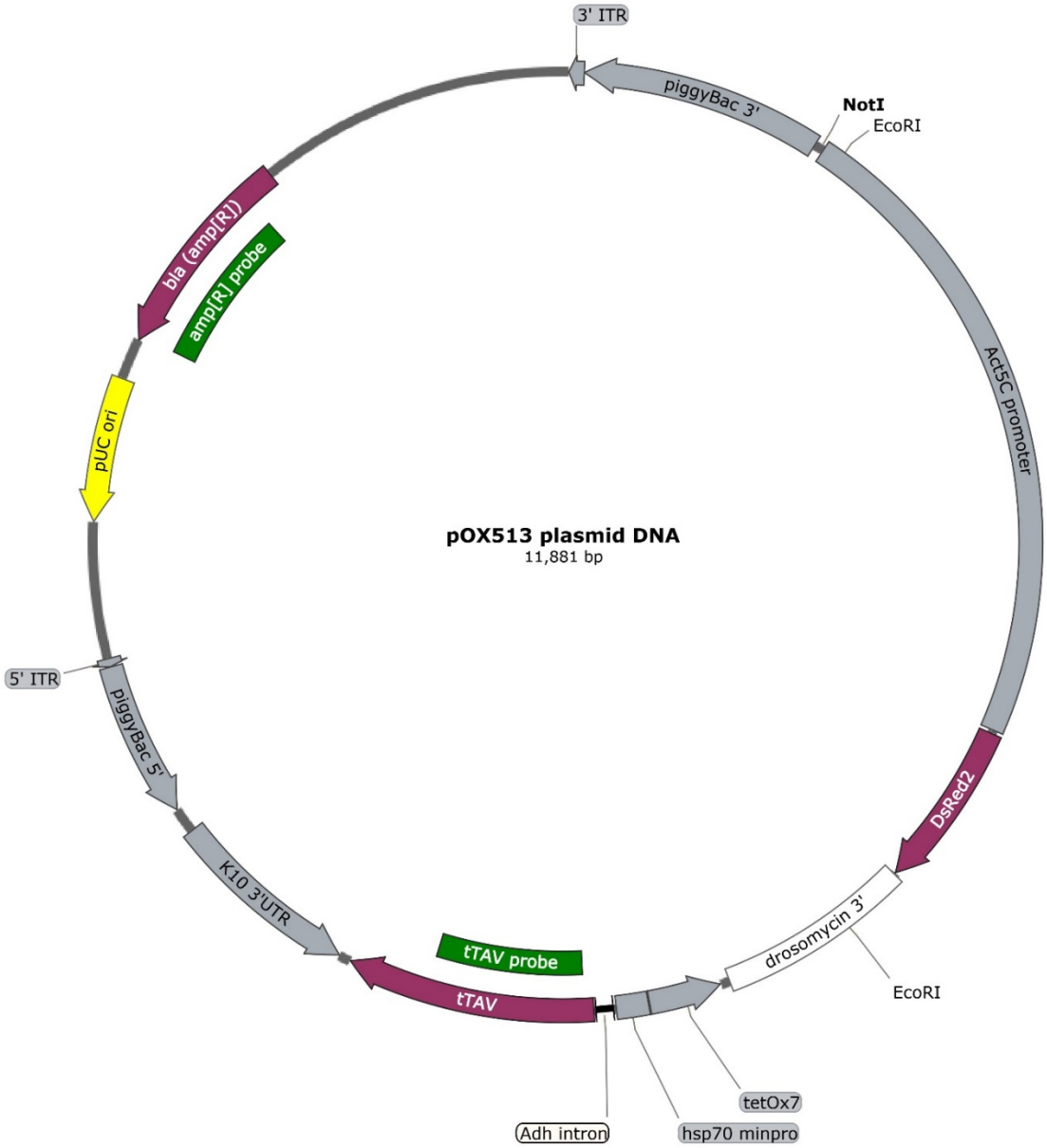

Figure 1. Schematic diagram of the pOX513 plasmid DNA indicating the positions of the hybridization probes used for the Southern blot.

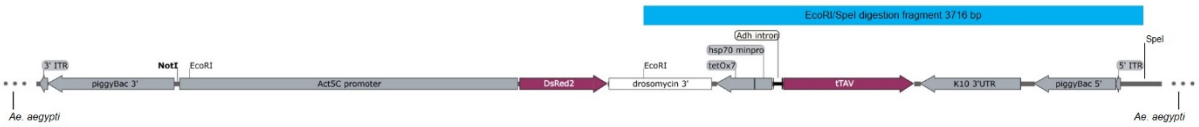

Figure 2. Schematic diagram of the OX513A-rDNA showing the position of the restriction sites used and the expected fragment on the Southern blot.

Restriction Enzyme	Expected Band size
EcoRI/SpeI	3716 bp in OX513A gDNA; unknown in Control strain (approx. 4000 bp on gel)
NotI	11881 bp in pOX513 plasmid DNA

