I2I Landscaping report: Lab efficacy testing of larvicides-Bacterial bioassay methods

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Acronym List

Bti	*Bacillus thuringiensis* subspecies
Bs	*Bacillus sphaericus*
IGR	Insect growth regulator
LC_{50}	Lethal concentration of the larvicide for 50% mortality
$LC_{90/95}$	Lethal concentration of the larvicide for 90/95% mortality
LSM	Larval Source Management
Mg/l	Milligrams per litre
s.l.	Sensu lato
WHO	World Health Organization
WHOPES	World Pesticide Evaluation Scheme

Summary

Aim and key questions addressed	- To evaluate the biological activity of a mosquito microbial larvicides *Bacillus thuringiensis* subspecies *israelensis (Bti)* and bacterium *Bacillus sphaericus (Bs)*
Context	- Laboratory
Test item	- Mosquito larvae
Mosquito population	- Field mosquitoes or laboratory reared
Number of mosquitoes per replicate	- 20/25
Endpoints measured	- Mortality
Exposure time	- 24 hours for *Bti*, 48 hours for *Bs*
Holding time	- 24 hours for *Bti*, 48 hours for *Bs*
Indicative of personal protection	- N/A
Suitable chemistries	- Bacterial larvicides
Appropriate controls	- Negative control is solvent used for preparation of test insecticides
Relevant stage of production pipeline	- Mosquito characterisation

Characterisation of output	- Endpoints well defined
Accessibility	- Materials and set up need to be sourced and training is required
Cost	- Low cost, non specialised equipment required and straightforward method to perform
Level of validation and characterisation of outputs	- Not identified any formal validation
Outstanding questions, gaps and priorities	- N/A
Key references, related SOPs, guidelines and publications	- Guidelines for 'Laboratory and Field testing of Mosquito Larvicides' developed by WHO in 2005 - Report of the WHO informal consultation on the evaluation and testing of insecticides (World Health Organization - WHO, 1996) - Larval source management: a supplementary measure for malaria vector control (World Health Organization, 2013) - I2I-SOP-001: Bacterial Larvicide Bioassay (i.e. Bacillus thuringiensis subsp. israelensis and Bacillus sphaericus) - I2I-SOP-042: Performing larval insecticide bioassays and larval susceptibility testing

Overview

Larval source management (LSM) is globally used to control malaria by targeting the immature stages of the mosquito vectors in their aquatic habitats preventing development and therefore reducing the abundance of adult vectors (World Health Organization, 2013). Integrating larval source reduction with adult mosquito control interventions e.g. insecticide treated bednets has been considered to be a highly effective strategy to control malaria.

There are four types of LSM: habitat modification, habitat manipulation, larviciding and biological control. Mosquitoes lay their eggs in various types of water bodies, depending on species, which can be man-made or natural, temporary, or permanent (WHO, 2013). Once laid, eggs develop through to larval and then pupal stages, before eventually emerging as adults and leaving the aquatic habitat. Larvicides have various modes of action, such as suffocating larvae, interfering with their nervous systems, causing starvation, or inhibiting metamorphosis (Tusting *et al.,* 2013). There are five main groups of larvicides: oils and surface agents; synthetic organic chemicals; bacterial larvicides; spinosyns and insect growth regulators (WHO, 2013).

The discovery and commercialization of the first microbial pesticide targeting mosquitoes occurred in the 1970s (Laird, 1985) of two naturally occurring, spore-forming bacterium widely found in soil and aquatic environments which are:

- *Bacillus thuringiensis* subspecies *israelensis (Bti)*
- *Bacillus sphaericus (Bs)*

Bti and *Bs* have a unique mode of action which is useful in managing mosquito resistance to chemical insecticides. When the mosquito larvae ingest the bacteria, insecticidal crystal proteins attack the gut lining causing cessation in feeding and resulting in mortality. *Bti* and *Bs* selectively kill mosquito larvae with no effect on non-target organisms. There has been recent evidence suggesting that LSM by applying bacterial larvicides is effective at lowering the density of mosquito vectors (Derua *et al.,* 2019). However, control efficacy of *Bti* and *Bs* has also been reported to vary due to certain variables such as mosquito species, age, density of larvae, larval

habitat conditions (temperature, water depth) and larvicide properties. Due to this it is possible that a larviciding strategy will be more effective for malaria control in some settings than others. To be effective the application of *Bti* and *Bs* need to be guided by adequate knowledge of targeted mosquito vectors, their ecology and the properties of the bacterial larvicide used (Derua *et al.,* 2019).

