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ANALYSIS OF SPECIALIZED PESTICIDE PROBLEMS

INVERTEBRATE CONTROL AGENTS - EFFICACY TEST METHODS

VOLUME VIII

MOSQUITOES, BLACK FLIES, MIDGES AND SAND FLIES

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REPORT TO THE

ENVIRONMENTAL PROTECTION AGENCY

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Report To The
Environmental Protection Agency

By The

American Institute of Biological Sciences
Arlington, Virginia 22209

EPA REVIEW NOTICE

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MOSQUITOES, BLACK FLIES, MIDGES AND SAND FLIES

Table of Contents

Table of Contents Continued

Table of Contents Continued

INTRODUCTION

This document presents test procedures for the assessment of the biological efficacy of pesticides against several groups of important insect pests which pose an economic and public health threat to man in urban, rural, agricultural and wilderness habitats. While these protocols do not cover all procedures and techniques employed to date, they, however, include the most widely used and accepted procedures by researchers in this field. Deviation from and modification of these procedures may be deemed desirable when required by specific conditions.

The insects for which evaluation procedures are outlined here, belong to four families: Culicidae (mosquitoes), Simuliidae (black flies), Ceratopogonidae (biting midges) and Chironomidae (nonbiting midges, including chaoborid midges). These insects inhabit a variety of ecological niches and show diverse biological, physiological and behavioral patterns. In the evaluation of any bioactive compounds, the unique features of each species must be taken into consideration.

The procedures and techniques outlined here are mere guidelines for securing meaningful information on the efficacy of pesticides for the control of pest and vector insects.

GENERAL CONSIDERATIONS

Members of the four families for which these protocols are developed, are mostly aquatic or semi-aquatic in their immature stages. The mature stage is terrestrial, dispersing beyond the immediate vicinity of its breeding source. Pesticides are evaluated against the immature (egg, larva, pupa), as well as the adult stage. In assessing the activity and efficacy of pesticides against these insects, the following factors should be considered and standardized. Deviations necessitated by test conditions are given in the separate sections of this document.

Biological Parameters

Species:--The insect should be identified to species whenever possible. If not, it should be determined at least to the generic level. Additional information as to subspecific level, strains, etc., should be noted.

Stage:--The susceptibility and response of living organisms to pesticides varies a great deal depending on the size, stage and instar of the insect. Methods and procedures utilized for assessment are dictated by the stage and habitat of the insect. Pesticides are usually evaluated against the larval and adult stages, while in certain situations, it will be necessary to gather data on the egg and pupal stages. In larval evaluations, the instar should be standardized and specified. Most laboratory tests in this referenced group employ fourth instars. In some situations, younger or older (if existing) instars are employed.

Age:--This factor plays an important role in influencing the performance of pesticides. The age of the test insects should be reported or an age range, if precise age is not known. In dealing with larvae, it is not essential to report the calendar age, if the larval instars are specified. Approximate age of adult test insects should be specified.

Sex:--Sex of the test insects should be specified. This is an important consideration in evaluation procedures against adults. It is difficult to determine the sex of the immature stages and generally this information is not needed.

Experimental Parameters

Experimental parameters should be developed to meet the needs of a specific situation. The procedures followed under laboratory and field conditions may be similar or quite different from each other. Some general guidelines for experimental design and data analysis are as follows:

Laboratory

Test procedures and conditions should be standardized in the laboratory, so that the results will be reproducible. In addition to specifying the biological parameters as discussed above, the following experimental conditions and techniques should be given due consideration and recorded:

Test Container:--The volume and size of the test container will vary according to the requirements of the species and the nature of the test. Guidelines are given under each group.

Contamination:--This is an important problem in the evaluation of pesticidal chemicals. Laboratories used for testing should be kept free of insecticidal contamination. Contamination is minimal where disposable equipment and containers are employed. If glass or other nondisposable items are used, they should be thoroughly washed, cleaned and decontaminated prior to reuse.

Test Solutions:--Solutions should be made in acetone, ethanol, or other appropriate organic solvents. Stock solutions should be sealed tightly and refrigerated to preclude degradation. If water is used as a solvent, diluent or carrier, fresh preparations should be made prior to each use.

Number of Organisms:--This will vary according to the species and availability of material for testing. In general, no less than 10 individuals should be employed per replicate. The most desirable number of test animals is 20-50 per unit.

Replications, Number of Tests and Dosages:--Each treatment (test concentration) preferably should be repeated on a minimum of three separate occasions, yielding a total of six replicates per treatment. Each compound should be evaluated at three to four discriminating dosages producing mortality in the range of 10-90 percent.

Physical Factors:--Temperature, relative humidity and photoperiod conditions should be standardized. These parameters will vary according to the species and test requirements. In general, insects during exposure and holding are subjected to normal daylight periods, 20-30°C temperature and 40-80% RH.

Duration of Exposure:--Varies according to species, age, stage and the mode of action of test compounds. For quick acting compounds, mortality is assessed 24 to 48 hours after exposure. In some tests, the insects are exposed for 15-60 minutes, then transferred to or held in untreated containers for 24-48 hours prior to reading of mortality. In the case of delayed acting substances, such as insect growth regulators, the test duration may involve the entire life stage or beyond.

Food Provision:--For short exposure periods, food is generally not required. However, for longer exposure or holding periods, food is provided

the test insects. In some situations, such as in the case of black fly larvae, food particles may be employed as a vehicle for the ingestion of stomach poisons.

Field

It is essential that bioactive compounds be evaluated against various species in a variety of biotopes. The following factors should be considered in an evaluation of pesticides under field conditions:

Sampling Techniques:--Various species and the different stages of the same species require specific sampling techniques. Applicable techniques for assessment of populations of test species or groups should be employed.

Plot Size:--This can vary from a few square meters to hundreds of hectares, depending on the objectives of the test, species and the type of control desired.

Experimental Design:--Each treatment and untreated check should be replicated at least twice. A minimum of three samples of the organisms should be taken per plot at each interval. Larger plots may require a greater number of samples. The treatments should be assigned to the plots at random, or in other manners as dictated by the test conditions.

Data Analysis

Data gathered in laboratory bioassay and field conditions should be anlayzed for significant differences. Standard statistical procedures and computer programs are available for expediting such an analysis (Busvine, 1957; WHO, 1970). For analysis and interpretation of the data, the following procedures should be followed:

In laboratory bioassay experiments, the extent of mortality in the checks will provide information on natural mortality. If check mortality is more than 20%, the tests should be discarded. If mortality in the checks is between 5-20%, mortality induced by the test compound should be corrected by Abbott's Formula:

$$\% \text{ corrected mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

Corrected mean mortalities may be plotted against log concentration on probit paper. The points for three or four discriminating concentrations of dosages showing activity are fitted with a straight line from which LC_{50} and LC_{90} values are read off. The data may also be treated and analyzed by computer and the confidence limits determined.

In the field, the data may be analyzed and interpreted in various ways. If no check plots are employed, then the extent of control is determined by comparing the mean values of posttreatment intervals with those obtained prior to treatment using Formula I:

$$\% \text{ Control of Inhibition of Emergence (EI)} = 100\left(\frac{T_2}{T_1}\right) 100 \text{ where:}$$

T_1 = mean number per sample prior to treatment

T_2 = mean number per sample after treatment

Where values for both treated and check plots for both pretreatment and posttreatment periods are available, the calculations for extend of control or inhibition of emergence are made by Mulla's Formula II (Mulla et al. 1971):

$$\% \text{ Control or Inhibition of Emergence (EI)} = 100-\left(\frac{C_1}{T_1} \times \frac{T_2}{C_2}\right) 100 \text{ where:}$$

C_1 = mean number per sample pretreatment in check

T_1 = mean number per sample pretreatment in treated

C_2 = mean number per sample posttreatment in check

T_2 = mean number per sample posttreatment in treated

If no counts are available for pretreatment periods, and both check and treated plot designs are utilized, then the extent of control and inhibition of emergence are calculated by Formula III (Mulla et al. 1975):

$$\% \text{ Control or Inhibition of Emergence (EI)} = 100\left(\frac{T}{C}\right)100 \text{ where:}$$

T = mean number per sample in treated

C = mean number per sample in check

Additional techniques and computer methodologies may be employed in the analysis of data as dictated by the condition of a given experiment. Detailed methodologies are outside the scope of this document.