8 RESULTS

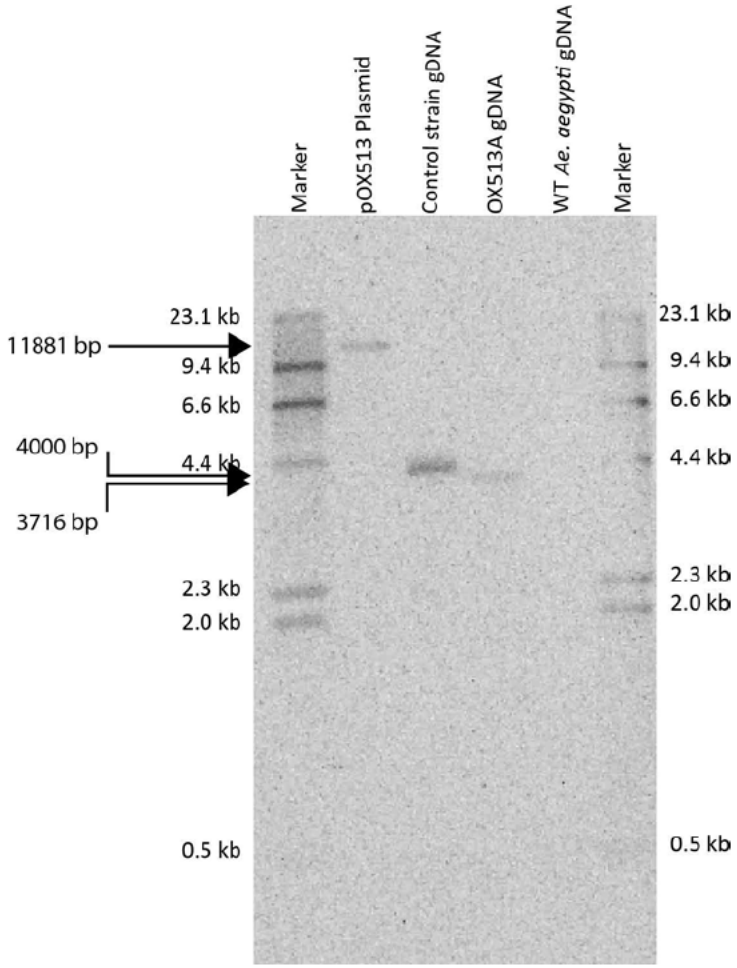

Figure 3. Confirmation of gDNA integrity. Southern Blot using probe annealing to the tTAV sequence showing the presence of the tTAV gene in the pOX513A plasmid DNA (11.9 kb), archival control strain, and OX513A strain (3.7 kb) while confirming its absence in the wild-type *Ae. aegypti*. Equivalent amounts of DNA in gene copy number were run for all samples.

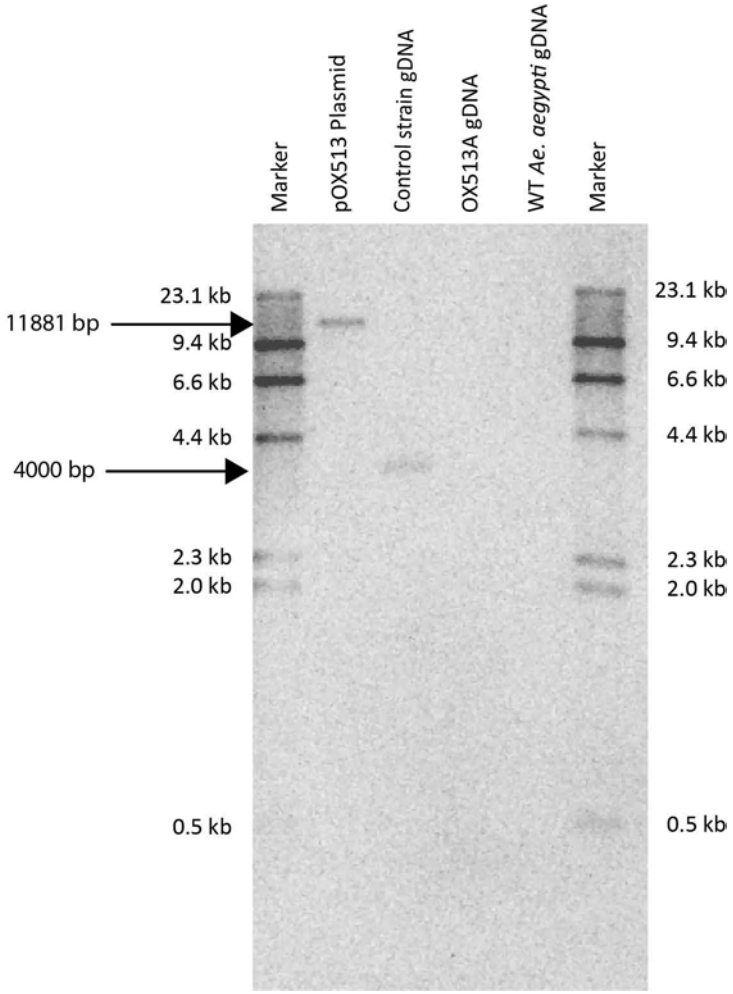

Figure 4. *bla* gene detection. Southern Blot using probe annealing to the *bla amp [R]* sequence showing the presence of the gene in the pOX513A plasmid DNA (11.9 kb) and the Control strain while confirming its absence in the OX513A and wild-type *Ae. aegypti*. Equivalent amounts of DNA in gene copy number were run for all samples.

9 REFERENCE TO LOCATION OF RAW DATA

Lab book OX284

10 DISCUSSION AND CONCLUSIONS

The results of the control Southern blotting using the tTAV hybridisation probe (Figure 3) show single bands matching the expected size in the pOX513 plasmid DNA, the archival control strain, and OX513A which all have the *tTAV* gene inserted, while no tTAV sequence is detected in the wild-type gDNA, confirming the integrity of the gDNA samples. The more intense band observed in the archival control strain compared to the one in OX513A is the result of the two tTAV hybridisation sites characterised in this strain while in OX513A the tTAV construct is present in a single copy.

When the same samples were probed with the *bla amp[R]* probe (Figure 4), a product of expected size was detected only in the pOX513 plasmid DNA and the archival control strain indicating that the *bla amp[R]* gene is not present in the OX513A and wild-type *Ae. aegypti* samples.

In conclusion, the Southern blot analysis indicates that the presence of the *bla amp[R]* gene in OX513A product in one or more copies is unlikely.

11 REFERENCES

Molecular characterisation and lineage of *Aedes aegypti* OX513A

12 APPROVALS

Approval below indicates agreement with information presented in this study report. Raw data has been checked against summary information presented within this report.

Name	Position	Signature	Date Signed
	Team Leader R&D		20 March 2017
	Regulatory Affairs		20 March 2017

Q2

Is it correct that within the rearing unit (male) mosquitoes are always reared and kept in their primary containers and that no activities with mosquitoes are performed in the rearing unit outside of the primary containers? If activities with mosquitoes outside of the primary container are performed in the rearing unit, these should be addressed in the SOPs.

