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GUIDELINES FOR LABORATORY AND FIELD TESTING OF MOSQUITO LARVICIDES

WORLD HEALTH ORGANIZATION
COMMUNICABLE DISEASE CONTROL, PREVENTION AND ERADICATION
WHO PESTICIDE EVALUATION SCHEME

CONTENTS

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1. INTRODUCTION

The purpose of this document is to provide specific and standardized procedures and guidelines for testing larvicides, including bacterial larvicides and insect growth regulators (IGRs), against mosquitoes. Its aim is to harmonize the testing procedures carried out in different laboratories and institutions to generate data for the registration and labelling of larvicides by national authorities.

The document is an expanded and updated version of the guidelines recommended by the WHO Pesticide Evaluation Scheme (WHOPES) Informal Consultation on the evaluation and testing of insecticides, held at WHO headquarters (HQ), Geneva, 7–11 October 1996 (*1*). The guidelines were reviewed and recommended by the Eighth WHOPES Working Group Meeting, held at WHO-HQ, Geneva, 1–3 December 2004 (*2*).

The document provides guidance on laboratory studies and small-scale and large-scale field trials to determine the efficacy, field application rates and operational feasibility and acceptability of a mosquito larvicide. The table below summarizes the sequence and objectives of the studies and trials. The procedures provide some information on the safety and toxicity of the larvicides for non-target organisms, but it is presumed that preliminary eco-toxicity and human assessments have been undertaken before any field study is carried out – detailed treatment and analysis of these extra data are beyond the scope of this document.

Table 1.1
Sequence of the stages of evaluation of mosquito larvicides

Phase	Type of study	Aim
Phase I	Laboratory studies	• Biopotency and activity • Diagnostic concentration and assessment of cross-resistance
Phase II	Small-scale field trials	• Efficacy under different ecological settings • Method and rate of application • Initial and residual activity • Effect on non-target organisms
Phase III	Large-scale field trials	• Efficacy and residual activity • Operational and community acceptance • Effect on non-target organisms

2. PHASE I: LABORATORY STUDIES

The objective of laboratory testing is to determine the inherent biopotency of the technical material or, in the case of formulated larvicides, their activity. It is assumed that the compound's mode of action has already been established. Information on the speed of activity is important, as this will determine the type of testing procedures to be employed.

To evaluate the biological activity of a mosquito larvicide, laboratory-reared mosquito larvae of known age or instar (reference strains or F1 of field-collected mosquitoes) are exposed for 24 h to 48 h or longer in water treated with the larvicide at various concentrations within its activity range, and mortality is recorded. For IGRs and other materials with delayed activity, mortality should be assessed until the emergence of adults. It is important to use homogenous populations of mosquito larvae or a given instar. These are obtained using standardized rearing methods (see Annex 1).

The aims of the tests are:

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

2.1 Determination of biological activity

2.1.1 Larvicides other than bacterial products and insect growth regulators

2.1.1.1 Materials required for testing

- One pipette delivering 100–1000 µl.
- Disposable tips (100 µl, 500 µl) for measuring aliquots of dilute solutions.
- Five 1 ml pipettes for insecticides and one for the control.
- Three droppers with rubber suction bulbs.
- The following materials to make a strainer: two wire loops, one piece of nylon netting (30 cm^2) and one tube of cement. It is suggested that two pieces of netting be cut and cemented to opposite sides of the larger end of the wire loops. More cement should then be applied around the edges of the loops to join the two pieces of netting. When dry, the netting may be trimmed with scissors.
  If a strainer is not available, a loop of plastic screen may be used to transfer test larvae into test cups or vessels.
- Data recording forms (see Annex 4).
- Disposable cups (preferred as they avoid contamination) or, if not available, glass bowls or beakers of two capacities: 120 ml (holding 100 ml) and 250 ml (holding 200 ml).
- Graduated measuring cylinder.
- Log–probit software or paper.

2.1.1.2 Preparation of stock solutions or suspensions and test concentrations

The technical materials of many organic compounds are insoluble in water. These materials have to be dissolved in appropriate organic solvents such as acetone or ethanol (the manufacturer should be consulted) in order to prepare dilute solutions for laboratory testing. The formulated materials are, however, miscible with water. Suspending or mixing these formulations in water requires no special equipment – homogeneous suspensions can be obtained by gentle shaking or stirring.

The volume of stock solution should be 20 ml of 1%, obtained by weighing 200 mg of the technical material and adding 20 ml solvent to it. It should be kept in a screw-cap vial, with aluminium foil over the mouth of the vial. Shake vigorously to dissolve or disperse the material in the solvent. The stock solution is then serially diluted (ten-fold) in ethanol or other solvents (2 ml solution to 18 ml solvent). Test concentrations are then obtained by adding 0.1–1.0 ml (100–1000 µl) of the appropriate dilution to 100 ml or 200 ml chlorine-free or distilled water (see Table A2.1). For other volumes of test water, aliquots of dilutions added should be adjusted according to Table A2.1. When making a series of concentrations, the lowest concentration should be prepared first. Small volumes of dilutions should be transferred to test cups by means of pipettes with disposable tips. The addition of small volumes of solution to 100 ml, 200 ml or greater volumes of water will not cause noticeable variability in the final concentration.