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CULICIDAE (Mosquitoes)

Mosquitoes rank as the most important insect pests and vectors of human diseases. They are prevalent throughout the United Stated causing discomfort not only to humans but also to domestic animals. Mosquitoes, if not controlled, may cause substantial economic losses to real estate, tourism and agricultural industries. Mosquitoes are well known as the vectors of human and equine encephalides. In 1975, over 2,000 cases of human encephalitis occurred in over 30 states and 30 deaths resulted. Mosquitoes are vectors of dog heartworm which occurs throughout the United States. Furthermore, the mosquito species which transmit malaria, dengue and yellow fever are present in many of our states. The reintroduction of these diseases remains a possibility because of the extensive world wide travel of our citizenry.

There are at least 260 organized mosquito control districts in the United States. New districts are being formed continually as the need for improved mosquito control is recognized by state and local governments. In addition to the organized mosquito control efforts, many cities, towns, communities and private citizens exercise chemical control of mosquitoes when mosquito annoyance becomes severe or when the threat of disease transmission exists. Most of these agencies utilize three basic methods of mosquito control. These are physical control (breeding source reduction or elimination), biological control and chemical control. At the present time, emphasis is placed on chemical control strategies because physical control measures are not always environmentally or economically feasible and biological control measures are not technically feasible for most species of mosquitoes.

Mosquito control operations rely heavily on larviciding. Since immature mosquito populations are confined to breeding areas, less chemical is required than in adulticiding programs. However, adulticiding forms an important part of most mosquito control programs since it is not always feasible to achieve satisfactory control with larvicides and the other available methods.

Development of resistance to chemicals in use has brought about a need to develop new classes of chemicals that are effective mosquito control tools. Furthermore, there is a need for the development of new chemicals that are more specific, have greater efficacy and pose minimal or no risk to humans and the environment when applied as mosquito control measures.

Larvae

Vector and pestiferous mosquito larvae in the United States breed in a variety of habitats, ranging from stagnant ponds to vast expanses

of coastal salt marshes and flood plains of rivers to rainwater caught in tin cans, tree holes and the leaf axils of plants. The control of these mosquitoes is generally best accomplished by measures directed during this somewhat confining and limiting period of larval development as opposed to measures directed at the free-flying adult stage.

Laboratory Evaluation

Laboratory testing of conventional mosquito larvicides, i.e. compounds causing mortality within 24-48 hours as opposed to insect growth regulators (IGR's) which may act more slowly, frequently begins with the exposure of a given number of larvae to serial dilutions of candidate materials in a suitable container. Larvae should be of uniform instar and may be obtained from laboratory-reared colonies. If a laboratory population is not available, field collected specimens may be utilized. The larval source in the field must be sufficient to provide 20-25 larvae per each of two replicates per treatment, including an untreated check. Larvae for testing should be third or early fouth instars, but earlier instars and eggs may also be collected in the field and used in laboratory tests. Field collected material should be carefully handled and transported to the laboratory to minimize injury. Packaging into secured containers in a cool ice chest is desirable. The larvae should be allowed an adjustment period of about one hour upon arrival to the laboratory after transport from the field. Any larvae showing abnormalities, such as a fuzzy or discolored appearance due to the presence of parasites, should be discarded. Washing of larvae, if necessary, may be accomplished by transferring the larvae to clean water or by straining larvae and then rinsing under slowly running water. Lots of 20-25 larvae should be selected by pipet, strainer, or screen loop and distributed into small beakers or containers, each containing 25 ml of water, which are transferred to larger containers for bioassay.

Technical and Emulsifiable Formulations:--Place 225 ml water into appropriate containers 7.5-10 cm in diameter such that the depth of water is between 2.5 and 7.5 cm. Glass containers (jars, bowls or 500 ml beakers) may be used, but they must be cleaned and decontaminated after each use. Waxed paper cups are acceptable but must be discarded after each use. Distilled water, rainwater or tap water may be used. Tap water may cause problems in some areas because of the addition of disinfectants that may have deleterious effects on the larvae. Distilled water, obtained commercially, may also contain traces of poisonous heavy metals. Certain species, such as salt-marsh or tree-hole mosquitoes, may suffer on transfer to relatively pure water, an effect that will be reflected in high control mortalities. In some cases, water from the breeding site should be used, provided that it is free from insecticides and care is exercised to exclude detritus. For salt-marsh mosquitoes, add sufficient salt to the solution. The average temperature of the water should be recorded and should be approximately 25°C; it must not be below 20°C or above 30°C.

Stock solutions are prepared from technical or formulated supplies. A 1% stock solution should be prepared using acetone, ethanol, or other

appropriate solvent, with serial solutions prepared from this stock solution which is stored under refrigeration in a sealed glass vial. Test concentrations are prepared by pipetting 1 ml or less of the appropriate solution under the surface of the water into each of the containers. In preparing a series of concentrations, the most dilute should be pipetted first. Each concentration should be duplicated with duplicate controls included for a solvent check (1 ml of acetone or ethanol) and a standard insecticide, if possible. Within 15-30 minutes of preparation of the test concentrations, add the mosquito larvae by tipping the contents of the small beakers into the vessels (WHO 1975, Mount et al. 1971, Glancy et al. 1969). A variation of this techinque can be accomplished by adding 20-25 larvae directly into 100 ml of water in waxed paper cups. To obtain the desired concentrations, 1 ml or less of test solutions are added to each cup (Mulla et al. 1966).

Mortality counts are made after 24-48 hours exposure. The number of moribund and dead larvae should be combined. Dead larvae are those that cannot be induced to move when probed with a needle in the siphon or cervical region. Moribund larvae are those incapable of rising to the surface within a reasonable period of time or of showing the characteristic diving reaction when the water is disturbed. If more than 20 percent of the control larvae pupate or die, then the test should be discarded and repeated.

Each material should be tested on three separate occasions. Percent mortality should be corrected with Abbott's formula for each treatment dosage. A minimum of three discriminating dosages must fall between 10 and 90 percent mortality. The mortality for each concentration is plotted on probit log paper and a dosage response line fitted through the points. From this line or from a computer program, the LC_{50} and LC_{90} for each material can be determined as described under Data Analysis.

Granules:--Insecticides incorporated onto granular carriers are highly successful as larvicides against mosquitoes, particularly when the vegetative cover is too dense for penetration by sprays and dusts. However, granulated formulations of insecticides are complex in nature and require a special knowledge of the basic physico-chemical characteristics of each of the ingredients. The size and composition of the granule, the solvent used for impregnation and various extrinsic factors have been found to influence the release rate and effectiveness of candidate insecticides (LaBrecque et al. 1956, Mulla and Axelrod 1960a, 1960b, 1962). Different techniques of evaluation are required for granules than for emulsions and evaluation of the toxicant is also generally accompanied by evaluation of the various factors of the particular carrier.