Evaluation of mosquito larvicides, described by the WHO (WHO, 2005), is in 3 phases: Phase 1: Laboratory studies, Phase 2: Small-scale field trials and Phase 3: Large-scale field trials. The scope of this report will focus on Phase 1: Laboratory studies, specifically bioassay methods for bacterial larvicides. The objective of laboratory testing is to determine the inherent bio-potency of the technical material, or activity of formulated insecticides. Laboratory bioassays are also conducted to assess the minimum effective dosage before *Bti* and *Bs* formulations are used in the field following WHO guidelines.

To evaluate the biological activity of a mosquito larvicide, laboratory-reared mosquito larvae of known age or instar are exposed to the bacterial larvicide and mortality is recorded. This data can in turn be used to:

- establish dose-response line(s) against susceptible vector species
- determine the lethal concentration of the larvicide for 50% and 90% mortality (LC_{50} and LC_{90})
- establish a diagnostic concentration for monitoring susceptibility to the mosquito larvicide in the field
- assess cross-resistance with commonly used insecticides

The list of Prequalified Vector Control Products (WHO, 2023) includes larvicidal products based on the active ingredients and Modes of Action detailed in Table 1.

Table 1: List of bacterial larvicidal products liked in Prequalified Vector Control Products (WHO, 2023)

Product Name	Active Ingredient/Synergist	Mode of Action
VectoBac GR	*Bacillus thuringiensis* subspecies Israelensis strain AM65-52	Bacteria – *Bacillus thuringiensis* subspecies produce insecticidal crystal proteins which, when ingested by larvae, attack the gut lining causing cessation of feeding and subsequent mortality.
VectoBac WG	*Bacillus thuringiensis* subspecies Israelensis strain AM65-52	Bacteria – *Bacillus thuringiensis* subspecies produce insecticidal crystal proteins which, when ingested by larvae, attack the gut lining causing cessation of feeding and subsequent mortality.
VectoMax FG	*Bacillus sphaericus* strain ABTS-1743, *Bacillus thuringiensis* subspecies Israelensis strain AM65-52	Bacteria – *Bacillus thuringiensis* subspecies. *israelensis* (Bti), and *Bacillus sphaericus* (Bs) produce insecticidal crystal proteins which, when ingested by larvae, attack the gut lining causing cessation of feeding and subsequent mortality.

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

- Guidelines for 'Laboratory and Field testing of Mosquito Larvicides' (WHO, 2005)
- Report of the WHO informal consultation on the evaluation and testing of insecticides (WHO, 1996)
- Larval source management: a supplementary measure for malaria vector control (WHO, 2013)
- I2I-SOP-001: Bacterial Larvicide Bioassay (i.e. *Bacillus thuringiensis subsp. israelensis and Bacillus sphaericus*)

Are these sufficiently detailed?

The document 'Larval source management: a supplementary measure for malaria vector control' (WHO, 2013) provides information on bacterial larvicides including modes of action and formulations available, but it does not describe how to carry out laboratory bioassays and only states to conduct them following WHO guidelines. The document references a link for information on WHOPES recommended formulations and bacterial larvicides, however this link is no longer accessible.

The 2005 WHO guidelines are an expanded and an updated version of the 1996 WHO guidelines, and do not include standardised procedures, so certain parameters are therefore open to interpretation. This document states that the laboratory bioassay procedures for bacterial products and chemical larvicides are the same. The 2005 WHO document gives additional details for the preparation of stock suspensions for bacterial larvicides along with instructions for dilutions and references an additional WHO document to assess cross-resistance.

I2I-SOP-001: Bacterial Larvicide Bioassay (i.e. *Bacillus thuringiensis subsp. israelensis and Bacillus sphaericus*), written by Peter DeChant et al, Valent BioSciences, describes in detail the bioassay procedure along with data generation and analysis.