The eclosion stage is the emergence of the flying adult insect from the pupal case and takes place entirely in the release device as described in *TD-SOP-00294 Male Eclosion in Release Device.* Following eclosion, release devices are transported to the outdoor release points and adult male OX513A are released as per *TD-SOP-00295 OX513A Releases*. The adult flying stages of male OX513A are thus not handled outside of the primary containers.

The process flow from egg receipt and storage to release is illustrated in Figure 4, *OX513A Technical Dossier Part B,* and as OX513A moves through the production process during egg, larvae and pupal stages, the points at which OX513A of any life stage is transferred to different primary containers requires the appropriate application of cleaning procedures to minimise the unintended transfer of viable material. This is currently addressed through training activities as referenced in *HS-SOP-00061 Rearing Unit Biosafety Procedures* where reference is made to *COR-SOP-00013 Training Procedures.*

As noted in Q8, final adjustment to existing SOPs and the development of final project specific SOPs for Saba will be undertaken as a matter of priority once a confirmation of the project is established through contractual agreement between Oxitec and the government of Saba. Once project specific SOPs are developed, a review of OX513A transfer control points will be made, and specific cleaning procedures will be explicitly referenced to ensure clarity.

Note: In the *OX513A Technical Dossier Part B* dated September 2016 v.1, reference is made to *00042 OX513A Egg Hatching for Release* within *00013_02 OX513A Egg Receipt and Storage* (Section 2.3 *Process Completion*); this is incorrectly noted and reference should read *TD-SOP-00194 OX513A Egg Hatching - For Release.*

Q3

The SOPs and ACL-2 mention that records should be kept on inadvertent escape within rearing units. Please indicate whether it can be concluded from these records that containment within the rearing units is adequate.

Reference is made in SOPs *HS-P-00057 Rearing Unit Emergency Procedures Policy*; and *HS-SOP-00061 Rearing Unit Biosafety Procedures* to possibilities for inadvertent release and are intended to report on any obvious breeches of containment that staff become aware of during routine operations, either through accidental or deliberate means (vandalism etc). The record keeping requirements are not however explicitly referenced, and it is thus proposed that the above noted SOPs are amended to specifically reference controlled records to be maintained in the case an inadvertent release is detected. These records would be intended to represent obvious breeches of containment such as damaged primary containers (for example) potentially arising from an incident involving human error, or equipment failure. These records may not capture less obvious breeches of containment. To provide a more conclusive and direct measure of the adequacy of containment in the primary containers, it is proposed that an adult trap(s) be placed in the lobby area of the mobile rearing unit and that an SOP and associated record keeping requirements be developed to detail required maintenance and record keeping requirements. In addition to stipulating daily inspection and recording requirements, the supplementary SOP would define parameters which would trigger an investigation and any subsequent corrective actions.

As noted in Q8 final adjustment to existing SOPs and the development of final project specific SOPs for Saba will be undertaken as a matter of priority once a confirmation of the project is established through contractual agreement between Oxitec and the government of Saba.

Q4

What extra measures are taken to prevent access to the rearing unit by unauthorized persons or to prevent vandalism, such as a fence?

The Standard Operating *Procedure HS-SOP-00061 Rearing Unit Biosafety Procedures,* specifies in Section 4 Containment, point 4.1 that the rearing unit is only accessible through a locked door or coded lock and accessible only to a limited number of authorised personnel.

It is additionally proposed that when project specific SOPs are produced for the proposal in Saba as noted in Q8, provision for appropriate signage at the exterior entrance/exit doors to the rearing unit, which are visible from the outside, be incorporated into the project specific rearing unit biosafety procedures SOP. It is proposed that the exterior signage indicate "authorised personnel only" or equivalent in any applicable local languages, and that an emergency contact number be provided on the exterior signs. This will mitigate the risk of entry by unauthorised personnel such as unscheduled visitors whom would be otherwise be unaware of any restrictions on entry.

It is also proposed that when project specific SOPs are produced, it is explicitly clarified in the project specific equivalent to SOP *HS-SOP-00059 Rearing Unit Entry and Exit*, that the doors are locked at the end of the day and that the intruder alarm be set as is stated in *HS-P-0057 Rearing Unit Emergency Procedures Policy* point 5.5.

The inclusion of a perimeter security fence with a lockable gate around the mobile rearing unit, combined with an intruder alarm would be a reasonable means to prevent unauthorised entry of persons whom may have intent to vandalize or commit malicious acts, for example, if this was deemed a risk. The need for any additional security measures such as a perimeter fence will be addressed through dialogue with local government and informed as community outreach and engagement activities are undertaken. As Saba does have an uncharacteristically low crime rate (personal communication with local public health and government communications officials), site security measures will be put in place appropriate to the community, and as recommended by local government.

Q5

Which measures are taken to safeguard the stability of the mobile rearing unit, for example in case of hurricanes?

The mobile rearing unit is to be fixed using a system similar to that in Figures 1 A and B. This is intended expressly to provide stability of the mobile rearing unit in adverse weather conditions such as hurricanes. Figures 2 A and B represent the current installation of these features in the Cayman Islands mobile rearing unit. A site survey will determine if the exact profile in Figure 1 A and B will be possible given the site features such as soil/rock type etc. If the exact profile presented below is not possible due to site specific considerations, the Oxitec engineering team will present an alternative design to local planning officials in Saba which will similarly provide the required stability.

