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

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Acronym List

Cm	Centimeter
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Net
LC_{50}	Lethal concentration that induces 50% mortality
LC_{90}	Lethal concentration that induces 90% mortality
LSM	Larval source management
mL	Milliliters
nAchR	Nicotinic acetylcholine receptor
PDMS	Polydimethylsiloxane
PPF	Pyriproxyfen
WHO	World Health Organisation
WHOPES	World Health Organisation Pesticide Evaluation Scheme

Summary

Aim and key questions addressed	– To evaluate the biological activity of a mosquito larvicide
Context	– Laboratory
Test item	– Mosquito larvae
Mosquito population	– Field mosquitoes or laboratory reared
Number of mosquitoes per replicate	– 25
Endpoints measured	– Mortality
Exposure time	– 24 hours
Holding time	– 24 hours
Indicative of personal protection	– N/A
Suitable chemistries	– 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' (World Health Organization- WHO, 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) – LITSOP011: The Performance of Larval Insecticide Bioassays and Selections. – I2I-SOP-042: Performing larval insecticide bioassays and larval susceptibility testing

Overview

Larval source management (LSM) targets the mosquito's immature, aquatic stages, preventing development and therefore reducing the abundance of adult vectors (World Health Organization, 2013). There are four types of LSM: habitat modification, habitat manipulation, larviciding and biological control. Larviciding refers to the regular application of microbial or chemical insecticides to water bodies or water containers to kill the aquatic immature forms of the mosquito – larvae and pupae. Mosquitoes lay their eggs in various types of water bodies, depending on species, and so can be man-made or natural, temporary, or permanent (Bruce-Chwatt., 1985). 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). The WHO recommends the use of larval source management (LSM) as a supplementary control measure (World Health Organization, 2013), and so larviciding is generally considered after environmental management has been used to eliminate as many breeding sites as possible.

When considering whether and how to deploy larvicides against a mosquito vector population, resistance status needs to be confirmed. Insecticide bioassays are frequently used to measure levels of insecticide resistance in mosquito populations, examining the ability of mosquitoes to survive exposure to insecticides. Larval insecticide bioassays are used to measure levels of insecticide resistance in field populations, in comparison with susceptible laboratory strains, using a range of concentrations that induce 0-100% mortality. This measures the toxicity of insecticides to mosquito larvae and determines the level of insecticide resistance.

The method for evaluation of mosquito larvicides established under WHOPES is in 3 Phases (WHO, 2005): 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, specific to insecticide bioassay methods which are not Insect growth regulators (IGR) or bacterial, this are detailed in separate landscaping reports. The objective of laboratory testing is to determine the inherent biopotency of the technical material, or activity of formulated insecticides.

To evaluate the biological activity of a mosquito larvicide, laboratory-reared mosquito larvae of known age or instar are exposed for 24 hours (or longer depending on the study protocol) to water containing various concentrations of an insecticide 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 (LC) of the larvicide for 50% and 90% mortality (LC_{50} and LC_{90}, Wang et al., 2023).
- establish a diagnostic concentration for monitoring susceptibility to the mosquito larvicide in the field
- assess cross-resistance with commonly used insecticides.

Larviciding is not widely used by malaria control programmes and is generally an under-researched area, with vector control methods that target the adult mosquito stages being prioritized, primarily indoor residual spraying (IRS) and insecticide treated bed nets (ITNs). The widespread use of ITNs and IRS interventions in many parts of Africa has led to new areas with low and focal malaria transmission that could be effectively targeted with larvicides (Derua *et al.*, 2019). This underlines the importance of larviciding as a component of an integrated vector control approach and the requirement for laboratory-based bioassays to evaluate effective resistance monitoring.

To date 21 larvicidal products have a listing as Prequalified Vector Control Products (WHO, 2023). Table 1 in the appendix details their active ingredients and the Modes of Action (MoA), limited to insecticides only.

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

- Guidelines for 'Laboratory and Field testing of Mosquito Larvicides' were developed by WHO in 2005. These are standardised procedures and guidelines recommended by WHO to evaluate efficacy of a larvicide.
- 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-042: The Performance of Larval Insecticide Bioassays and Selections.

Are these sufficiently detailed?

The document 'Larval source management: a supplementary measure for malaria vector control' (World Health Organization, 2013) does not describe how to carry out laboratory bioassay and only states to conduct them following World Health Organization (WHO) guidelines. The WHO, 2005 guidelines are an expanded and an updated version of the WHO, 1996 Guidelines.