The effectiveness of granular insecticides (less than 0.5% in concentration) can be determined by adding various quantities of the formulation to water, adding larvae and assessing mortality after 24-48 hours. Granules are dropped into quart jars containing 900 ml water. High concentrate granules cannot be tested in this manner unless diluted with the inert carrier. The concentration of the active ingredient should be 2X to 3X the LC_{90} level. Do not stir the water containing the

granules and add 20-25 third or early fourth instar larvae, without food (LaBrecque et al. 1956). The test should be conducted at a constant temperature, within the range of 20-30°C, since temperature markedly influences the rate and extent of release (Mulla and Axelrod 1960a). This test procedure may be modified by adding the test organism to the water after addition of granules at intervals of 24 hours or longer and assessing the mortality.

Diffusion of the toxicant can be determined by siphoning from the top, without disturbing the portion remaining in the bottom of the jar, portions to be jar tested. Twenty to 25 larvae should be exposed to each portion and mortality recorded after 24 hours or longer (LaBreque et al. 1956).

Effectiveness and release of toxicants from granules can be determined by the aliquot testing method. The rate of release of the toxicant is measured by withdrawing aliquots of treated water at various time intervals after addition of the granules. One gallon jars containing 3,500 ml of water are used in evaluating the rate of release of granular formulations (Mulla and Axelrod 1960a). Aspirate 10 ml of water each from the top and bottom portions (one inch above granules), combine and add varying volumes to 100 ml water in cups containing larvae to obtain theoretical concentrations equaling 1-5X the LC_{90} concentration of the active ingredient. Record larval mortality after 24 hours (Mulla and Axelrod 1960a, 1962). The % release for each formulation is calculated from the observed mortality of larvae at a given concentration in the cups. The amount of toxicant corresponding to the observed mortality is determined from standard dosage mortality lines established for the toxicant against larvae of that species. Knowing this concentration, the extent of release can be determined in the jar.

Larvae requiring a plant substrate for attachment, such as *Mansonia perturbans* (Walker), may be used in bioassay tests with granular insecticides. They may be collected from field sources and shaken from the roots into water. The clean plants should then be placed in battery jars, or other appropriate vessels, containing 2 liters of water. Add 20-25 fourth instar larvae and allow them to reattach. Add various amounts of granules to jars and record mortality at 24 hours or longer (Chapman 1955).

Briquettes:--Insecticidal briquettes are used as mosquito larvicides to obtain a longer lasting residual action than afforded by other formulations. Such residual action is important in specialized uses involving standing water in small containers such as cemetery urns, street catch basins and stagnant ponds and, in some cases, to dispense low levels of toxicant to flowing streams. Briquettes are made of plaster and sawdust, sand and cement, charcoal, polymers and various combinations of plaster of paris (Raley and Davis 1949, Elliott 1955, Barnes et al. 1967, Nelson et al. 1973).

The length of effectiveness of briquettes can be measured by periodically exposing briquettes to water and then bioassaying with mosquito larvae. Removing aliquots of the treated water after various exposure intervals to the briquettes and bioassay as mentioned above.

Petroleum Oils:--Petroleum hydrocarbon formulations have been used for a long time to control mosquito larvae. Although these oils are primarily larvicidal, most formulations also induce mortality in the eggs and pupae. The oils are applied to the surface of water where they form a thin film. Larvae and pupae coming to the surface for breathing receive toxic action.

The biological activity of petroleum hydrocarbons is influenced by the characteristics of the films. Uniform film, having long duration, generally results in greater mortality of immature mosquitoes coming in contact with the film. Films consisting of patches, lenses and globs do not result in good contact with the larvae, and therefore, show low biological activity (Hagstrum and Mulla 1968). Due to the unique feature of petroleum hydrocarbons, their biological assay is somewhat different from that of the other insecticides.

Techniques developed for evaluation of petroleum oils mostly use glass or enamel containers. Coated or plastic containers absorb oils and should not be used. Oil formulations have to be applied without any dilution which will affect film characteristics. Glass beakers holding 250 ml water with 20-25 third or early fourth instar mosquito larvae are treated by releasing droplets of oil from a micro-syringe over the water. The test containers are held for 24-48 hours at 20-30°C and mortality recorded (Micks et al. 1967, 1968).

Another variation of the above method is the use of shallow pie glass dishes. These dishes have a capacity of 1-liter, when filled to the lip, and thus provide greater surface area than beakers and simulate field conditions in terms of the evaporation pattern of the oils. Since the volume of water used here is greater than that in the beakers, 50 larvae may be employed per unit.

Petroleum oil formulations are evaluated against the pupae in exactly the same manner as against the larvae, while against eggs, a slight variation may be used. Assessment of ovicidal activity is determined by counting larvae (Micks et al. 1967), or assessing the number of hatched ova (with the operculum popped out) where larval counts are not possible (Mulla 1964, Mulla and Chaudhury 1968). The eggs are normally exposed for 48-96 hours before mortality is assessed. In order to assess the extent of hatch of eggs laid in an egg raft (laid by *Culex, Culiseta, Mansonia* species and others), the egg rafts, after desired exposure, are placed in 2 ml of 1% KOH in a screw cap vial for 12-24 hours. The volume of KOH is then brought to 10 ml with water, the vial capped and shaken vigorously for one minute. The KOH disintegrates the raft into individual eggs. For counting the hatchability, the egg suspension is streaked out on strips of filter paper, observed and counted under a dissecting microscope. Hatched eggs are easily distinguished from unhatched eggs by the operculum opened out. Eggs laid individually need not be treated in this manner (Mulla 1964).

Insect Growth Regulators and Antimetabolites;--The development of compounds which inhibit mosquito development has occurred in the recent past which have been called the "third generation pesticides" (Williams 1967). These synthetic compounds display juvenile hormone-like activity which is characterized by inhibition or prevention of emergence of adults by mimicking the biological activity of the natural insect hormones. The use of the term "juvenile hormone" originally was used to describe this group of compunds, but it is no longer appropriate because it may connote potentially undesirable interpretations, and because other compounds not so closely related to the natural juvenile hormone have shown antimetabolic effects on growth development. For the purpose of this discussion, both insect growth regulators (IGR's) and antimetabolites will be collectively termed IGR's.

The criteria for effectiveness of IGR's are different from those used for measuring larval and pupal response to conventional larvicides. In general, IGR's induce a response in stages beyond that treated. In evaluating the effects if IGR's, developmental events are monitored to the stage of the adult emergence. In some cases, it might be necessary to assess the longevity and fecundity of the resulting adult population. Criteria for "normal" adults, or completely separated pupal exuviae, must be established. Normal adults are those displaying no abnormalities in physical appearance or behavior.

Laboratory assays are conducted primarily in glass containers, but other types may be employed. Paper cups are inadequate because they deteriorate due to the length of the duration of the test. Place 200-250 ml of water into each container. Add 25-50 fourth instar larvae from standardized larval source. Add food in quantity and selection as determined for each test species. Pipet from stock solution the desired amount of test compound, not exceeding 1 ml of solution. Place test units at constant temperature 20-30°C, and relative humidity, ca. 40-80%. Mortality of each stage is assessed every two or three days and dead or moribund organisms are removed. Normal adult emergence is assessed by counting and removing completely separated pupal exuviae and collecting adults in emergence units. The overall effectiveness is determined by calculating the inhibition of adult emergence in the treatments by formula III as given under Data Analysis (Hsieh and Steelman 1974, Jakob 1972, Jakob and Schoof 1971, 1972, Mulla et al. 1974, Schaefer and Wilder, 1972). A variation of this technique involves the use of larger containers and the removal of pupae to cages for assessment of eclosion (Dame et al. 1976).