A

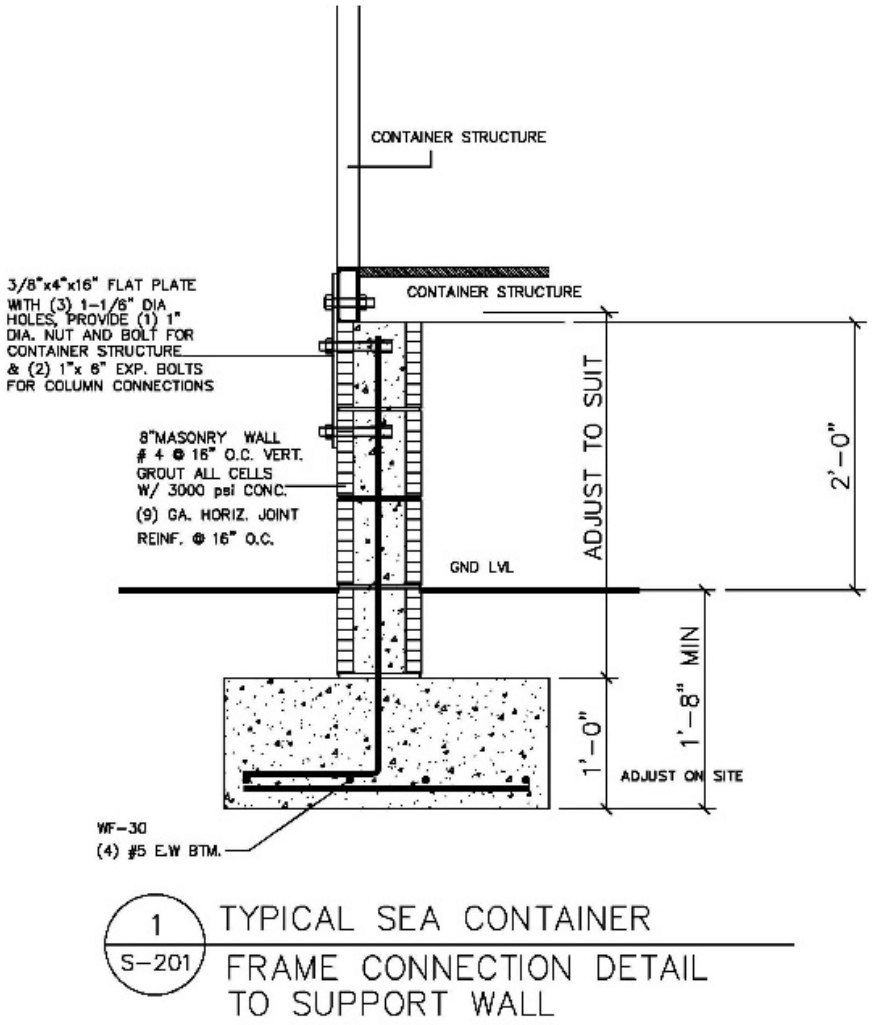

Figure 1A. Details of mobile rearing unit (MRU) anchoring system. End wall feature (A) is a footing supporting a filled concrete block wall running the width of the MRU at each end of the MRU.

B

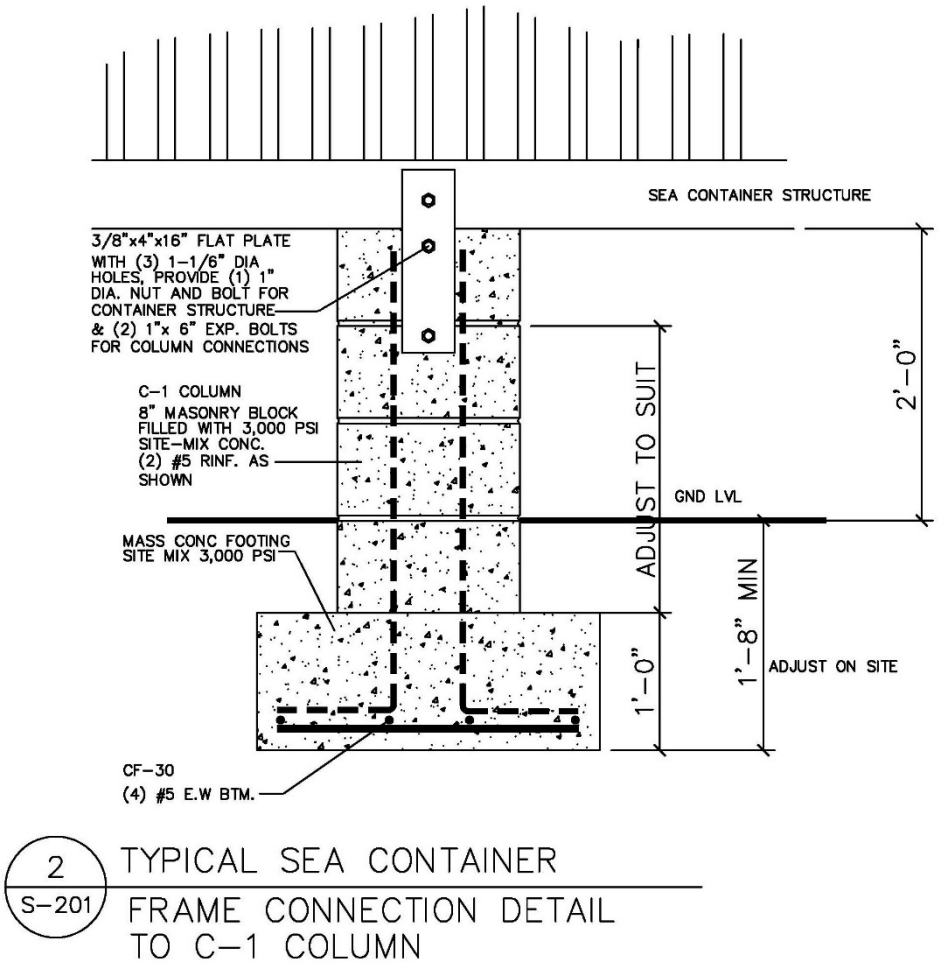

Figure 1B. Details of mobile rearing unit (MRU) anchoring system. Column support feature (B) is a footing which supports each of 2 single columns in approximately the middle along the length of the shipping container on each side.

A.

B.

Figure 2. Current mobile rearing unit (MRU) anchoring system installation in Grand Cayman. End wall feature (A) and column feature (B).

Q6. In several SOPs there is reference to inactivation of biological material by freezing or chemical decontaminants before material is discarded. Please indicate if efficacy of decontamination and inactivation has been validated.