When a test is carried out using formulated materials, distilled water is used in the preparation of the 1% stock solution or suspension and in subsequent serial dilutions, according to the content of the active ingredient.

2.1.1.3 Bioassays

Initially, the mosquito larvae are exposed to a wide range of test concentrations and a control to find out the activity range of the materials under test. After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h) is used to determine LC_{50} and LC_{90} values.

Batches of 25 third or fourth instar larvae are transferred by means of strainers, screen loops or droppers to small disposable test cups or vessels, each containing 100–200 ml of water. Small, unhealthy or damaged larvae should be removed and replaced. The depth of the water in the cups or vessels should remain between 5 cm and 10 cm; deeper levels may cause undue mortality.

The appropriate volume of dilution is added (see Table A2.1) to 100 ml or 200 ml water in the cups to obtain the desired target dosage, starting with the lowest concentration. Four or more replicates are set up for each concentration and an equal number of controls are set up simultaneously with tap water, to which 1 ml alcohol (or the organic solvent used) is added. Each test should be run three times on different days. For long exposures, larval food should be added to each test cup, particularly if high mortality is noted in control. The test containers are held at 25–28 ^{o}C and preferably a photoperiod of 12 h light followed by 12 h dark (12L:12D).

After 24 h exposure, larval mortality is recorded. For slow-acting insecticides, 48 h reading may be required. Moribund larvae are counted and added to dead larvae for calculating percentage mortality. Dead larvae are those that cannot be induced to move

when they are probed with a needle in the siphon or the cervical region. Moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. The results are recorded on the form provided (Fig. A4.1), where the LC_{50}, LC_{90} and LC_{99} values, and slope and heterogeneity analysis are also noted. The form will accommodate three separate tests of six concentrations, each of four replicates.

Larvae that have pupated during the test period will negate the test. If more than 10% of the control larvae pupate in the course of the experiment, the test should be discarded and repeated. If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula (*3*):

$$\textit{Mortality}\ (\%) = \frac{X - Y}{X}\ 100\ ,$$

where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

2.1.1.4 Data analysis

Data from all replicates should be pooled for analysis. LC_{50} and LC_{90} values are calculated from a log dosage–probit mortality regression line using computer software programs, or estimated using log–probit paper. Bioassays should be repeated at least three times, using new solutions or suspensions and different batches of larvae each time. Standard deviation or confidence intervals of the means of LC_{50} values are calculated and recorded on a form (Fig. A4.1). A test series is valid if the relative standard deviation (or coefficient of variation) is less than 25% or if confidence limits of

LC_{50} overlap (significant level at $P < 0.05$). The potency of the chemical against the larvae of a particular vector and strain can then be compared with the LC_{50} or LC_{90} values of other insecticides.

2.1.2 Insect growth regulators

Testing methods for the juvenile hormone (JH) analogues (juvenoids) and the chitin synthesis inhibitors differ. JH analogues interfere with the transformation of late instar larvae to pupae and then to adult, whereas chitin synthesis inhibitors inhibit cuticle formation and affect all instars and immature stages of the mosquito. The delayed action of IGRs on treated larvae means that mortality is assessed every other day or every three days until the completion of adult emergence. The effect of both types of IGR on mosquito larvae is expressed in terms of the percentage of larvae that do not develop into successfully emerging adults, or adult emergence inhibition (IE%).

2.1.2.1 Preparation of stock solutions or suspensions and test concentrations

The preparation of the test solutions or suspensions and bioassay set-ups are the same as for the fast-acting compounds (see Sections 2.1.1.1 and 2.1.1.2). Technical materials are generally soluble in organic solvents and stock solution (1%) should be made by dissolving 200 mg in 20 ml. Formulated materials should be diluted with water and serial dilutions made in the same manner.

2.1.2.2 Bioassays

Third instar larvae are used for testing JH analogues and chitin synthesis inhibitors. The accurate initial count of larvae is essential because of the cannibalistic or scavenging behaviour of larvae during the long exposure period. The long duration of the test also means that the larvae have to be provided with a small amount of food (finely ground yeast extract, rabbit pellets, or ground fish or mouse food) at a concentration of 10 mg/l at two-day intervals until mortality counts are made. The food powder should be suspended in water and one or two drops added per cup. The larvae in the control are fed in the same manner as those in the treated batches. If necessary, all the test and control cups should be covered with netting to prevent successfully emerged adults from escaping into the environment. Mortality or survival is counted every other day or every three days until the complete emergence of adults. The test containers are held at 25–28 ^{o}C and preferably for a photoperiod of 12L:12D.

At the end of the observation period, the impact is expressed as IE% based on the number of larvae that do not develop successfully into viable adults. In recording IE% for each concentration, moribund and dead larvae and pupae, as well as adult mosquitoes not completely separated from the pupal case, are considered as "affected". The number of successfully emerged adults may also be counted from the empty pupal cases. The experiment stops when all the larvae or pupae in the controls have died or emerged as adults. Data are entered on a form (Fig. A4.2). Any deformities or morphogenetic effects that occur in either the moulting immature mosquitoes or the emerging adults are also recorded.