Field Evaluation

Liquid Formulations:--Compounds with proven laboratory effectiveness are tested out-of-doors in small replicated man-made or naturally occurring ponds. An important feature of such experimental ponds is knowing the volume of water for administering exact application rates.

Laboratory outdoor ponds have been constructed by digging holes 3 m long by 1.5 m wide and .75 m deep with sloping sides. The ponds are lined with a double layer of 8-mil polyethylene plastic film which laps over the sides. About 15 cm of soil and sod are placed over the film and 1,100 liters of water added. Manure and other organic materials are added. Water hyacinths are placed in the ponds to shade the water and keep it cool. The ponds are allowed several days to stabilize. Measurements are made of final length and width at predetermined levels to calculate surface area and pool volume. The ponds are filled to the desired depth the day before testing, and the water level is kept constant throughout the test (Bailey et al. 1970).

Other ponds are constructed in sizes of 5 X 5 X 0.3 m and 3.5 X 7 X 0.3 m with an automatic water replenishment device triggered by a float valve. These ponds have natural bottom, vegetation, and fauna, and support wild populations of three to four species of mosquitoes. For sampling sites, each pond is provided with five bundles of straw, one in each corner and one in the center, as a source of organic matter (Mulla and Darwazeh 1971). Organisms may also be sampled along the vegetated margins or from other portions of the ponds. Fifty-five gallon drums are also used for outdoor bioassay (Brooks and Schoof 1965).

The pond systems are employed to evaluate compounds against indigenous mosquito populations. Pre-treatment counts of larvae are made by dips in areas of highest larval concentration. This sampling procedure is biased and not random by design because larvae tend to clump and a true random sample would be less definitive. Samples from the margins of the pools or next to artificial sampling sites yield the least variable results (Mulla et al. 1975). Post-treatment samples should be collected at regular intervals and mortality calculated according to the appropriate formula given in the Data Analysis section.

At times, it may be necessary to evaluate the longevity of a larvicide by treating water in outdoor ponds and bioassaying it against laboratory populations. In this scheme, collect water samples prior to and at 2, 24 and 48 hours, or other intervals after treatment, return to the laboratory and strain through a fine mesh screen to remove detritus, naturally occurring mosquito larvae and other organisms if present. Place one sample into a container along with 25-50 early fourth instar larvae. Evaluate mortality as determined in laboratory tests (Dame et al. 1976).

Evaluation of mosquito larvicides at field scale level requires special considerations. Care should be exercised in calculating dosage levels. Sampling should be conducted by the same individual throughout the trial. The larger plot, the greater the number of samples required. Sample at the same time of day in the same general area. Sample those areas most likely to have the largest concentration of larvae, i.e., along margins of grassy shore, near organic accumulations or in the shade. Count only third and fourth instar larvae and pupae to avoid recently hatched eggs not exposed to toxicant.

Tests in irrigated pastures with naturally occurring larvae can be conducted in 1/32 acre, or longer, plots. Small dikes may be employed to separate the plots. Mix the required amount of toxicant with 1,000 ml of water and apply evenly with a hand sprayer. Ten to 15 dips per plot are taken prior to and 24 hours after treatment (Mulla et al. 1975, Mulla and Darwazeh 1975a). Effectiveness is determined by comparing mean number of larvae in posttreatment with those in pretreatment of the same plots (Formula I).

Plots may also be established in river bottoms or other breeding sources by diking and treating as above (Mulla et al. 1960). Rice fields with established levees can be divided into plots for trials. Treat each plot with the proper dosage of chemical with a hand sprayer. Assessment is made by comparing pre- and post-treatment counts of mosquitoes. Depending on the size of the plot, 20 samples per plot may be sufficient (Gahan et al. 1976). Effectiveness is determined by comparing larval counts in the post-treatment samples with that of the pre-treatment (Formula I).

Granules:--Evaluation of granular insecticides under small field plot trials can be accomplished as reported for liquid formulations. Pre-and post-treatment larval samples are taken from treated and untreated plots. Roadside ditches have been used as granular test sites. Ten to 20 foot plots are treated and 10 dipper samples taken pre-treatment and 24 hours post-treatment (LaBreque et al. 1956). Granular formulations may be evaluated against *Mansonia perturbans* (Walker). Determination of control is made by pre- and post-treatment collections of larvae. A standard quantity of plant material supporting larval populations is collected and quickly transferred to white pans one-half full of water. The plants are shaken into the water to dislodge the larvae for counting (Chapman 1955).

Briquettes:--The field effectiveness of briquettes is measured in the same manner as for liquid formulations, except that evaluation periods are extended. Treated ponds are assayed by placing 25 early fourth instar larvae into floating cages in the pond. The cages are made of 32-ounce waxed paper cups that have the bottoms replaced with a fine mesh screen. Fishing floats are attached for flotation. Assays can be made at regular intervals until the formulation ceases to give control (Bailey et al. 1970). Field water from the plots can also be bioassayed against larvae of known susceptibility in the laboratory.

Petroleum Oils:--The efficacy of oils can be assessed as reported under liquid formulations.

Insect Growth Regulators and Antimetabolites:--IGR's are field tested in man-made ponds or small naturally occurring pools and other breeding sources similar to those described for field testing conventional larvicides. When appropriate modifications as described for laboratory testing of IGR's are employed, which measure their delayed effects, these breeding sources can provide useful field trial information. It is necessary to follow a representative portion of the population through to emergence,

and this is accomplished by means of a floating cage. The cage affords constant exposure of the test organism to the treated water, and yet, permits removal of the test population for recording of developmental events. The cage consists of a 1-quart polystyrene cup fitted inside a styrofoam ring float. The container is covered with lid screen to permit ventilation. Holes are cut in the lower section of the cup and are covered with a fine mesh screen to allow water and food particles to pass through, but not the larvae (Mulla et al. 1974).

Ponds treated with IGR's at various rates are assayed at various intervals by isolating a portion of the natural populations of larvae in two or more floating cages per pond. Each floating cage contains 20 fourth or other instar larvae. Emergence and mortality are monitored every other day, or at other appropriate intervals and they may be provided with food. The percent inhibition of emergence based on the number of larvae corrected by the formula given in Data Analysis is then calculated (Mulla et al. 1974).

Effectiveness of treatments in ponds or other breeding sources can also be determined by collecting larval and pupal samples with a dipper and assessing the density of the various instars. This is especially needed for the assessment of compounds that induce mortality at the interstadial period or during molting. In these situations, disappearance or absence of the pupal stage provides a good criterion of effectiveness (Mulla et al. 1975). Water from treated ponds or other breeding sources can also be returned to the laboratory for bioassay. After filtering through organdy or cheesecloth (discard cloth after each use to avoid contamination), the filtrate is assayed using the standard laboratory techniques described for IGR's (Dame et al. 1976, Schaefer and Wilder, 1972). Field population in the treated and untreated water may be brought into the laboratory for assessment.

Natural field pools are utilized as testing units by dividing them by dikes into replicated plots. Emulsifiable concentrates, microencapsulated concentrates and wettable powder formulations are applied by mixing into 1,000 ml of water and applying by hand sprayer over the plot. Percent reduction of populations is determined by five dips per plot (1/32 acre plots) prior to and 24 and 48 hours after treatment. Percent emergence inhibition is determined by isolating 20 fourth instar larvae or pupae in floating cages as described above. Emergence of pupae may be assessed by placing 20 per 100 ml water in paper cups. The number of dead pupae and adults is recorded after completion of emergence. The % EI is determined as given in the Data Analysis section (Mulla and Darwazeh 1975).