The WHO, 2005 guidelines state it produces standardised procedures for testing larvicides. Information on materials, preparation of stock solutions and test concentrations and data analysis is included in the guidelines. There are, however, parameters in these methods open to interpretation, and testing methods may vary between studies. Changes to testing methods could impact measured efficiency and efficacy of larvicidal products. The WHO 2005 guidelines also include a set of standardised guidelines for rearing methods of *Aedes* and *Culex* to ensure testing of homogeneous mosquitoes. There are no standardised methods for *Anopheles* mosquitoes.

For laboratory testing the I2I-SOP-042: The Performance of Larval Insecticide Bioassays and Selections is sufficiently detailed, providing clear and detailed instructions on performing a larvicide assay. Details about data analysis are not included.

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

Specialised training is required for the preparation of insecticide concentrations, and the identification of 1st-4th instar larvae from mosquito colony to be tested. Mosquito handling skills, practice in conducting the assay, and knowledge of the methods of data analysis (use of log-probit software) are required.

The WHO, 2005 guidelines also detail information on making a customised strainer to transfer test larvae. Specific materials required for the I2I protocol are a 'Drop' measuring spoon for feeding to provide a standardised measurement of diet.

Are there issues with the methods or their interpretation?

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

- The methods provide standardised instructions for rearing *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 the 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 WHO, 2005 instructions state to add 0.1 or 1mL of the appropriate dilution to 100/200mL of water. The procedures state that this will not cause any noticeable variability in the final concentration. It is inconclusive if this has been tested, however. In comparison the I2I methods have standardised instructions and state to use a final volume of 200mL by adding 1mL of the appropriately diluted insecticide to 199mL of water.
- 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 insecticides 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 insecticides and controls.
- A larval feeding regime is not included within the guidelines. There is no guidance for the quantity and frequency of larval food to supply. Specifying the material, amount, and intervals of feeding will help replicate results between studies. The I2I methods in comparison instruct a standardised method of feeding 20mg of ground fish flakes every 24 hours. The WHO, 2005 recommends 'disposable test cups or vessels' for the types of containers 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 disposable deli pots.

What AIs or combinations of AIs have the tests been used for?

A range of insecticides have been tested across studies. The following list of active ingredients are listed in Prequalified Vector Control Products, specific to insecticides (WHO, 2023):

- Temephos
- Pirimiphos-methyl
- PDMS (Polydimethylsiloxane)
- Diflubenzuron
- Spinosad

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, number of mosquitoes per test and recommended larval instar for testing. The overall sample size and mosquito-insecticide combinations are determined by the study protocol.

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?

The I2I SOP states to test for 24 hours or longer depending on the study protocol, a longer time period is required for slow-acting insecticides. The WHO, 2005 guidelines state to record mortality at 24 hours and 48 hours for slow-acting insecticides.

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

- I2I-SOP-042: The Performance of Larval Insecticide Bioassays and Selections.
- LITSOP123- Test preparation, detailing the set-up of equipment required.
- LITSOP142-Equipment Cleaning in the LITE Laboratory Area, detailing general cleaning after testing.
- 2I-SOP-027: Field Evaluation of Microbial Mosquito Larvicide Efficacy.
- I2I-SOP-028: Insect Growth Regulator Larvicides.
- I2I-SOP-001: Bacterial Larvicide Bioassay (i.e. *Bacillus thuringiensis subsp. israelensis and Bacillus sphaericus*). This SOP focuses on bacterial larvicides only.

Define Current Use Practices

Does everybody use the same SOP?

The 2005 WHO guidelines are commonly used as 'guidance' when performing laboratory testing. This is reflected in the literature where the WHO, 2005 guidelines are commonly used and adapted for individual studies.

Are there differences of interpretation of the method?

Manufacturers regard current guidelines as flexible, adapting them as needed. The literature suggests that the guidelines are flexible and 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 there results obtained largely consistent between studies?

Laboratory larvicide studies often test with different mosquito strains and different concentrations of active ingredients making comparability between studies difficult.

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

The WHO guidelines have not been updated since 2005. There is a need to update them considering the advancements in vector control and consideration of product types which have been developed since then.