2.1.2.3 Data analysis

The data from all replicates of each concentration should be combined. Total or mean emergence inhibition can be calculated on the basis of the number of third stage larvae exposed. The overall emergence of adults reflects activity. IE% is calculated using the following formula (*4*):

$$IE(\%) = 100 - \left(\frac{T \times 100}{C} \right) ,$$

where T = percentage survival or emergence in treated batches and C = percentage survival or emergence in the control.

If adult emergence in the control is less than 80%, the test should be discarded and repeated. Where the percentage is between 80% and 95%, the data are corrected using Abbott's formula (see Section 2.1.1.3). IE values obtained at each concentration should be subjected to probit regression analysis to determine IE_{50} and IE_{90} values (using computer software programs or estimated from log–probit paper). The data analysis procedures stated in Section 2.1.1.4 should be followed.

2.1.3 Bacterial larvicides

The laboratory bioassay procedures for bacterial products are the same as those for chemical larvicides, except in the preparation of stock suspensions.

2.1.3.1 Principles

The biopotency of the material is first examined by comparing mosquito larval mortality produced by the product under test with the mortality produced by the corresponding reference standard or other technical or formulated product. The toxicity of preparations based on *Bacillus thuringiensis* subsp. *israelensis (B. thuringiensis* subsp. *israelensis)* can be determined against a standard product that has been calibrated using *Aedes aegypti (A. aegypti)* larvae. The potency of products tested is determined by the following formula:

$$\text{Potency of product "X"} = \frac{\text{Potency standard (ITU)} \times LC_{50}\ \text{(mg/1) standard}}{LC_{50}\ \text{(mg/l) of "X"}}$$

When the international reference standard is used, potency is expressed in International Toxic Units per milligram (ITU/mg). The biopotency of products based on *B. thuringiensis* subsp. *israelensis* is compared with a lyophilized reference powder (IPS82, strain 1884) of this bacterial species using early fourth instar larvae of *A. aegypti* (strain Bora Bora). The potency of IPS82 has been arbitrarily designated as 15 000 ITU/mg powder against this strain of mosquito larva.

The biopotency of products based on *Bacillus sphaericus* (*B. sphaericus)* is determined against a lyophilized reference powder (SPH88, strain 2362) of this bacterial species using early fourth instar larvae of *Culex pipiens pipiens (C. pipiens pipiens)* or *Culex quinquefasciatus*. The potency of SPH88 has been arbitrarily set at 1700 ITU/mg of powder against this mosquito strain.

The use of other bacterial larvicide reference powders and/or alternative strains of mosquito in this test is possible but must be approached warily, because it is inevitable that different results will

obtain. Such alternatives must be the subject of careful cross-calibration with the reference powders and strains identified above. Ideally, such cross-calibration should be conducted by a group of independent expert laboratories. The alternative powders or strains, and the cross-calibration data that support them, should be made available to anyone who wishes to use, or check, the test.

In general, it is not necessary to calibrate with or test against the standard if comparing the activity of a bacterial product with other larvicide products. Bioassay results providing LC_{50} and LC_{90} values of products are sufficient to enable comparison among different products.

2.1.3.2 Additional materials required for testing

- Top-drive homogenizer or stirrer for lyophilized products
- Ice bath (container of crushed ice) for grinding or sonication
- Micropipette
- 10 ml pipette
- 12 ml plastic tubes with stoppers or caps
- 120 ml or 250 ml plastic or wax-coated paper cups to hold 100 ml or 200 ml water

2.1.3.3 Preparation of reference standard suspensions for calibration of the bioassays

To prepare a “stock suspension”, weigh 200 mg or 1000 mg of the solid product, place in a vial (30 ml) or volumetric flask, and add 20 ml or 100 ml distilled water, yielding 1% stock suspension, or 10 mg/l. Most powders do not need blending or sonication. Vigorous shaking or stirring will facilitate suspension. If placed in

tubes, the stock suspension can be frozen for future bioassays. Frozen aliquots must be homogenized thoroughly before use, because particles agglomerate during freezing.

From the "stock suspension", any necessary subsequent dilutions (see Table A2.1) are prepared by serial dilution. Plastic or paper cups are filled with 100 ml deionized water. Twenty-five late third or early fourth instar larvae of *A. aegypti* or *C. pipiens* (depending on the bacterial species to be tested: *Aedes* larvae for *B. thuringiensis* subsp. *israelensis* and *Culex* larvae for *B. sphaericus*) are added to each cup. Using micropipettes, 400 µl, 300 µl, 200 µl, 100 µl, 80 µl and 50 µl of a given suspension (see Table A2.1) are added to the cups and the solutions mixed to produce final concentrations of 0.04 mg/l, 0.03 mg/l, 0.02 mg/l, 0.01 mg/l, 0.008 mg/l and 0.005 mg/l, respectively, of the reference standard powder. Four or more replicate cups are used for each concentration and the control, which is 100 ml deionized water.