Rice field mosquitoes can be assayed by dividing the field into small plots prior to flooding. Several days after flooding, a natural population is treated at various rates with candidate compounds. Following treatment, 20 larvae are collected from each plot and placed in floating cages, as described above, back into the same plots.

Observations for mortality and adult emergence are made 24, 48, and 72 hours post-treatment. Larvae may also be transported back to the laboratory for observation and assessment of mortality and emergence of adults (Steelman et al. 1975, Schaefer and Wilder 1972). When the test insects reach the pupal stage in the field, cubic foot cages (with one side removed) are placed over concentrations of pupae in the various tests and control plots to obtain adult emergence (Schaefer and Wilder 1972).

Adults

Adulticides are important tools used by most mosquito control districts since this method can be employed when other methods are either not feasible or have failed to provide satisfactory control. Adulticides are primarily applied as ground or aerial aerosols and sprays. In recent years, ultralow volume aerosols and sprays have become popular because of substantial economic and environmental advantages inherent with this method. Ground aerosols are useful in controlling adult mosquitoes in urban and suburban areas where road networks allow adequate coverage. Aerial applications are useful in large-scale adulticiding and in target areas inaccessible to ground equipment. Both aerosols and sprays are applied at relatively low dosages which do not provide residual activity. Thus, their effect is of a temporary nature and applications must be repeated as reinfestation is indicated by mosquito surveys.

Laboratory Evaluation

An important consideration in testing insecticides against adult mosquitoes is susceptibility variation among species and strains of the same species. New insecticides should be evaluated against a wide range of species and especially against the more important target species. Variations in the susceptibility of strains usually depend on their history of exposure to various classes of insecticides. Thus, the most useful data are those obtained by using either laboratory strains of known susceptibility (or resistance) or from field collected specimens. If field specimens are used, they must be collected from areas which have not received recent insecticide applications.

Test specimens may be collected as eggs, larvae or pupae and reared to adults using standard rearing techniques (AMCA 1970). Immature mosquitoes may be transported long distances if they are kept in insulated jugs to protect them from excessive cold or heat. They should be kept in their breeding water until transfer to appropriate rearing media in the laboratory. In the field, adult mosquitoes can be collected by aspiration or baited traps and transferred to the laboratory in insulated chests containing moist cotton and also canned ice if being transported during hot weather.

In general, adult female mosquitoes are less susceptible than males; therefore, the former are usually utilized in laboratory testing program since they represent the field problem. If both sexes are used, data for each sex should be recorded and analyzed separately for maximum statistical reliability.

The age of test specimens should be as uniform as practicable within a range of two to eight days since it does, to some extent, govern physiological condition. Other than age, the physiological condition of test specimens is controlled by using standard rearing techniques which require consistent handling, environment and nutrition.

There are two widely used test methods available that permit the rapid evaluation of large numbers of insecticides against adult mosquitoes with a standardized equipment, solvents and handling procedures. These are the filter paper residue method and the wind tunnel contact aerosol method. The topical application method has also been used for many years, but it has never gained popularity because it is relatively slow and tedious.

Filter Paper Residue Test:--The insecticides to be tested are dissolved in acetone (w/v) and pipetted (1 ml) onto Whatman No. 2 9-cm diameter filter paper placed horizontally on pin points. After five minutes of drying, a section ca. 5 cm wide at the widest point is trimmed off one side of the paper and the latter is rolled and placed inside a shell vial (2.1 X 8.4 cm) lining the sides almost completely. Approximately one hour later, the mosquitoes are anesthetized with cold or CO_2, counted into groups of 25 and transferred to the test vials. The vials are covered at the open end with cheesecloth and placed flat on their side for a one hour exposure period. After exposure, the mosquitoes are anesthetized and transferred into holding cages (0.47 liter unwaxed paper cups fitted with a net cover (Georghiou and Metcalf 1961, Georghiou and Gidden 1965).

Wind Tunnel Aerosol Test:--The wind tunnel test simulates field aerosol applications in the laboratory, thus it is a useful method of testing chemicals intended for use in aerosol programs.

Test chemicals are diluted in acetone or other suitable solvents and pipetted as small aliquots (0.25-0.5 ml) into a nozzle designed for total delivery into the wind tunnel tube. Tunnel air velocities are 3-4 mph to simulate natural wind currents. Adult female mosquitoes are exposed in 14-18 mesh screen wire cages in groups of 25. After exposure, the mosquitoes are anesthezied with CO_2 and transferred to clean holding cages for knockdown and mortality observations (Mount and Pierce 1975, Mount et al. 1976, Rathburn 1969, Boike and Rathburn 1975).

Topical Application Test:-- Application of diluted (usually in acetone) chemicals is made with a calibrated repeatable microsyringe. Volumes applied are usually 0.3-0.5 µl/mosquito. Specimens can be anesthetized with either CO_2 or cold during actual application. For consistent treatment, the droplet of insecticide should be applied to

the same body area (usually the mesonotum) each time. One refinement that is sometimes employed with this method is the coordination of dosage with mosquito weight. Adequate results can be obtained without using this additional step, especially when working with mosquito populations of uniform size and weight. After treatment, the mosquitoes are held in clean cages and provided a sucrose solution on cotton (Ludwick 1953, Georghiou and Metcalf 1961, Mount and Pierce 1975).

Since test specimens are treated individually with uniform dosages, the repeatability of results with this method is somewhat higher than with the two previously described methods. Past results indicate that reliable lethal doses may be obtained with an exposure of a minimum of 160 specimens per chemical. This total would be made up of four discriminating doses and 40 specimens per dose with 10 specimens per replicate.

In all procedures, mosquitoes that have been exposed to insecticides should be held in clean cages and supplied with 5-10% sugar water solution on cotton pads or wicks. These moistened pads will supply food, water and additional humidity to the mosquitoes.

Mortality observations should be made 24 hours or longer after exposure to the insecticides. Additional knockdown and mortality observations can be made at various intervals, if desired. Mosquitoes are counted as dead if unable to walk when the cage is jarred. The same criterion should be used in determining knockdown. Knockdown refers to short interval post-treatment effects (one hour or less). Quick knockdown is a desirable, but not essential, characteristic of mosquito adulticides. With some insecticides, mosquitoes may recover from the knockdown effect, therefore, the 24 hour mortality observation is considered essential.

Each laboratory test should include cages of mosquitoes not exposed to insecticides. These mosquitoes should be exposed to the insecticide solvents, the identical handling procedures and the same laboratory environment as those exposed to insecticides. The inclusion of mosquitoes not treated with insecticides in each test will reveal any mishandling, insecticide contamination or physiological weakness of the mosquitoes. When untreated mosquitoes show more than 20% mortality, the test should be discarded and repeated once the cause for excessive mortality has been resolved. Corrections for untreated mosquito mortality of 5-20% can be made by Abbott's Formula. Data from laboratory tests should be reported as lethal concentrations at the 50% and 90% levels (LC_{50} and LC_{90}) as described under Data Analysis.

The primary function of laboratory tests with insecticides against adult mosquitoes is to determine their relative toxicities. Therefore, in testing and in reporting data, a standard insecticide should be included for comparison.

Field Evaluation

Ground Applications:--Ground applications include ultralow volume aerosols and high volume thermal and cold aerosols. Regardless of the

application equipment used, the basic methods of evaluation are essentially the same.

Commercial aerosol generators should be maintained and operated according to recommendations of the manufacturer. Exceptions to this could be variance in atomization requirements for particular insecticide formulations. Testing with custom constructed generators should be preceded by research showing adequate atomization capabilities.

Aerosol generators should be calibrated for liquid flow rates at approximately the same ambient temperature at which actual applications will be made. This is especially critical when using generators equipped with flowmeters calibrated with a float. The float position can change dramatically with changes in viscosity as influenced by temperature. This problem is solved in actual practice by use of temperature correction data for each insecticide.

