Standard Operating Procedure (SOP)

Field Evaluation of Microbial Mosquito Larvicide Efficacy

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1. **Purpose**
   a. To provide instructions on how to perform field trials to evaluate the efficacy of liquid and granular larvicide products at application rates identified during laboratory trials.

2. **Type of Field Evaluation**
   a. There are two types of field evaluation- namely outdoor microcosm or outdoor natural habitat. According to availability and convenience either evaluation method can be chosen.
      i. Outdoor Microcosm – Artificial Colonization
      ii. Outdoor Microcosm – Natural Colonization

3. **Outdoor Microcosm- Artificial Colonization**
   a. Artificial habitats are created outdoors where natural colonization of mosquitoes is not expected or is not desirable for ethical reasons. This type of evaluation is convenient when natural habitats are hard to find and or distant from the institution where the evaluation will be conducted. For studies using artificial colonization, microcosms will need to be screened to prevent natural oviposition, or the study will need to be conducted in a screen house.

   b. Preparation
      i. Equipment and Materials
         1) Plastic tubs (0.5 – 1m in diameter, approximately 30 cm deep). Total number required 10.
         2) Fine mesh netting (Mesh size small enough to prevent larvae from escaping)
         3) Plastic dippers (350 ml) for sampling larvae from the tubs
         4) Map of area
         5) Small, sealable containers for transport of test species
         6) Cooler or bin for transport of test species
         7) Permanent marker
         8) Prescribed personal protective gear, to include nitrile gloves
         9) Formulated larvicide
         10) Application equipment (if required)

11) Turkey baster or bulb pipette
12) Small plastic disposable pipettes for transferring larvae
13) White plastic or other trays for sorting larvae
14) Third instar lab reared larvae
15) Milligram balance for weighing larvicide doses.
16) Flasks and transfer pipettes if serial dilution is needed for dosing microcosms.

ii. Pre-Test Procedures

1) Make small drain holes of about 1cm diameter at the top of the plastic tubs just below the soil level and cover the hole with the mesh. This is to prevent larvae from escaping when there is a rain event
2) Line the plastic tubs with 1cm of soil from known larval habitat if possible and if soil is not available or if the test mosquitoes are from the *Culex* genus then add 0.5 grams of rabbit chow or hay to promote microorganism growth
3) Flood the tubs to the level of drain hole
4) Randomly allocate 5 plastic tubs for each formulation and rate as treatment tubs and 5 as control tubs
5) Cover all the tubs with non-insecticide treated mosquito netting or other netting that would prevent mosquitoes from colonizing the tubs
6) Leave the tubs undisturbed for 4 days

iii. Test System

1) Acquire laboratory-reared 3rd instar larvae of similar size and nutritional state and verify their identification as the target genus and species.
2) Ensure 50 3rd instar larvae are available for each replicate of each application rate, and for each control.
3) Immediately prior to the tests, transfer larvae and water from the rearing container into small, sealable containers for transport. Avoid direct sunlight on containers or excessive heat (>32ºC) if traveling in a covered vehicle. Place containers in an insulated cooler with lid if the above requirements cannot be achieved. Place data logger with probe in the cooler or vehicle to monitor temperature.
4) Label test system containers using a permanent marker. Each container will indicate test site, application rate, and test/control replicate number.

iv. Ensure Proper Application Rates

1) Application rates (dosages) to be tested will be based on previously conducted laboratory trials or on rate range as directed on the product label.