2.1.3.4 Preparation of suspensions of the product to be tested

For bioassays of technical (solid or liquid) products of unknown potency, an initial homogenate is made simply by mixing without reducing particle size. For assays of liquid formulations, 20 ml water is added to 200 mg in a vial. Serial dilutions are made and cups and larvae are prepared as described in the previous section.

Range-finding bioassays are performed using a wide range of concentrations of the product to determine its approximate toxicity. The results are then used to determine a narrower and more refined range of concentrations for precise bioassay.

2.1.3.5 Bioassays

To prepare a valid dose–response curve, only concentrations giving values between 10% and 95% mortality should be used. A minimum of two concentrations above and two below the LC_{50} level must be used. Each bioassay series should involve at least four concentrations; and each concentration should be tested in four replicates of 25 late third or early fourth instar larvae per replicate.

No food is added to larval vessels when the exposure period is 24 h. Food may be required if the exposure period is longer. Finely ground yeast extract or ground mouse or rabbit pellets suspended in water (1.5 mg) is added to the water in test vessels at 10 mg/l. Mortality is determined at 24 h for *B. thuringiensis* subsp. *israelensis* and 48 h for *B. sphaericus* by counting the live larvae remaining. The results of the tests at different concentrations (including LC values) are entered on the form (Fig. A4.1). If more than 10% of larvae pupate, the test is invalidated because late instar larvae do not ingest 24 h before pupation and too many larvae may have survived simply because they are too old. All tests should be conducted at 25–28 °C, preferably with a 12L:12D photoperiod.

2.1.3.6 Data analysis

If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula (see Section 2.1.1.3). Tests with control mortality greater than 20% or pupation greater than 10% should be discarded. A mortality–concentration regression is made using log–probit analysis software or log–probit paper. Bioassays should be carried out at least three times and the validity of the results assessed as for the other

larvicides. LC values (Fig. A4.1) are determined and compared to examine the activity of one product versus another.

2.2 Determination of the diagnostic concentration

The diagnostic or discriminating concentration is determined from the dose–response regression lines of testing a technical material against susceptible vector species according to the procedures outlined in Section 2.1. The diagnostic concentration is double that of the estimated $LC_{99..9}$ value.

2.3 Cross-resistance assessment

New, candidate larvicides are tested simultaneously against a small number of distinct, multi-resistant mosquito strains and a susceptible strain, according to the procedures outlined in Section 2.1. If cross-resistance is detected, its exact nature will be determined by testing the larvicide against strains that each possess a single resistance mechanism. The mechanism of resistance may be assessed following the procedures outlined in the WHO document *Techniques to detect insecticide resistance mechanisms (field and laboratory manual)* (*5*).

Susceptible strains of some mosquito species are kept in laboratories. Otherwise, any susceptible strains should be collected in the field (if truly susceptible populations still exist). If not, susceptible strains may be artificially selected using bioassays, assays for individual resistance mechanisms and selection between lines derived from individually mated females.

The resistant strains should be identified using well established assay techniques. The strains should preferably be homozygous for one or more known resistance mechanisms. If homozygosity cannot be achieved, periodic selection is usually necessary to prevent natural selection favouring the susceptible at the expense of the resistant. Established reference strains should be regularly monitored by bioassays and biochemical and/or molecular assays so that any changes in resistance or underlying mechanisms can be assessed and rectified by selection.

3. PHASE II: SMALL-SCALE FIELD TRIALS

Larvicides that show promise in laboratory studies (Phase I) may be subjected to small-scale field testing (Phase II). In Phase II, field trials of formulated products are performed on a small scale against target mosquitoes, preferably in representative natural breeding sites or, where such trials are not feasible, under simulated field conditions (see Section 3.2).

Evaluation procedures should be selected on the basis of the breeding sites and the behaviour of mosquitoes. The formulations are tested at three–five concentrations and the Phase I studies will guide the dosages chosen for use in the Phase II trials. Usually, this will be multiple concentrations of LC_{90} for the target species. Treatment concentrations are calculated on the basis of the amount of active ingredient per volume of water (if known or measurable) or surface area of the habitat.

The objectives of small-scale field trials are:

- to determine efficacy, including residual activity, against different mosquito vectors in different breeding sites and ecological settings;
- to determine the optimum field application dosage(s);
- to monitor abiotic parameters that may influence the efficacy of the product; and
- to record qualitative observations on the non-target biota cohabiting with mosquito larvae, especially predators.

3.1 Trials in natural breeding sites

The field efficacy of the larvicide under various ecological conditions is determined by selecting representative natural breeding habitats of the target species. These include stagnant drains (cement lined and unlined), soakage pits, cesspits, cesspools, domestic service tanks collecting sewage water, pools, wetlands, irrigated fields and unused wells for *Culex* spp.; cement tanks, drums, cisterns, water storage containers and air coolers for *A. aegypti*; and disused wells, garden pits, ponds, curing yards, rice plots, stream pools, wetlands, marshes, irrigated fields and seepages for *Anopheles*spp.