It is essential to test the actual insecticide formulation to be registered for use. This is especially critical with ultralow volume equipment since droplet size may change significantly with change in viscosity and surface tension.

When applying insecticides, it is important to monitor dispersal by the use of remote instruments and/or correlate actual insecticide discharge with application time. It is also important to either monitor vehicle speed or time each application with a stopwatch to insure accuracy and even coverage.

The efficacy of aerosols of insecticides is evaluated by exposing either caged or natural infestations of adult female mosquitoes downwind of the application. Caged mosquitoes can be from either a laboratory colony of known insecticide susceptibility or wild mosquitoes collected from target areas. Laboratory reared mosquitoes should be fairly uniform in age within a range of two to eight days old.

Tests with caged mosquitoes can be conducted over various types of terrain. Level, open terrain enchances even coverage and thus minimizes experimental variation. Extremely hilly or densely vegetated terrain is not suitable for this kind of testing. Tests in urban or suburban areas better simulate actual application conditions, but require somewhat greater sampling than tests in open areas.

Meteorological conditions needed for aerosol testing are usually found during twilight or at night when adult mosquitoes are active. Ideal test condiditons include ground winds of 1-10 kilometers per hour and a ground-based inversion. Good application conditions also exist during isothermals if winds are 5-10 kilometers per hour. These stronger winds tend to drift the aerosol across the plot before a great deal of vertical mixing takes place. With regard to temperatures, this type of testing should be done "in season", i.e., when mosquitoes are likely to be prevalent in target areas. This could range from 15-40°C, depending on latitude.

Mosquito exposure cages should be constructed of 14-18 mesh screen wire. Specimens are anesthetized with either CO_2 or cold for sexing and counting into cages. One type of exposure cage is 15 cm diameter X 2.5 cm deep (Rathburn and Boike 1975). These are covered with 14 X 18 mesh screen on both circular surfaces and hung vertically on poles with screen surfaces facing into the wind. With this type of cage, the exposed mosquitoes are returned to the laboratory, anesthetized and then transferred to clean holding cages.

A combination exposure-holding cage for aerosol tests is 4.5 cm diameter X 15 cm long and is constructed entirely of 16-mesh, galvanized screen wire (Mount et al. 1975). This exposure cage is threaded into one side of a plastic slide. A 4.5 cm diameter X 14 cm long plastic tube lined with clean paper is threaded into the opposite side for use as a holding cage. During exposure to the aerosols the holding tube is sealed with masking tape. Immediately after exposure, the tape is removed and the mosquitoes are gently blown from the screen wire cage to the holding cage through a hole in the movable slide separating the two cages. This type of cage has the advantage of immediate transfer of the mosquitoes to a clean holding cage. The need for transfer is to keep the mosquitoes from being confined to a treated screen wire surface. The object of this type of space treatment is to kill by direct contact rather than indirect contact on a treated surface.

After aerosol exposure, the mosquitoes should be returned to the laboratory for a holding period of 12-24 hours. Mortality counts should be made at the end of the holding period; additional knockdown and mortality counts can be made at any time desired. During the holding period the mosquitoes should have access to 5-10% sugar water solution on cotton pads placed on the screened tops of the holding cages. The sugar water provides food, water and added humidity to the mosquitoes. While in the field, mosquitoes should be maintained in clean insulated containers at ca. room temperature and provided moist cotton for humidity. Excessive heat and low humidity are very detrimental to adult mosquitoes.

Most aerosol applications are made with swaths of ca. 100 m (one city block). Small-scale tests with caged mosquitoes consist of one to three swaths of 300-400 m. Cages of mosquitoes should be exposed in two rows 50 m apart and at downwind distances of 45-50 m and 90-100 m to demonstrate swath coverage. A minimum of four cages of 24 mosquitoes of each species should be exposed in each test (Mount and Pierce 1972, Mount et al. 1975, Rathburn and Boike 1972, Taylor and Schoof, 1968).

Wide swath (up to 1,600 m) applications are preferred for ground aerosol applications against adult mosquitoes in the western United States. Testing methods for extended swaths are the same except that additional cages of mosquitoes and/or counting stations are needed. A minimum of two rows of cages at downwind intervals of 200 m are needed to adequately demonstrate complete swath coverage. Thus, 16 cages of mosquitoes would be needed to sample a 1,600 m swath (Gillies et al. 1972, Sjogren et al. 1973, Womeldorf et al. 1973).

Tests with natural infestations of mosquitoes are not usually feasible on a small scale (< 400 hectares). Exceptions to this can be made if short interval post-treatment evaluations are made (this can only be done with insecticides that produce rapid kill) or rapid reinfestation (overnight) will not occur. Overnight reinfestation is likely to take place in small plots with most species of mosquitoes.

Methods of determining the efficacy of insecticidal aerosols against natural infestations of mosquitoes include the use of caged wild mosquitoes and a variety of pre- and post-treatment sampling methods. These include landing and biting rates on humans, New Jersey light traps, CDC battery traps, CO_2 traps, animal bait traps, suction traps and truck traps. Landing counts on humans are most commonly used since they yield a direct indication of mosquito biting potential and can be made quickly, thus providing a means of short interval pre- and post-treatment sampling. A minimum of five counting stations should be established for each plot to be treated and untreated check plots. These stations should be arranged in one or more rows perpendicular to swath lines. Because of natural fluctuations in mosquito activity due to time of day or night, it is essential to count mosquitoes in the untreated check plots at ca. the same time as for the treated plots. In large-scale tests (400 hectares or more), a minimum of 10 counting stations should be used (Mount et al. 1972, Mount and Pierce 1974).

In small-scale tests, each insecticide dosage should be replicated at least three times and three or more discriminating doses are needed to establish the minimum effective dose. Minimum effective doses at the 90% or the 95% level should be based on either a probit analysis or an eye-fitted line on logarithmic probability graph paper.

Test series with new insecticides should include tests with a standard insecticide for comparison. The inclusion of a standard yields an additional check on mosquito sampling procedures and environmental conditions present during testing.

Furthermore, each test should contain a replication of untreated mosquitoes to reveal any mishandling or physiological weakness of the mosquitoes. Untreated mosquitoes should be exposed to the same handling and environment (both laboratory and field) as those treated with insecticides. Caged mosquito tests in which untreated mosquitoes exhibit more than 20% mortality should be discarded and repeated. Corrections for untreated mosquito mortality from 5-20% can be made by using Abbott's Formula.

In tests with natural infestations, untreated samples should be replicated equally with each insecticide treatment to reflect variance in mosquito density due to natural causes. The % control can then be determined by the use of Formula II.

Check plots may not be feasible in some large scale tests against wild mosquitoes. In these cases, the efficacy may be obtained by comparing pre- and post-treatment observations using Formula I given in

Data Analysis, provided pre- and post-treatment counts are made at the same time of the day or very close to each other in time.

Aerial Applications:--Aerial applications of aerosols include ultralow volume and diluted sprays. The test methods are the same for both types of application.

Aircraft nozzles must be maintained and operated according to recommendations of the manufacturer. Spray systems must be kept clean and in excellent operating condition throughout the tests. The spray system should be calibrated for insecticide formulation flow rates at ca. the same ambient temperature as anticipated during test applications.

It is essential to test the same insecticide formulation to be registered for use. This is especially critical with ultralow volume sprays since droplet size may change significantly with change in viscosity and surface tension of the formulation.