2) Use the surface area of the microcosm (Plastic tubs) to determine the amount of larvicide needed for each. Refer to manufacturer's recommendations.
   a. Note: 1 gram per square meter = 10kg/ha
   b. WG, WP, AS, and EC formulations should be applied diluted in aqueous suspension with sufficient water to evenly cover the microcosm with small drops from a plastic pipette.
3) Pre weigh the application amount needed in the lab for each microcosm, prepare aqueous suspensions if needed and label containers.

c. Test Procedure

i. Test System Placement

1) Add 50 3rd instar larvae from the transport container into each microcosm (Plastic Tub). Larvae number can be increased as necessary depending on size of microcosm (i.e., if larger than 1m diameter).
2) Allow the mosquito larvae to acclimate in habitat for three hours prior to larvicide application.
3) Measure and record water temperature and pH from the beginning of the acclimation period.

ii. Larvicide Application

1) Dress in prescribed personal protective gear, including gloves as recommended by manufacturer of product
2) Evenly dispense the amount of larvicide calculated over the entirety of each microcosm (Plastic Tub).
3) While treating, monitor for drift and adjust application as necessary. Do not apply into the wind but downwind.
   a. If applications are made close to the water surface and "raindrop" size drops are used for aqueous materials, drift should not be an issue.

d. Post Test Procedure

i. Monitor Mosquito Mortality and Emergence

1) Examine mosquito mortality and emergence post-treatment at 24 hours, 48 hours, and every 48 hours thereafter until 7 days.
2) Wear clean gloves while checking for mosquito mortality and emergence at each tub to avoid contamination.
3) In order to determine mortality and emergence use the plastic dipper to dip 5 times per plastic tub (4 opposing points and center). Transfer the contents of the dipper to the tray.

4) It will be necessary to allow the tubs to "rest" between dips so that diving larvae re-surface. This may be accomplished by taking one dip from the first point in each tub for a specific treatment, then returning for the second from that treatment and continuing in this manner until all five dips have been recorded for all tubs for the selected treatment.
5) Record time, cumulative number of live larvae and pupae, number of dead larvae and pupae at each evaluation time interval.
6) After counting, retain pupae for emergence monitoring and return all the contents to the same plastic tub from which it was collected.
    a. Live pupae should be retained in plastic or Styrofoam cups with lids and taken to the laboratory in a separate ice chest to monitor for %Emergence.
7) To avoid contamination between the tubs, use separate dipper, tray and pipette for each plastic tub
8) Measure and record water temperature and pH at each mortality and emergence evaluation time interval.

ii. Monitor Residual Activity

1) Weekly (7 days) after the first application, transfer 50 new larvae per plastic tub for transport as described in and transport them to the test sites and add them to the plastic tubs.
2) Monitor mortality and emergence as described above.
3) Continue adding larvae every week until total mortality including emergence inhibition drops below 80% for any given treatment/dose. Microcosms which received that treatment/dose should then be removed from the study. Infestation and monitoring should continue for microcosms which received treatments that continue to exhibit >80% total mortality.
4) Stop experiment when all treatment (those that received larvicide application) tubs have <80% mortality

4. Outdoor Microcosm- Natural Colonization

This evaluation is like the above-mentioned outdoor microcosm but instead of artificially stocking the tubs, microcosms are allowed to naturally colonize with mosquito larvae. Keep ethical considerations in mind when deciding where to conduct experiments with regard to naturally occurring vector-borne disease in the community.

a. Preparation

i. Equipment and Materials

1) Plastic tubs (0.5 – 1m in diameter, approximately 30 cm deep). Total number required 10 (minimum and dependent on test design).
2) Fine mesh netting (Mesh size small enough to prevent larvae from escaping)
3) Plastic dippers (350 ml) for sampling larvae from the tubs
4) Map of area
5) Small, sealable containers for transport of pupae to lab
6) Cooler or bin for transport of pupae to lab
7) Permanent marker
8) Prescribed personal protective gear, to include nitrile gloves
9) Formulated larvicide
10) Application equipment (if required)
11) Anemometer
12) Turkey baster or bulb pipette
13) Small plastic disposable pipettes for transferring larvae
14) White plastic or other trays for sorting larvae