A minimum of three replicates of each type of habitat should be randomly selected for each dosage of the formulation, with an equal number of controls. The size of the plot should be recorded, taking account of surface area and depth. As far as possible, the plots selected should be similar and comparable. Each of the confined breeding sources or containers can be considered as a discrete plot or replicate. Habitats such as drains, irrigation canals, irrigated fields, rice fields, streams and seepages may be divided into discrete areas of 4–50 m^2 and replicated for treatment and control.

Pretreatment immature abundance (first and second instar larvae, third and fourth instar larvae, and pupae) should be recorded in both experimental and control sites (minimum of two observations at equal intervals). The sampling method should be appropriate to the type of breeding habitat, and the appropriate number of samples should be taken from each habitat based on the type and size of the habitat. Larval instars and pupae from each sample are counted and recorded. At least three different dosages of the larvicide should be applied to the breeding habitats. These can be applied using small atomizers, compression sprayers or, in most cases, plastic squeeze bottles. Granules, pellets, tablets and briquettes can be manually broadcast or thrown in the water.

Post-treatment immature abundance (all stages) should be monitored on day 2 and then weekly until the density of fourth instar larvae (or pupae in the case of IGRs) in the treated habitats reaches a level comparable to that in the control. Data are recorded on the form (Fig. A4.3).

Characterization of the habitats in terms of abiotic and biotic factors aids the interpretation of results. Rainfall and any change in water level or other parameters, such as algal bloom or predators in the habitats, should be recorded.

The efficacy and residual activity of the larvicide at different dosages are determined from the post-treatment counts of live larvae and pupae in treated and control sites compared with the pretreatment counts or the control, taking into consideration the dynamics of change occurring in the treated and the control batches (see below).

The assessment of an IGR's efficacy is based on the level of inhibition of emergence of adults and the percentage reduction in larval and pupal densities. Larvae and pupae are sampled as described above. Adult emergence can be monitored directly in the field by floating sentinel emergence traps in treated and untreated habitats (see Fig. A4.4), by pupal isolation, or by sampling and counting pupal skins. Adult emergence may also be assessed by collecting pupae (20–40 per replicate) and bringing them to the laboratory in glass containers with the water from the respective habitats, then transferring them to small cups inside the holding cages. Dead larvae and pupae found in the cups should be removed and any morphological abnormalities recorded.

When monitored directly in the field, the pretreatment and post-treatment data on adult emergence in treated and untreated habitats are analysed for IE%. The following expression (*6*) is used to calculate IE% values:

$$IE(\%) = 100 - \left(\frac{C1}{T1}\right) \times \left(\frac{T2}{C2}\right) \times 100 \quad ,$$

where $C1$ is the number of adults emerged in control habitats before treatment, $C2$ the number of adults emerged in control habitats at a given interval after treatment, $T1$ the number of adults emerged in treated habitats before treatment and $T2$ the number of adults emerged in treated habitats after treatment.

When adult emergence is monitored in the laboratory using pupae collected from treated and untreated habitats, IE% is calculated using the following formula, on the basis of determining adult emergence from the number of pupae isolated (see also Section 2.1.1.3):

$$IE(\%) = \left(\frac{C - T}{C} \right) \times 100 \ ,$$

where C = percentage emerging or living in control habitats and T = percentage emerging or living in treated habitats.

3.1.1 Data analysis

The mean number of pupae or larvae collected per dip for each replicate of each treatment and the control is calculated for each day of observation. The percentage reduction in larval and pupal densities, or the IE% on post-treatment days, will be estimated for each replicate of each treatment using Mulla's formula. The difference between treatments treatments can be compared by two-way analysis of variance (ANOVA) with treatment and number of days as independent factors. The ANOVA should be carried out after transforming the percentage reduction to arcsine values.

The post-treatment day up to which 80% or 90% reduction is observed for each treatment or dosage will then be compared to determine the residual effect and optimum application dosage (see Section 3.3).

3.2 Simulated field trials

In these trials, multiple artificial containers (jars, bucket, tubs, cylinders, etc.) of water are placed in the field or under simulated field conditions and the materials are tested against laboratory-reared or field-collected larvae. The type and size of the container will depend on the natural larval habitat of the target mosquito species. The water-filled containers are given at least 24 h for

conditioning or ageing. A batch of 25–100 laboratory-reared third instar larvae of the mosquito species to be tested is released into each container or replicate and larval food is added. After 2–3 h of larval acclimation, the containers are treated with selected dosages in a randomized manner using pipettes or appropriate hand atomizer sprays, or by broadcasting solid materials over the water surface. The containers are covered with nylon mesh screen or solid covers to prevent other mosquitoes or other insects from laying eggs and to protect the water from falling debris. The water level in the containers must be sustained. A minimum of four replicates of each dosage and four controls are to be used. For fast-acting agents all the containers are examined after 48 h and live larvae are counted to score post-treatment larval mortality. For slow-acting materials, such as IGRs, the survival of larvae, pupae and pupal skins is assessed seven days or more after treatment, by which time all larvae would have pupated and emerged as adults. The pupal skins provide the best gauge of final or overall effectiveness. To test residual activity, a new batch of laboratory-reared, late third instar larvae of the same mosquito species is introduced to each container, and mosquito larval food is added on alternate days or weekly. Larvae survival is assessed 48 h post addition, and pupal skins are counted seven days or more after addition. This process continues until no mortality is noted.