Larviciding is not widely used by malaria control programmes and is generally an under-researched area, with vector control methods that target the adult mosquito stages being prioritized. Several larvicides have different modes of action to currently available adulticides and can therefore target resistant mosquitoes where major classes of

adulticide insecticides are resisted. Larviciding is particularly crucial in order to control *Anopheles stephensi,* which thrives in urban settings making larviciding effective at controlling it.

Updated WHO guidelines providing standardised guidelines would be beneficial to ensure robust data from testing at the Phase 1 laboratory stage.

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 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.
- The insecticide solutions are made up for testing by the user. The WHO, 2005 methods state to add 0.1 or 1mL of the appropriate dilution to 100 or 200mL of water. This could potentially cause variation in results e.g. 1mL of solution added to 100mL of water in comparison to 0.1mL in 200mL of water could produce differences in the final concentration and volume of water.
- Feeding larvae. This is not specified in the WHO, 2005 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 mortality results and fitness of the mosquitoes being tested.
- 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 WHO, 2005 guidelines.)

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

There are many different products that have been developed since 2005. Guidelines for efficacy testing of larvicides were developed using old insecticides and focus on granules or liquid formulations. The guidelines state to measure mortality at 24 hours, and 48 hours for slower acting active ingredients. To evaluate the full impact of a product measuring beyond these time points may be required to evaluate slow-release products such as tablets. It is important for the length of trial to ensure it has enough longevity to detect any loss of efficacy in the active ingredient and information on the speed of activity is also important as it will determine the type of testing procedures to be employed.

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

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

N/A

References

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Appendix

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

Product Name	Active Ingredient/Synergist	Mode of Action
Abate 1 SG	Temephos	Inhibits cholinesterase and affects the central nervous system.
Abate 500 EC	Temephos	Inhibits cholinesterase and affects the central nervous system.
Actellic EC	Pirimiphos-methyl	Inhibits cholinesterase and affects the central nervous system.
Aquatain AMF	PDMS (Polydimethylsiloxane)	It has a physical, not chemical mode of action. It works by lowering the water surface tension affecting all stages of the mosquito life cycle; it is ovicidal, larvicidal, pupicidal and adulticidal.
Device 25WP	Diflubenzuron	Insect growth regulator (IGR)- interferes with insect metamorphosis and prevent adult emergence.
Dimilin GR	Diflubenzuron	Insect growth regulator (IGR)- interferes with insect

		metamorphosis and prevent adult emergence.
Du-Dim 2 DT	Diflubenzuron	Insect growth regulator (IGR)- interferes with insect metamorphosis and prevent adult emergence.
LIMITOR 5 GR	Pyriproxyfen	Insect growth regulator (IGR)- interferes with insect metamorphosis and prevent adult emergence.
Mosquiron 100EC	Novaluron	Insect growth regulator (IGR)- interferes with insect metamorphosis and prevent adult emergence.
MOZKILL 120 SC	Spinosad	Acts on nicotinic acetylcholine receptor and gamma-aminobutyric acid receptors, producing mortality by hyper-excitation of the insect nervous system.
Spinosad 0.5% GR	Spinosad	Acts on nicotinic acetylcholine receptor and gamma-aminobutyric acid receptors, producing mortality by hyper-excitation of the insect nervous system.

Spinosad 20.6% EC	Spinosad	Acts on nicotinic acetylcholine receptor and gamma-aminobutyric acid receptors, producing mortality by hyper-excitation of the insect nervous system.
Spinosad 25 Extended-Release GR	Spinosad	Acts on nicotinic acetylcholine receptor and gamma-aminobutyric acid receptors, producing mortality by hyper-excitation of the insect nervous system.
Spinosad 7.48% DT	Spinosad	Acts on nicotinic acetylcholine receptor and gamma-aminobutyric acid receptors, producing mortality by hyper-excitation of the insect nervous system.
Spinosad Monolayer DT	Spinosad	Acts on nicotinic acetylcholine receptor and gamma-aminobutyric acid receptors, producing mortality by hyper-excitation of the insect nervous system.
Sumilarv 0.5G	Pyriproxyfen	Insect growth regulator (IGR)-interferes with insect metamorphosis and prevent adult emergence.

Sumilarv 2MR	Pyriproxyfen	Insect growth regulator (IGR)- interferes with insect metamorphosis and prevent adult emergence.
Temeguard	Temephos	Inhibits cholinesterase and affects the central nervous system.
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.

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