When applying insecticides by aircraft, it is essential to correlate insecticide volume with the area treated to determine the actual dosage applied. Aircraft velocity and altitude should be monitored during application. Aircraft flight lines (swaths) should be marked to insure even coverage. In large scale tests electronic guidance systems could be used to identify flight lines.

The efficacy of aerial sprays of insecticides is evaluated by exposing either caged of free-flying (natural infestations) adult female mosquitoes. Caged mosquitoes can be from either a laboratory colony or from natural infestations.

Aircraft tests may be conducted over various types of terrain. Efficacy will be higher in tests over level, open terrain than in uneven, densely vegetated terrain because of greater penetration and more even coverage. An attempt should be made to select test areas that either resemble or are within actual target areas.

The minimum test plot size should be 16 hectares for small fixed wing and rotary wing aircraft flying at relatively low altitudes (15-25 m). Tests with aircraft flying at an altitude of 46 m or more should be conducted on plots of at least 400 hectares.

Meteorological conditions needed for successful aircraft application are usually found during twilight hours when many species of mosquitoes are active. Ground winds should be less than 10 kilometers per hour to avoid excessive drift away from experimental plots. Ambient temperatures near ground level may range from 15-40°C, depending on latitude. Tests with caged mosquitoes should coincide with the mosquito season within a geographic area.

Mosquito cages and handling techniques used in aircraft spray testing are essentially the same as those given previously for ground aerosol testing. Cages of mosquitoes should be aligned perpendicular to flight lines and parallel with prevailing winds. One-half of the cages of mosquitoes should be exposed at or near ground level. The other half of the cages should be exposed at levels of 1-2 m above the ground. A minimum of eight cages of 25 mosquitoes each should be exposed for each replication. Laboratory reared mosquitoes used in these tests should be fairly uniform in age within a range of two to eight days old (Mount et al. 1970, Rathburn et al. 1969, 1971).

The most commonly used method of assaying aerial sprays has been the use of pre- and post-treatment landing counts in treated and untreated plots (Knapp and Gayle 1967, Knapp et al. 1976, Mount and Lofgren 1967, Mulla et al. 1973, Stevens and Stroud 1967). If small plots (16 ha) are used, the applications must be made during the early morning so that post-treatment counts can be made before possible nighttime reinfestation takes place. A minimum of five counting stations should be established on small plots. These stations should be aligned in one or two rows perpendicular to flightlines. Ten or more stations are needed on large plots (440 ha or more) for adequate sampling. Post-treatment counts should be made at the same time of day as pre-treatment counts since landing rates vary with the time of day. These counts should be made at 1-12 hours post-treatment with additional counts at 24 hours or longer made as needed to indicate duration of control. Other sampling methods that can be used in area-wide evaluations include New Jersey light traps, CDC battery traps, CO_2 traps, animal bait traps, suction traps and truck traps.

Each insecticide dosage should be replicated at least two times and three or more discriminating doses are needed to establish a minimum effective dose. At least five samples should be taken per plot. Minimum effective dosages at the 90% or the 95% level should be based on either a probit analysis or an eye-fitted line on logarithmic probability graph paper as given in Data Analysis.

Tests in which untreated mosquitoes exhibit more than 20% mortality should be discarded and repeated. Corrections for untreated mosquito mortality of 5-20% can be made by using Abbott's Formula.

In tests with natural infestations, untreated plots should be replicated equally with each insecticide treatment to reflect variance in mosquito density due to natural causes. In each plot, a minimum of five samples should be taken. The % control can be determined by the use of Formula II.

Check plots may not be feasible in some large scale tests against wild mosquitoes. In these cases, the efficacy may be obtained by comparing pre- and post-treatment observations using Formula I.

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SIMULIIDAE (Black Flies)

Black flies are important vectors of disease and bloodsucking pests of man and livestock in many parts of the world. Although they are not known to carry human disease in the United States, they are at times abundant and annoying, especially in the forested mountainous regions where the immature stages breed in clean, unpolluted streams. They are occasionally pests near large rivers such as the Potomac in Virginia and, before extensive pollution, in states bordering the Mississippi River. In some areas they are serious pests of turkeys and ducks, interfering with commercial production of these birds because they transmit lethal or debilitating protozoan parasites. They may also reduce milk or meat production of livestock or egg production of hens when they are abundant (Jamnback 1973). The construction of extensive networks of irrigation channels and dams in recent years in the western United States has provided new breeding sites for nuisance species (Mulla and Lacey 1975). The decreasing pollution of many United States rivers also appears to be favoring a resurgence in populations of some species of annoying black flies (Sleeper 1975).

Black flies breed only in moving water of rivers, streams, brooks, irrigation channels, etc. The moving water automatically distributes and transports the insecticide. Under these conditions, many factors influence the effectiveness of a given insecticide in addition to its inherent toxicity. These include the speed of the current, turbulence of the stream, duration of exposure to the toxicant, specific gravity, solubility and irritant effect (as it influences larval feeding) of the toxicant (WHO 1968, 1973).

Black fly larvae are more susceptible to most insecticides than the eggs or pupae. They are also restricted to running water, and so are far less widely distributed than the adults. For this reason, most control programs are directed against the larvae.

Larvae

Laboratory Evaluation

It should be noted that black flies have not been adapted to laboratory culture and so studies of their susceptibility to chemicals are hampered by the need for using wild larvae with all their inherent variablility. Laboratory tests, depending on their nature, may evaluate the inherent toxicity of a pesticide or the effectiveness of a given formulation. There are 4 basic techniques: jar, cloth bag, flushing and draining, and the trough techniques.

Jar Technique:--The jar test is especially adapted for testing the inherent toxicity of a chemical in an acetone or alcohol solution. It is less useful for testing formulations which may settle or rise in the exposure jar. Clumps of grass or stones with black fly larvae attached can be collected from streams. These can be transported between plastic sheets or in plastic bags. The larvae must be kept moist, but they survive poorly if transported in jars or bags partially filled with water. If they are to be transported long distances in hot weather, they should be kept in a portable cooler. Although larvae will survive for several days, if kept cool in a refrigerator, freshly collected specimens should be used for testing. Alternatively, field collected egg masses may be brought into the laboratory for hatching and subsequent testing of the larvae.

On arrival in the laboratory, the material collected in the field is immersed in water in large containers. Samples of the larvae are gently picked up with forceps, eye dropper, brush or syringe, and transferred to the test jars. It is advisable to aerate the large containers by directing a jet of air against the lower part of the inside wall. The larvae will migrate to this zone of maximum aeration, thus permitting rapid collection. Medium to large size larvae are required, but not those of the last stage with dark gill-spots, which are liable to pupate during the test. Place approximately 250 ml of clean water, preferably obtained from the breeding place, into each of 12 cylindrical glass jars or beakers about 15 cm high and 5 cm in diameter. The water in each jar is aerated by a steady stream of compressed air directed against the bottom of the jar through a glass tube, drawn out into a fine point. A standard sample of 25-50 larvae is placed in each test jar. The larvae become attached to the walls of the jar and to the aeration tube near its tip. After about 30-60 minutes, any detached and damaged larvae are removed with a pipet.

The insecticide suspensions are prepared by pipetting the required quantity of standard solution into jars for a total volume of 250 ml of solution. The control should be prepared by the addition of 1 ml of ethanol or acetone with 249 ml of water. There should be two replicates of each concentration and two for the control.