Data are recorded on the form in Fig. A4.2. For the IGRs under test, pupae are removed from the treated and control containers every other day and put into vials or cups with water from the respective containers, then placed in cages and adult emergence is recorded. Another precise method of assessing emergence is to count and remove pupal skins from containers (Fig. A4.4). Adults not freed from pupal skins are considered dead. The test is terminated when there is no statistically significant residual activity in terms of larval mortality or inhibition of adult emergence when comparing the

treated (at the highest dosage tested) batches and the untreated controls. Values of pH and water temperature are recorded throughout the evaluation.

Alternatively, tests can be conducted by exposing third instar larvae in small natural breeding sites to selected dosages of larvicides using screened floating cages (minimum of three replicates, two cages per replicate). These cages should have screened portholes to allow the movement of water and food into the cage from outside. For each dosage, at least three treated and three untreated control habitats are selected. The habitats are treated with the selected dosages of the material to be tested. Twenty-five laboratory-reared or, preferably, field-collected third instar larvae are placed in each cage. The number surviving is counted every other or every third day until all larvae have pupated and emerged. Percentage mortality or IE% is calculated. To test residual activity, 25 third instars are set weekly in treated and untreated control cages. As with the initial batches of larvae, assessments of mortality should be made every other or every third day post introduction. The weekly settings of larvae continue until no difference in mortality is recorded between untreated controls and treated batches.

3.2.1 Data analysis

The method given in Section 3.1.1 can also be used to analyse data collected under simulated trials. However, since the denominator is known for simulated trials, a probit or logistic regression analysis is more suitable than ANOVA and is described below.

The data on the number of live and dead larvae and pupae from all replicates of each dosage on one day should be combined and percentage mortality or IE% calculated. Logistic or probit

regression of the percentage mortality or IE% on dosage and number of post-treatment days can be used to determine the post-treatment day (and its 95% CI) up to which 80% or 90% (the desired level of control) is achieved for a given dosage. This analysis can be done using appropriate statistical software packages.

3.3 Selection of optimum field application dosage

From the dosages tested against a target species in the small-scale or simulated field trials, the minimum dosage at which the maximum effect (immediate as well as residual) is achieved should be selected as the optimum field application dosage for each type of habitat. The frequency of larvicidal treatment is determined based on the reappearance of fourth instar larvae or pupae, in the case of common larvicides and bacterial larvicide products, or the day reduction in inhibition of emergence falls below 90% for IGRs.

4. PHASE III: LARGE-SCALE FIELD TRIALS

The efficacy of larvicides found to be suitable in small-scale field trials (Phase II) should be validated in larger scale field trials against natural vector populations in natural breeding habitats. In this phase, the larvicide is applied to the breeding sites of the target mosquito at the optimum field dosage(s) selected in the small-scale field trials using appropriate application equipment, depending on the formulation.

The objectives of the trial are:

- to confirm the efficacy of the larvicide at the selected field application dosage(s) against the target vector when applied to large-scale plots in natural breeding sites;
- to confirm residual activity and application intervals;
- to record observations on the ease of application and dispersal of the insecticide;
- to observe community acceptance;
- to record any perceived side-effects on operators; and
- to observe the effect of the treatment on non-target organisms.

4.1 Selection of study sites

The experimental plots selected will depend on the type of larval habitat and the environment. Care should be taken that all the representative habitats of the target vector species are included in the trial. A minimum of 25–30 replicates or plots of each type of larval habitat of the target species should be selected for control and then again for treatment. Just as for the small-scale trials, each confined habitat can be considered as an individual replicate; larger habitats can be subdivided into replicates of about 10 m^2.

4.2 Assessment of pretreatment density

Pretreatment larval and pupal abundance (and adult emergence in the case of IGRs) in the treatment and control habitats should be carried out for a week on at least two occasions before treatment. The immature population and adult emergence should be estimated in different types of larval habitat by using appropriate sampling devices (as in the small-scale field trials with natural populations).

4.3 Application of larvicide

All the breeding sites within the unit should be treated at the optimum field application dosage determined in Phase II, using equipment that is appropriate to the formulation and its operational use. The optimum dosage for the major or most important larval habitat of the target species in the area can be used for all the habitats. Where small-scale trials found wide variation between optimum dosages for each type of habitat, the specific optimum dosage should be applied to each type of habitat.

4.4 Assessment of post-treatment density

The impact of larvicidal treatments on the larvae and pupae of mosquitoes (and the inhibition of adult emergence) should be evaluated by sample collection at 48 h and then at weekly intervals using a fixed number of dips or sentinel cages. Sampling procedures are similar to those followed for small-scale trials conducted in natural breeding habitats. Data should be recorded on the relevant form (Figs. A4.3 or A4.4).