Do these methods require specialised/non-standardised equipment and/or training?

Specialised training is required for the preparation of stock concentrations, the identification of 1st-4th instar larvae from mosquito colony to be tested, mosquito handling, and conducting the assay, and methods of analysis (log-probit software).

The 2005 WHO laboratory bioassay procedures for bacterial products are the same as those for chemical larvicides, except in the preparation of stock suspensions. Specialised additional materials required for testing are:

- Top-drive homogenizer or stirrer for lyophilized products
- Ice bath for grinding or sonication

Are there issues with the methods or their interpretation?

The 2005 WHO guidelines refer to the laboratory bioassay procedures for bacterial products to be the same as for chemical larvicides, except in the preparation of stocks solutions.

There are specific parameters that are not specified in detail in the 2005 WHO guidelines, as detailed below:

- The methods provide instructions for standardised rearing of *Aedes* and *Culex* spp. mosquitoes to ensure that homogenous batches of mosquito larvae are tested. For other species it is stated to use these procedures subject to any necessary modifications for their biological requisites. Consequently, there are no guidelines for other species, leaving the rearing methods open to interpretation and variations may occur between test sites. The I2I SOP is targeted at *Aedes aegypti* and *Culex quinquefasciatus* mosquito larvae specifically.
- The procedure specifies to maintain a depth of 5-10cm in the containers and that deeper water may cause mortality. Evaporation may occur in a procedure ongoing beyond 24 hours resulting in a loss of water volume. There is no instruction on maintaining this depth without altering the intended insecticide concentration.
- Distilled/non-chlorinated water is recommended for testing larvicides and tap water for controls. This difference in water type could be a confounding variable in any mortality results obtained in the control test. In comparison the I2I SOP states to use deionised water for testing both treatments and controls.
- A larval feeding regime is not included within the guidelines. WHO methods for bacterial larviciding specify to add no food when the exposure period is 24 hours, but that food can be added if longer. There is no standardised method included for the quantity or

frequency of feeding. Specifying the material, amount, and intervals of feeding will help replicate results between studies.

- The 2005 WHO guidelines recommend 'disposable test cups or vessels' to be used in testing. Specifying a particular container that has been validated, ensuring that no active ingredient is absorbed would be useful. The I2I methods specify wax coated cups.

What larvicidal products have the tests been used for?

The list of Prequalified Vector Control Products (WHO, 2023) includes the larvicidal products containing *Bs* and *Bti* outlined in Table 1. In addition, (Derua *et al.,* 2019) conducted a review of published literature which conducted laboratory testing on *Bti* and *Bs* in sub-Saharan Africa, and identified the range of bacterial formulations listed below:

- VectoBac (WDG) *(Bti)*
- VectoLex (WDG) *(Bs)*
- Bactimos (PP) *(Bti)*
- Teknar (FC) *(Bti)*
- 1593 IF-119 (SD) *(Bs)*
- 2362 IF-118 (SD) *(Bs)*
- Spherimos (FC) *(Bs)*
- IPS-82 *(Bti)*
- SPH-88 *(Bs)*

Are they validated, for which AIs/entomological effects, and to what extent?

This method is reported as being used frequently in published literature, however we have not identified any formal validation data.

What inputs need to be characterised? e.g., samples, mosquitoes, equipment

For laboratory testing the WHO, 2005 guidelines and I2I SOP sufficiently detail the bioassay requirements, including the equipment required, quantity of replicates, and recommended larval instar for testing. Additional inputs that need to be characterized are detailed in 'Are there any issues with the method and their interpretation?'

Are endpoints clearly defined and appropriate? Who were they defined by?

Within 24 hours of ingestion larvae would be killed with *Bti*. Due to this rapid action the endpoint of measuring mortality at 24 hours is appropriate. *Bs* has a slower mode of action and therefore mortality is recorded at the longer time point of 48 hours. The I2I SOP states measuring mortality between a 3 hours window of 17-20 hours for Aedes *(Bti)* and 42-45 hours for Culex *(Bs)*.

Are their supporting SOPs? e.g., cleaning SOPs, mosquito rearing SOPs required.