Please find attached report SR-00042 ED 1.a *Biological Waste Process Validation for Aedes aegypti.* It is reported that quantities of approximately 10,000 OX513A eggs, and approximately 5,000 OX513A adults were subject to separate freezing treatments at below -15°C for 2 time points; 7 hours, and >12 hours. In all cases, no viable OX513A adults or eggs were observed following treatment.

OXITEC

Biological Waste Process Validation for *Aedes aegypti*

SR-00042 ED 1.a

Effective Date: 10 March 2017

Printed: 10 Mar 2017

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1 STATEMENT OF DATA CONFIDENTIALITY

The identity of these research strains is highly proprietary and their disclosure could be expected to under-mine the developer Oxitec's competitive position as the market leader.

Oxitec has developed innovative new solutions to controlling harmful insect pests. If competitors knew what methodologies Oxitec has developed or what materials it uses in those methods, often after significant investment of time and resources, those competitors could adopt these proprietary materials and methods for their own benefit without themselves having to expend the same level of resources that Oxitec devoted to develop these materials and methods. Oxitec therefore maintains this type of information in secrecy and considers information about its research efforts to constitute confidential business information.

2 STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

3 ASSOCIATED PERSONNEL

Name	Affiliation	Role/Tasks
Zoe Barnes	R&D Team Leader Milton Park, UK	Study supervisor, experimental design, report writing
Mary Bancroft	Lab Manager Milton Park, UK	Data collection and recording
Gwilym Phillips	Technician Milton Park, UK	Data collection and recording

4 TEST FACILITY

This research was performed at the laboratory of 37C Innovation Drive, Milton Park, Abingdon, OX14 4RX. UK

5 OBJECTIVES

1. To test the biological waste procedure to ensure that OX513A mosquitoes adults and eggs which are frozen at below minus 15°C for 12 hours or more are killed.

6 INTRODUCTION AND SUMMARY

Oxitec's biological waste procedure states that all waste should be frozen at below minus 15°C for over 12 hours to ensure that all biological material is killed, before being disposed of. To validate this process, an experiment was performed to ensure that OX513A *Aedes aegypti* eggs and adults, frozen at minus 15°C for 7 hours and over 12 hours, are not be viable. This would demonstrate that freezing for over 12 hours at minus 15°C is a reliable procedure to follow to ensure that all insects are killed before being disposed of. The results of this experiment confirm that after undergoing this waste procedure, that the biological material is killed. This confirms that the freezing process is a suitable method to dispose of biological waste.

7 MATERIALS AND EQUIPMENT

7.1 Mosquito adult cage equipment

- Data logger
- Vacuum chamber
- Vacuum pump
- Magnetic stirring plate and flea
- Glass beaker

8 METHODS

8.1 Sample preparation

Weigh two samples of 0.1 g (estimated to be 10,000 eggs) of < 1 month old OX513A homozygous eggs from the same batch as used for setting up production of the week.

Set up 2 cages of > 5,000 OX513A adults.

8.2 Biological Waste Freezing process

Place a data logger into the freezer to record the temperature.

Place both the adult cage and the samples of the eggs into a freezer. Record the time.

Determine the time to take the samples out of the freezer so that they would have been in the freezer for 7 hours and >12 hours.

Remove the samples and record the time.

Remove the data logger and download the data. Attach to results below.

8.3 Assessment process

Place the adult cages in the insectary overnight and determine if any adults have survived the freezing process by inspecting for movement after the cage has returned to room temperature.

Take the sample of eggs and follow the standard hatching procedure – (TD-SOP 0002 ED4.a OX513A Egg Hatching). In brief, add 6.6 mL water to the eggs. Agitate for 20 seconds. Allow to soak for 30

mins. Place into the vacuum chamber and reduce pressure to 0.8 bar. Leave for 1 hour. Remove egg sample and transfer to a deli pot. Place into the insectary overnight.

The following day, visually inspect the pot for hatched larvae. If larvae are present estimate the hatch rate by the standard procedure – (TD SOP 0002 ED4.a OX513A Egg Hatching). In brief, place the contents of the deli pot into a glass beaker. Add water to ~ 500 mL. Take 3 samples of 10 mL. Using a microscope, count the number of hatched eggs and the total number of eggs under three microscope views. Aim for approximately 30-50 eggs per view.

Record the hatch rate of the control egg sample (hatched for production) to confirm the expected hath rate before freezing, hatched using the standard procedure.

9 RESULTS

Date: 21st February 2017

Time sample A, eggs and adults >12 hours in freezer: 20 hours 30 mins

Time sample B, eggs and adults >7 hours in freezer: 7 hours 0 mins

Freezer data downloaded and attached here? Yes (Figure 1,2 & 3)

Did any adults survive the freezing process, 7 hours? No

Did any adults survive the freezing process,>12 hours? No

Did any larvae hatch from the frozen eggs > 12 hours? No

Did any larvae hatch from the frozen eggs, 7 hours? No

Hatch rate from control (not frozen) egg sample – 87%

Table 1 Hatch rate data from control (not frozen) egg sample. This data represents the expected hatch rate of the egg sample which was frozen.

Weigh boat	Sample	Hatched	Unhatched
1	1	34	7
	2	24	3
	3	24	3
2	1	22	3
	2	25	6
	3	22	2
3	1	19	4
	2	31	0
	3	26	7