4.5 Effect on non-target organisms

Specific, separate trials have to be carried out to assess the impact of larvicides on non-target organisms. However, during the large-scale trial, and where appropriate, non-target organisms cohabiting with mosquito larvae can be counted and examined for impact of treatments while sampling mosquito larvae. Larvivorous fish, snails, polychaetes, shrimps, cray fish, crabs, mayfly naiads, copepods, dragonfly naiads, coleopterans and heteropterans,

ostracods and amphipods are some of the non-target organisms that coexist with mosquito fauna.

4.6 Operational and community acceptability

During the trial, observations should be made on the ease of storage, handling and application of the insecticide formulation on the breeding sites, and of the effects of the insecticide formulation on the proper functioning of application equipment such as nozzle tips and gaskets, rotors, blowers, etc.

Observations are also recorded on the acceptability of the insecticide treatments to the residents of the area, particularly on domestic and peridomestic breeding sites.

4.7 Data analysis

The mean number of pupae or larvae or non-target organisms collected per dip on each day of observation is calculated for each replicate in treatment and control. The statistical analysis to determine residual efficacy – including the number of post-treatment days over which the desired level of control is achieved at the selected dosage – is carried out following the method described in Section 3.1.1.

REFERENCES

(*1*) *Report of the WHO Informal Consultation on the evaluation and testing of insecticides*. Geneva, World Health Organization, 1996 (CTD/WHOPES/IC/96.1).

(*2*) *Report of the Eighth WHOPES Working Group Meeting.* Geneva, World Health Organization, 2005 (WHO/CDS/WHOPES/2005.10).

(*3*) Abbott WS. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 1925, 18:265–267.

(*4*) Mulla MS, Darwazeh HA. Activity and longevity of insect growth regulators against mosquitoes. *Journal of Economic Entomology*, 1975, 68:791–794.

(*5*) *Techniques to detect insecticide resistance mechanisms (field and laboratory manual)*. Geneva, World Health Organization, 1998 (WHO/CDS/CPC/MAL/98.6).

(*6*) Mulla MS et al. Control of chironomid midges in recreational lakes. *Journal of Economic Entomology*, 1971, 64:301–307.

ANNEX 1
PRODUCTION OF TEST LARVAE

Use of homogenous batches of mosquito larvae is of prime importance in laboratory studies and is crucial in determining the activity and biopotency of synthetic larvicides, IGRs, bacterial larvicides and natural products. The following standard procedure is proposed for rearing *A. aegypti* and *Culex* spp. Other species may be reared according to these procedures, subject to any modifications necessary to fit the biological requisites of the test species.

For *A. aegypti*, eggs are laid in a cup lined with filter paper strips and one third filled with deionized or tap water. About one third of the paper strip should be in water. This will keep the strips moist where the eggs are laid above the water line. The paper strips are dried at room temperature and stored at room temperature for several months in a sealed plastic bag. When larvae are needed, the paper strip is immersed in de-chlorinated or distilled water. To synchronize and promote hatching, add larval food to the water 24 h before adding the eggs. The bacterial growth will de-oxygenate the water and this triggers egg hatching. This process usually induces the first instars to hatch within 12 h of hydration. The hatched larvae are then transferred to shallow pans or trays containing 2 l de-chlorinated water. The aim is to create a population of 500 to 700 larvae per container. Larval food may be flakes of protein as used for aquarium fish, rabbit pellets, chicken mash or powdered cat biscuit. The containers are held at 25 $\pm$ 2 °C. It is important that the amount of food is kept low to avoid strong bacterial growth (which kills the larvae), increasing food provision as the larvae grow. Several feeds at intervals of one or two days and daily observation of the larvae are optimal. Provision of solid pellets (chicken mash or rabbit pellets) prevents turbidity and scum. If the

water becomes turbid (in the case of powdered food), replace all water by filtering out the larvae and then transferring them to a clean container with clean water and food, a process that may result in larval mortality. A homogenous population of late third or early fourth instars (5 days old and 4–5 mm in length) should be obtained five to seven days later.

The materials and procedures necessary to rear *Culex* larvae, especially those that are severe pests or vectors of disease, are essentially the same as for *A. aegypti*, except that *Culex* eggs are deposited on water as egg rafts and will hatch 1–2 days after deposition. They require no conditioning and cannot be dried. If they do not hatch in two days they will die. It is more difficult to obtain a homogenous population of third or fourth instars of *Culex* spp. larvae. First, a large number of egg rafts must be laid and collected on the same day. These can be stored at 15–18 °C in order to accumulate more eggs for hatching over a day or two. The first instars are fragile and thus should not be handled. Development to the second instar usually takes 3–4 days at 25 $\pm$ 2 °C after the eggs are hatched. In trays containing 2–3 l de-chlorinated water at 4–6 cm depth, 400–600 larvae per tray are reared. Food (see above) is provided as needed. Early fourth instars suitable for testing are usually obtained within 7 days, although sometimes 8 or 9 days are required.