- LITSOP123: Test preparation, detailing the set-up of equipment required.
- LITSOP142: Equipment Cleaning in the LITE Laboratory Area, detailing general cleaning after testing.
- I2I-SOP-027: Field Evaluation of Microbial Mosquito Larvicide Efficacy.
- I2I-SOP-028: Insect Growth Regulator Larvicides.
- I2I-SOP-042: The Performance of Larval Insecticide Bioassays and Selections. This SOP details information about conducting insecticide bioassays only and does not include information on bacterial bioassays.

Define Current Use Practices

Does everybody use the same SOP?

Derua *et al.,* (2019) details laboratory trials using *Bs* and *Bti*. All studies that tested *Bti* measured mortality at 24 hours in line with the 2005 WHO guidelines. There was variation in the exposure time in the studies that tested with *Bs*. Despite the 2005 WHO guidelines suggesting a 48-hour exposure, 6 studies used this time frame and 4 used 24 hours. This suggests that different procedures are used during testing and that the 2005 WHO guidelines are commonly used as 'guidance' when performing laboratory testing.

Are there differences of interpretation of the method?

The methodologies reported in published literature suggest that the guidelines have been adapted dependent on the study protocol, revealing inconsistencies in specific methodological details used for laboratory testing. Key parameters which may impact results of testing have listed in the section 'Are there issues with the methods or their interpretation?'

Are the results obtained largely consistent between studies?

Derua *et al.,* (2019) compared the activity of *Bti* and *Bs* in laboratory settings across ten studies which showed variation in the results obtained. For *Bti*, the lethal concentration value that caused 50 and 90/95% mortality of *An. gambiae (s.l.)* larvae (LC_{50} and LC90/95) ranged between 0.006–0.662 mg/l and 0.132– 1.743 mg/l, respectively. For *Bs,* the LC50 and $LC_{90/95}$ values for the same mosquito species ranged between 0.002–0.342 mg/l and 0.018–1.807 mg/l, respectively. For *An. funestus,* LC_{50} and LC_{95} values after 48 hours of exposure to *Bs* were 1.0 mg/l and 6.0 mg/l, respectively.

Is further development, refinement or validation of the method required? Based on priority, significance, and relevance of method.

Laboratory testing of bacterial larvicides is routinely used across multiple sites to assess the bio-potency of *Bti* and *Bs,* and to generate mortality data to determine e.g. LC_{50} and LC_{90} values. Updated WHO guidelines providing standardised instructions would be beneficial to ensure that robust data from testing at the Phase 1 laboratory stage can be obtained which is comparable across studies.

Identify Potential Sources of Variation

What are the sources of variability in the method and are there means to minimise or characterise these.

There are various methodological factors that would benefit from being standardised to prevent variability between studies. These are detailed below:

- Testing homogenous batches of mosquitoes: the 2005 WHO guidelines provide standardised methods for mosquito rearing but do not include *Anopheles*. Variations in rearing methods can cause variations in the fitness of the mosquito, affecting mortality.
- Feeding larvae: this is not specified in the 2005 WHO guidelines and left to the user to decide. A larval feeding regime detailing the quantity and frequency of food supply would be beneficial. Over or underfeeding would impact the fitness of the mosquitoes being tested and so potentially mortality.
- Using the same water type for insecticide testing and controls would be beneficial to ensure that conditions are standardised. Distilled/non-chlorinated water is recommended for testing insecticides and tap water for controls in the 2005 WHO guidelines.

Do current method/s need to be adapted for new active ingredients/MoA/types of tool?

There are separate landscaping reports available for the different methods for lab efficacy testing of larval insecticides specific to insect growth regulators and insecticide bioassay methods. These are:

- I2I Landscaping report: Lab efficacy testing of larvicides-Insecticide bioassay methods
- I2I Landscaping report: Insect growth regulator (IGR) bioassay methods

Are new methods required? Identify areas where current method/s are not suitable or sufficient.

N/A

Gaps in biological or other understanding that hinder method development or validation

N/A

Is Prioritisation – is there an issue that needs to be addressed, what specifics, how urgent is the need?

N/A

References

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