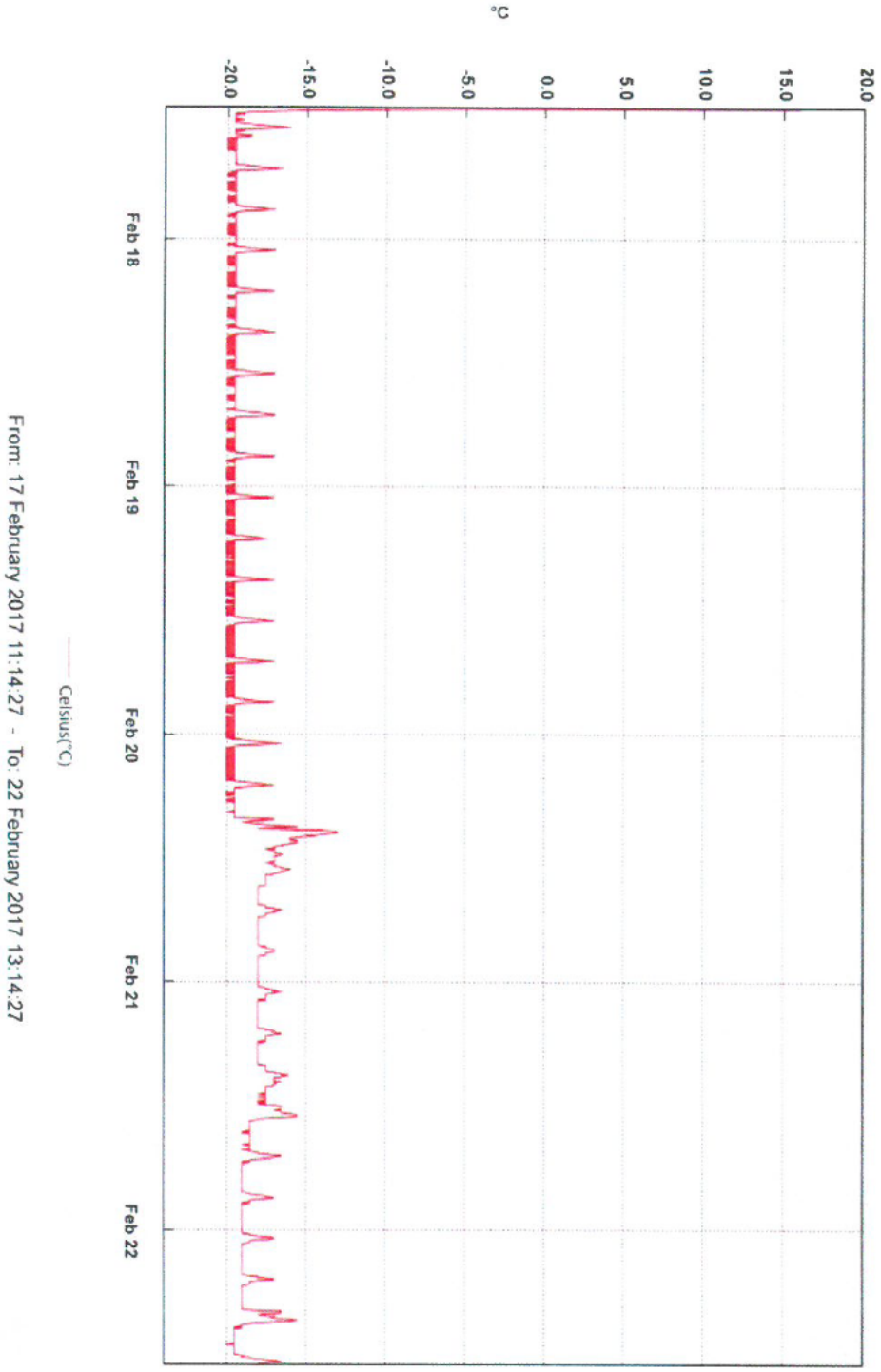

Figure 1 Data logger recording of the temperature in the freezer for samples >12 hours. Spike in temperature on 20th February to approximately -13°C is because of opening the freezer door.