ANNEX 2
DILUTIONS AND CONCENTRATIONS

Table A2.1
Aliquots of various strength solutions added to 100 ml water to yield final concentration

Initial solution		Aliquot (ml)[a]	Final concentration (PPM) in 100 ml
%	PPM		
1.0	10 000.0	1.0	100.0
		0.5	50.0
		0.1	10.0
0.1	1 000.0	1.0	10.0
		0.5	5.0
		0.1	1.0
0.01	100.0	1.0	1.0
		0.5	0.5
		0.1	0.1
0.001	10.0	1.0	0.1
		0.5	0.05
		0.1	0.01
0.0001	1.0	1.0	0.01
		0.5	0.005
		0.1	0.001
0.00001	0.1	1.0	0.001
		0.5	0.0005
		0.1	0.0001

[a] For 200 ml double the volume of aliquots.

ANNEX 3
MEASUREMENTS AND CONVERSIONS

Volume
l l = 1000 ml
1 ml = 1000 µl
1 cubic foot = 7.5 gallons = 28 l
1 gallon = 4 quarts = 8 pints = 128 ounces = 3785 ml

Surface
1 ha = 10 000 m^2 = 2.2 acres
1 acre = 43 560 square feet
1 square foot = 0.111 square yard = 0.105 m^2

Length
1 km = 0.62 miles = 1093 yards
1 m = 39.7 inches
1 inch = 2.54 cm = 0.0254 m
1 foot = 0.333 yards = 0.3048 m
1 yard = 91.44 cm = 0.9144 m
1 mile (statute) = 1760 yards = 5280 ft = 1609.3 m

Weight
1 pound = 0.454 kg
1 kg = 2.2 pounds
1 g = 0.035 ounces

Conversion factors
Square inches to square centimetres, multiply by 6.5.
Square yards to square metres, multiply by 0.8.
Square feet to square metres, multiply by 0.09.
Acres to hectares, multiply by 0.4.
Square miles to square kilometres, multiply by 2.6.

ANNEX 4
DATA RECORDING FORMS

Fig. A4.1

Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: _____ Investigator: _____ Location: _____ Treatment date: _____

Material: _____ Formulation: _____ Temp: _____ Lighting: _____

Species: _____ Larval instar: _____ Larvae/cup or vessel: _____

Water: Tap/Distilled Volume of water: _____ ml Food: _____ Date stock solution made: _____

		No of dead larvae at various conc. (mg/L) post exposure (hr.)													
		24 hr							48 hr						
Date	Replicate	0.00							0.00						
	1														
	2														
	3														
	4														
	5														
	6														
	7														
	8														
	9														
	10														
	11														
	12														
	Total														
	Ave.														
	% mortality														

LC50 (CL 95%): _____ LC50 (CL 95%): _____

LC90 (CL 95%): _____ LC90 (CL 95%): _____

LC99: _____ LC99: _____

Slope: _____ Heterogeneity: _____ Slope: _____ Heterogeneity: _____

Fig. A4.2

Laboratory evaluation of the efficacy of insect growth regulators against mosquito larvae

Experiment No: ____________ Investigator: _________________ Location: ___________________ Treatment date: ___________

Material:__________________________ Formulation:_________________ Sampling technique: ____________________

Species: ___________________ Larval instar: ________________ No. of larvae released/exposed: __________ Setting date: ______________

Cumulative number of dead / alive mosquitoes after treatment (date or days pre or posttreatment or setting) L=larvae, P=pupae, A=adults

	Date:											Grand total	
Conc.		Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
(mg/L)	Rep	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A
0.0	1												
	2												
	3												
	4												
	Total												
	Mean												
T1	1												
	2												
	3												
	4												
	Total												
	Mean												
T2	1												
	2												
	3												
	4												
	Total												
	Mean												
T3	1												
	2												
	3												
	4												
	Total												
	Mean												
T4	1												
	2												
	3												
	4												
	Total												
	Mean												
T5	1												
	2												
	3												
	4												
	Total												
	Mean												

Fig. A4.3

Small-scale field testing and evaluation of larvicides against mosquito larvae

Experiment No: _____ Starting date: _____ Location: _____ Investigator: _____
Assessment date: _____ Pre or days posttreatment: _____ Types of Habitat:_____Species _____

Live larvae (L3-4) and pupae (P)/sample							
Treatments		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Grand total
Dosage ( )	Sample	L3-4* P*	L3-4 P	L3-4 P	L3-4 P	L3-4 P	L3-4 P
Control	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T1	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T2	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T3	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T4	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T5	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						

Fig. A4.4

Small-scale field testing and evaluation of insect growth regulators against mosquito larvae

Experiment No: _____ Starting date: _____ Location: _____ Investigator: _____
Assessment date: _____ Pre or days posttreatment: _____ Type of Habitat: _____ Species:_____

		Live larvae (L3-4), pupae (P) and adult emergence (A) /sample or cage or trap												
Treatments		Rep 1			Rep 2			Rep 3			Rep 4			Rep 5			Grand total			Visual count	
Dosage ( )	Sample	L3-4*	P*	A*	L3-4	P	A	L3-4	P	A	L3-4	P	A	L3-4	P	A	L3-4	P	A	Pupae	Pupal skins
Control	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T1	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T2	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T3	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T4	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	
T5	1																				
	2																				
	3																				
	4																				
	5																				
	Total																				
	Mean																				
	%red																			IE%	