ii. Pre-Test Procedures

1) Make small drain holes of about 1cm diameter at the top of the plastic tubs just below the soil level and cover the hole with the mesh. This is to prevent larvae from escaping when there is a rain event
2) Bury the plastic tubs at least 25 cm below soil surface
3) Make sure the drain holes or slightly above the soil surface
4) Line the plastic tubs with 1cm of soil from known larval habitat if possible and if soil is not available or if the test mosquitoes are from the *Culex* genus then add 0.5 grams of rabbit chow or hay to promote microorganism growth
5) Flood the tubs to the level of drain hole
6) Randomly allocate 5 plastic tubs to each formulation/dose as treatment tubs and 5 as control tubs
7) Allow natural oviposition by mosquitoes (approximately 8 days)
8) Prior to assignment of treatments, all tubs should be pre-sampled to determine relative population densities, larval stages, and species composition. If larval densities of target test species are not homogeneous prior to testing, a stack-ranking of the tubs should be created based on their larval densities. This stack-ranking should be used to assign treatments in a randomized complete block (RCB) design using density-based blocks in order to compare similar initial densities across all treatments. For example, if four treatments are being

compared with five replicates, block #1 would consist of the four most highly populated tubs and treatments would be randomized within that block. The four tubs with the next highest larval densities would become block #2 and likewise for the remaining 3 blocks.

iii. Ensure Proper Application Rates

1) Application rates (dosages) to be tested will be based on previously conducted laboratory trials or on rate range as directed on the product label.
2) Use the surface area of the microcosm (Plastic tubs) to determine the amount of larvicide needed for each. Refer to manufacturer's recommendations.
    a. Note: 1 gram per square meter = 10kg/ha
    b. WG, WP, AS, and EC formulations should be applied diluted in aqueous suspension with sufficient water to evenly cover the microcosm with small drops from a plastic pipette.
3) Pre weigh the application amount needed in the lab for each microcosm, prepare aqueous suspensions if needed and label containers.

b. Test Procedure

i. Larvicide Application

1) Dress in prescribed personal protective gear, including gloves as recommended by manufacturer of product.
2) Evenly dispense the amount of larvicide calculated over the entirety of each microcosm (Plastic Tub).
3) While treating, monitor for drift and adjust application as necessary. Do not apply into the wind but downwind.
    a. If applications are made close to the water surface and "raindrop" size drops are used for aqueous materials, drift should not be an issue.

c. Post Test Procedure

i. Monitor Mosquito Mortality and Emergence

1) Examine mosquito mortality and emergence post-treatment at 24 hours, 48 hours and every 7 days thereafter.
2) Wear clean gloves while checking for mosquito mortality and emergence at each tub to avoid contamination.
3) In order to determine mortality and emergence use the plastic dipper to dip 5 times per plastic tub (4 opposing points and center). Transfer the contents of the dipper to the tray.
4) It will be necessary to allow the tubs to "rest" between dips so that diving larvae re-surface. This may be accomplished by taking one dip from the first point in each tub for a specific treatment, then returning for the second from that treatment

and continuing in this manner until all five dips have been recorded for all tubs for the selected treatment.

5) Record time, cumulative number of live larvae and pupae, number of dead larvae and pupae at each evaluation time interval.
6) After counting, retain pupae for emergence monitoring and return all the contents to the same plastic tub from which it was collected.
    a. Live pupae should be retained in plastic or Styrofoam cups with lids and taken to the laboratory in a separate ice chest to monitor for % Emergence.
7) To avoid contamination between the tubs, use separate dipper, tray and pipette for each plastic tub
8) Measure and record water temperature and pH at each mortality and emergence evaluation time interval.
9) Continue adding larvae every week until total mortality including emergence inhibition drops below 80% for any given treatment/dose. Microcosms which received that treatment/dose should then be removed from the study. Infestation and monitoring should continue for microcosms which received treatments that continue to exhibit >80% total mortality.

5. Data Analysis

a. For each day, add the mean mortality data including emergence inhibition for all replicates of an application rate together to determine the percent mortality of the treatment and of the control groups.

b. Appropriate statistical tests should be applied to compare mortality means of each treatment to confirm separation of means and p values.

c. Abbott’s formula can be used to determine a corrected percent mortality:

$$\text{Corrected \%} = \left(\frac{\text{Mortality \% in treated group} - \text{Mortality \% in control group}}{100 - \text{Mortality \% in control group}} \right) * 100$$