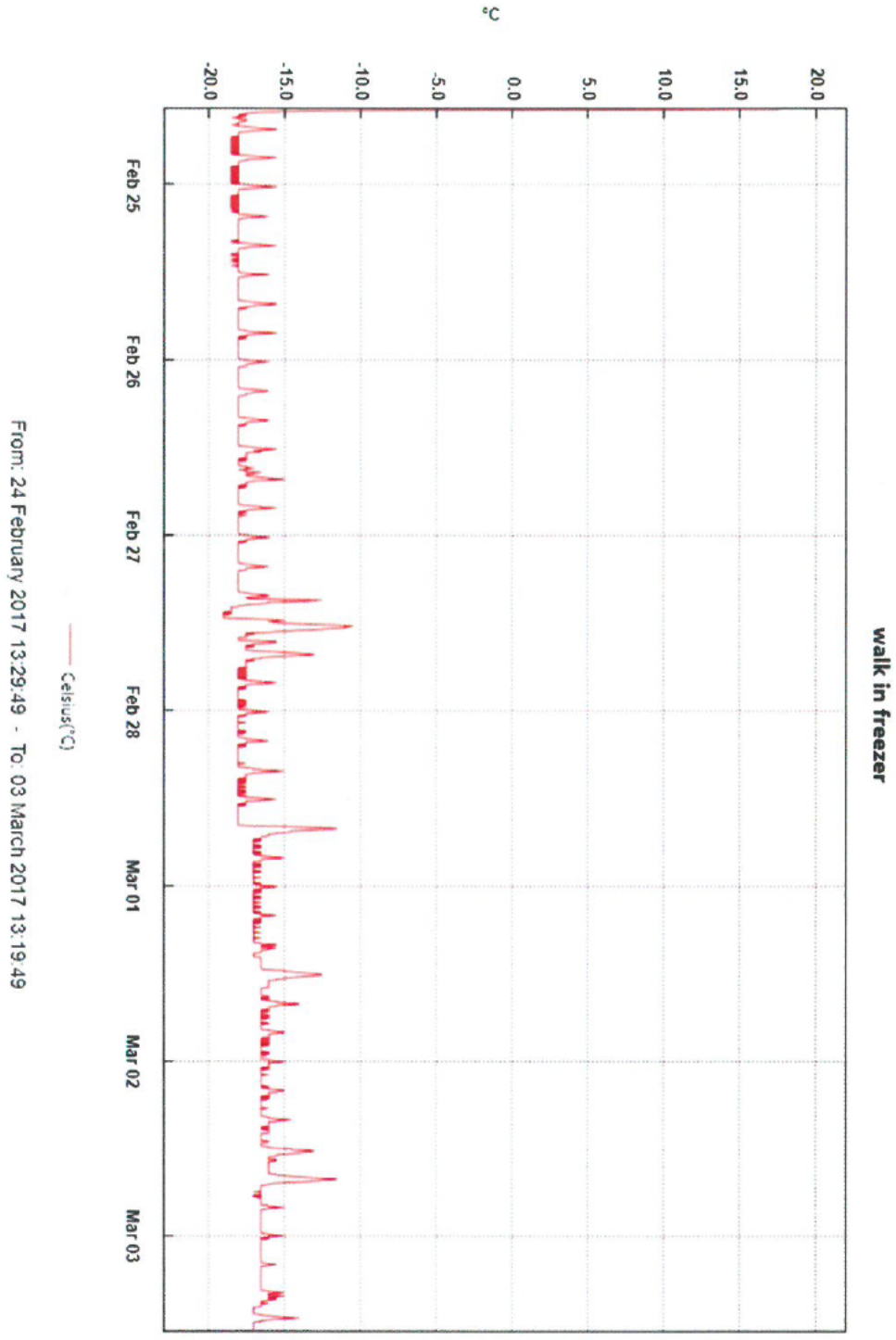

Figure 2 Data logger recording of the temperature in the freezer for eggs frozen for 7 hours.

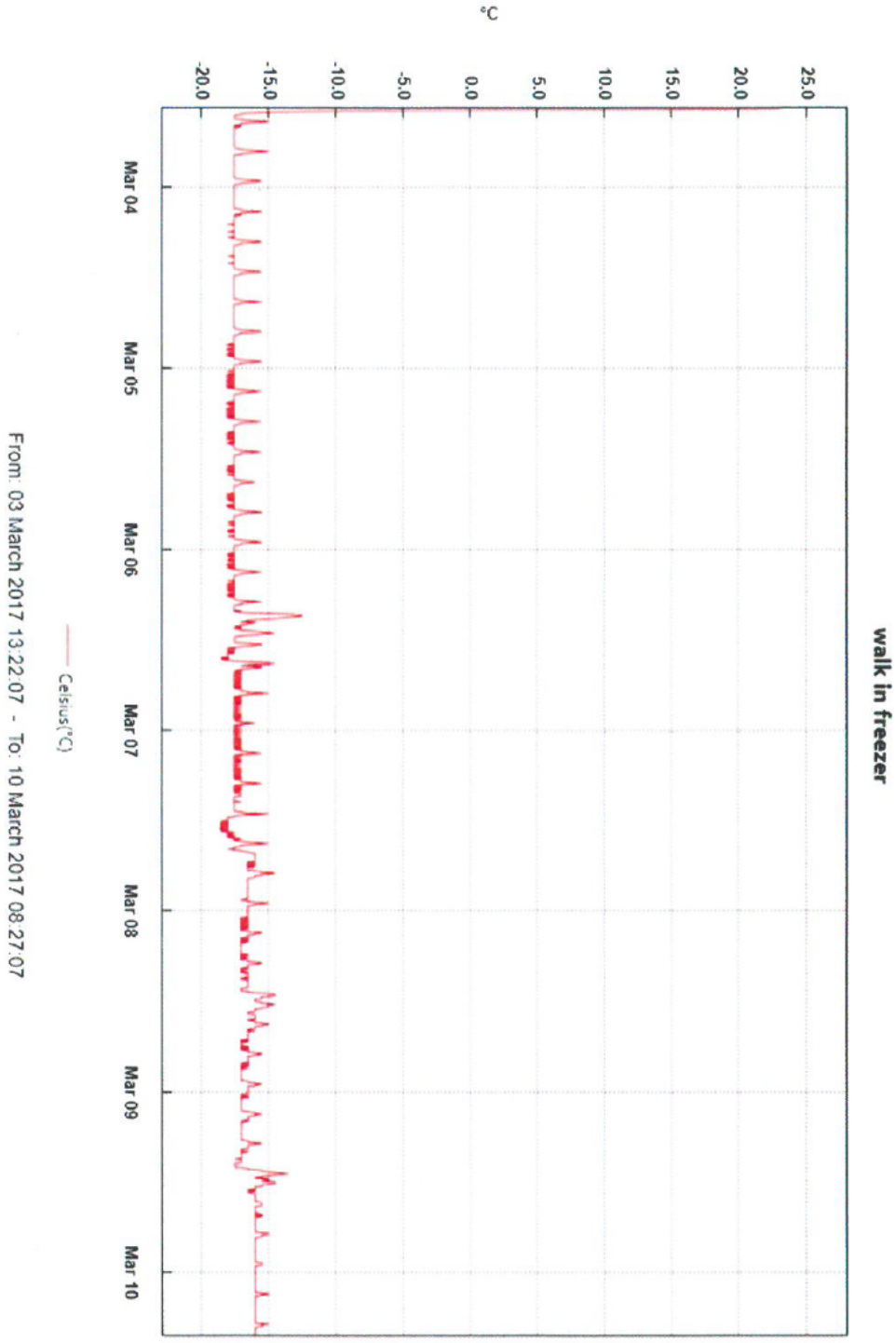

Figure 3 Data logger recording of the temperature in the freezer for adults frozen for 7 hours.

10 REFERENCE TO LOCATION OF RAW DATA

Lab book OX-190, pages 73-79.

11 DEVIATIONS

None

12 DISCUSSION AND CONCLUSIONS

The purpose of the biological waste procedure is to ensure that the insect material is killed before being disposed of. The results of this experiment confirm that the process of freezing OX513A eggs and adults for only 7 hours kills the insects. This therefore confirms that the procedure for freezing waste for 12 hours at below minus 15°C or more is a suitable method to dispose of waste material.

13 LIST OF ACRONYMS, ABBREVIATIONS AND TECHNICAL TERMS

None

14 REFERENCES

None

15 APPROVALS

Approval below indicates agreement with information presented in this study report. Raw data has been checked against summary information presented within this report.

Name	Position	Signature	Date Signed
Kelly Matzen	R&D Group Leader		10 Mar 2017
Amandine Collado	R&D Team Leader		10 MAR 2017

Q7

Please indicate whether dead insects remaining after cleaning of cages are frozen before they are treated as waste, like is done for all other biological material.

Dead insects remaining after cleaning of eclosion/release devices ("cages") are frozen at -15°C or colder for ≥ 12 hours and are treated as biological material as described in section 2 *Disposal of Insect Waste* in *HS-SOP-00053 Cleaning and Waste Procedures*, and also referenced in Section 3 *Waste Disposal* in *HS-SOP-00061 Rearing Unit Biosafety Procedures.* It is proposed that when project specific SOPs are developed for Saba as noted in Q8, specific reference to post release treatment of release devices be made in order to avoid misinterpretation.

Q8

It is noted that not all SOPs have yet been adjusted for use in Saba, for example who will be contacted in case of an accident or injury (the Saba Department of Agriculture, Hygiene & Vector Control?), how room/area decontamination will take place, whether an air curtain or an inner door will be used etc. Please indicate if and when these SOPs will be adjusted.

Final adjustment to existing SOPs and the development of final project specific SOPs for Saba will be undertaken as a matter of priority once a confirmation of the project is established through contractual agreement between Oxitec and the government of Saba.

Finalisation includes adjustments to biosecurity SOPs such as decontamination protocols, elements of which are currently addressed principally in *HS-SOP-00053 Cleaning and Waste Procedures; HS-P-00057 Rearing Unit Emergency Procedures Policy;* and *00054_01 OX513A Hurricane Preparedness Policy.* As well, the project specific rearing unit design which includes physical biosecurity features such as the air curtain (addressed in Q9; see note below) will be finalised and a detailed floor plan/schematic diagram with all relevant engineering details will be provided for review by local authorities.

At the time a project commitment is confirmed, overall project timelines and planning requirements will be established, and an assessment of all record-keeping and documentation requirements for the Quality Management System (QMS) and regulatory compliance will be identified. As SOPs are developed or amended, consultation with local government will inform appropriate contact information in the event of any breech of compliance, or accident or injury, or other events identified through consultation, and documentation will be adjusted to reflect local government input and requirements. Additionally, a project specific compliance plan for records will be developed to ensure records are readily available if needed for inspection by local authorities.

Note: In *HS-SOP-00061 Rearing Unit Biosafety Procedures*, Section 4.4, there is reference to the potential fitting of an air curtain above the door entrance to the rearing room please see Q9 for additional clarification on details of this feature.

Q9

It is mentioned that in some mobile rearing units an air curtain is fitted above the entrance door to the rearing room. Is the air curtain instead of an inner door in agreement with ACL-2?

In *HS-SOP-00061 Rearing Unit Biosafety Procedures*, Section 4.4, there is reference to the potential fitting of an air curtain above the door entrance to the rearing room. The language in the SOP may be somewhat vague in that it could be inferred that the air curtain is potentially *in place* of a door. An air curtain as described would be *in addition* to a door, as a supplementary feature. The integrity of containment would thus be enhanced by the addition of an air curtain above the existing physical containment provided by the door, thus would not alter the biosecurity profile of the passageway in the context of ACL-2.

Q10

Which measures are taken to prevent spillage of tetracycline stocks from the rearing unit into the environment?

In current operations the master tetracycline stock is stored as a powder in the freezer and a 10 mg/ml working stock solution is made up as detailed in *SOP 00039_02 Chlortetracycline HCl Working Solution Preparation* at the time the larval rearing tray system is set up as detailed in *SOP TD-SOP-00291 OX513A Larval Rearing (Tray System).* Current practice represents a deviation from *SOP 00039_02* in that only the required amount of stock tetracycline is prepared directly from the powder at the time the larval rearing trays are setup. Concentrated stock is thus not retained in storage, and the most likely route of potential environmental exposure would be through accidental spillage of the working concentrations of 30/ug/ml in the larval rearing trays. The spillage of a single tray (approximately 4.5 litres) by human error in handling, albeit unlikely to occur, would be the most foreseeable scenario. In this case a general purpose spill kit would be used to absorb the spill. The rearing unit itself also has features inherent to the containment design which would restrict the seeping of any spilled materials into the environment. Additionally, for Chlortetracycline hydrochloride hazard statements under EC Regulation 1272/2008 are as follows:

H315 Causes skin irritation
H319 Causes serious eye irritation
H335 May cause respiratory irritation

Thus appropriate precautions are taken to minimize exposure to staff working with tetracycline stocks.

Q11

In part B 1.4 it is stated that conditions for transit via St Maarten will need to be explored with local regulatory officials of Saba and St Maarten, and has been identified with Saba legal affairs officials. This may require support from RIVM for attestation of adequate containment of 0X513A in transit. Which support of RIVM is meant?

Throughout the course of an OX513A *Aedes aegypti* suppression project, regular shipments of OX513A eggs (1-2 X per month) would be required from the UK to support the project activities in Saba. It has been discussed with the Government Lawyer for Saba, that upon local approval of a project, it would be necessary to undertake a review of the regulatory requirements in St Maarten regarding the transit of viable GM mosquito eggs via St Maarten to Saba by air. This would be required as the most likely shipping route is either from the UK or Continental Europe by air direct to St Maarten, and further moving on to Saba by air transit. If a transit permit or a similar form of regulatory permission is required for the transit of the shipment via St Maarten, it could be anticipated that demonstration of adequate containment measures will be a requirement of an application. If an application or transit permission is applicable, the regulatory authority may not have experience with GMO containment measures.

If a project was approved, and a contractual agreement was established between Oxitec and the government of Saba, Oxitec will work with the Saba government Lawyer to establish specific requirements, and at that time, containment and packaging specifications may be proposed. A review by RIVM of any proposed containment and packaging specifications to be used for the air transit of OX513A eggs, and any technical recommendations on packaging, may be of assistance to St Maarten; this is however speculative and will be informed though dialogue with officials in St Maarten and Saba if a project contract is secured.