When the insecticide suspensions have been prepared, aeration of the test jars is stopped, and the water is poured out and immediately replaced by the insecticide suspension. The aeration tubes are left in the test jars during the exposure period (although aeration is not carried out) since larvae have usually settled on them. After a 30-minute or other appropriate exposure period, each test solution is poured into a different tray, and the jars are rinsed and refilled with water as before. Detached larvae are collected from the trays and returned to the jars, which are aerated again throughout a 24-hour recovery period. At the end of the 24 hours, the mortality is recorded. Larvae are considered dead if they do not move when probed with a needle. Moribund larvae may show discoloration, unnatural positions, tremors or inability to stay attached. Each dosage should be run in duplicates along with duplicate controls on 3 different occasions. The LC_{50}'s and LC_{90}'s are calculated as given under Data Analysis.

All larvae that have pupated during the test are discarded. If more than 10% of the control larvae have pupated, or if control mortality is 20% or more, the test should be discarded.

The living and dead larvae exposed to each insecticide concentration should be preserved separately in 70% alcohol. After they have been identified by microscopic examination, the mortality for each species is recorded. The susceptibility level of any one species is regarded as adequately tested when 100 or more larvae have been exposed to each concentration (WHO 1975).

Cloth Bags Technique:--A modification of the jar test involves the use of cloth bags. About 30 larvae are transferred individually with a broad-tipped medicine dropper from a holding pan to a white organdy cloth bag. Only uninjured normal larvae are selected. The bag containing larvae is then closed and fastened at the top with a wire pipe cleaner. The bags are immersed for 20 minutes or other appropriate period in jars containing 3,000 ml of nonchlorinated, nonaerated water to which an appropriate amount of pesticide in acetone solution has been added and mixed just prior to the test. Acetone is added to the check jar in a volume equal to the greatest amount of acetone solution added to any of the treated jars. Alternatively, small disposable paper cups may be used (Jamnback and West 1970). After 20 minutes, or longer period if necessary, the bags are rinsed in fresh water and transferred to 3,000 ml of untreated water. The mortalities are determined after a 24-hour holding period in the aerated water. A minimum of two replicates of each of three to five discriminating concentrations of insecticide are run and the average are used in determining the dosage-response line and the LC_{50} and LC_{90} with confidence limits.

Laboratory tests with insect growth regulators can be carried out in the same way as for conventional larvicides, except that after exposure, larvae are reared to adults with larval, pupal and adult mortalities or abnormalities recorded. Troughs may be particularly useful for IGR's because the larvae survive for long periods with less mortality than in jars. Testing to date has been confined to jars and long exposure periods of 24 hours or more (Dove and McKague 1975).

Flushing and Draining Technique:--This technique employs features of both the jar technique and to some extent that of the trough technique. The use of this technique approximates field conditions much more so than the jar technique (Mulla and Lacey 1976). This system can be used for rearing of *Simulium* larvae, as well as bioassay of short-term and delayed acting larvicides.

The test units consist of glass tubing (10 cm diameter) connected to pyrex glass funnels. The capacity of the unit approximates 1 liter. These glass units are provided with inflow arms (allowing water flow from a water line or reservoir) and outflow arms situated about 2-5 mm below the level of the inflow orifice on the opposite side. An air stone is affixed into the unit 10-15 mm above the edge of the funnel. Stem of the air stone is connected to air coming from an air pump or compressed air.

An important feature of these units is drainage facilitated through the stem of the funnel pointed downward. A rubber tubing, with a hose-cock clamp fitted over the funnel stem, can regulate the amount of water drained out. When not draining, the clamp can be tightened to shut the water off from draining.

If glass tubing is not available, the test units can be made from pint-size polystyrene cups. The bottom of the cups is removed, the narrow end glued to polyethylene funnel using silicone caulking compound. The overflow, inflow, aeration and drainage tubing are incorporated into the units as described for the glass units.

Water current in the units is provided with air passed through an air stone. The rearing chambers are placed in batteries of 4-6 units in wooden racks in a room maintained at 20-25°C.

For evaluation of quick and slow acting larvicides, 20-30 larvae of a given instar are placed in each unit, provided with 35 mg of food (2% aqueous suspension of ground up lab chow and brewer's yeast 3:1). After 4-24 hours of equilibration, dead and diseased larvae and pupae are removed. The surviving larvae are counted, the units are flushed with excess water for 5 minutes at the rate of 1 liter/min., in such a way that water is drained both from the overflows orifice and the drain stem of the funnel. After flushing and draining (larvae are never exposed out of water in this process), the larvae are provided with 35 mg of food. One hour later, the units are treated with various concentrations or different materials. After appropriate exposure period (60 minutes for quick acting larvicides, longer periods for slow acting agents), the active agents are eliminated from the units by flushing with excess water (3 liter/min) for 5 minutes. After flushing, the larvae are provided with 35 mg of food. Once or twice daily, the units are flushed and drained (1 liter/min) for 5 minutes and provided with food. Flushing and draining eliminates pollutants and toxic metabolites which induce mortality in blackfly larvae. It should be pointed out that in the flushing and draining process, the units remain full all the time and the larvae do not get exposed out of water (Mulla and Lacey 1976).

Mortality is assessed either 24 or 48 hours after treatment in the case of quick acting compounds. For the evaluation of *Bacillus thuringiensis* and insect growth regulators and antimetabolites, the mortality or level of emergence may be assessed 1-2 weeks after treatment.

The dosage response line, using 3-4 discriminating dosages, is established and the LC_{50} and LC_{90} levels are determined. If check mortality is more than 20%, the test should be discarded. For check mortalities greater than 5%, data should be corrected with Abbott's Formula.

Trough Technique:--Trough tests are useful in evaluating the effectiveness of formulations particularly in shallow water, but require modification for testing effectiveness in deeper water. Because the troughs are short, settling is minimal and so "carry", i.e., the distance downstream that an insecticide remains effective is not evaluated.

Sticks, stones, leaves, etc. with attached larvae are collected as outlined above and placed in a trough, typically 1 m long, 0.5 m wide and 15 cm deep, through which running unchlorinated water is flowing. The larvae migrate to a lip at the downstream end of the trough about 15 cm wide and 10 cm long. Water flows (21 cm/sec.) over this lip in a narrow sheet 0.5-0.8 cm deep through which the reactions of individual larvae to insecticide can be closely monitored. Known concentrations of larvicide are poured or dripped into the upper end of the troughs over standardized exposure periods. Larval behavior and detachment rates are recorded. Large numbers of formulations can be subjected to preliminary screening, sufficient tests and replicates can be carried out to establish accurate 50% detachment levels using procedures commonly applied to mosquito larvicide testing (Jamnback and Frempong-Boadu 1966, Frempong-Boadu 1966).

A few larvicides have a knockdown effect that is followed by subsequent recovery. These can be detected by using two troughs in a series with the lower trough (downstream) initially without larvae and determining whether larvae that are dislodged from the upper trough detached and carried to the second trough can recover and reestablish themselves. Alternatively, detached larvae can be captured in a cloth bag or screen and held for 24 hours in aerated water to determine whether or not they recover. There are also a number of variations of the trough test technique.

Larvae can be tested in sloping v-shaped gutters supplied with water by a pump or gravity. These are typically about 2 m long and 4 cm deep, made of sheet metal and are v or u shaped. The rate of flow can be varied by altering the angle of the gutters. The gutter is lined with a sheet of polyethylene film. This is covered on top with brown wrapping paper, a good attachment material for larvae. The polyethylene and paper are disposable. Water swirls through funnels into the upper end of the gutter, insecticide is added by dripping into the funnels where it thoroughly mixes with the water. Larvae that detach are captured in a screen at the bottom of the trough and held in a bag in a nearby stream for 24-48 hours mortality counts. Larvae that do not detach are dislodged and collected in nylon sieves which can be folded to form bags and held in a stream for 24-48 hours evaluation (Wilton and Travis 1965).

Field Evaluation

Single Stream Evaluations:--Promising larvicides can be tested in single stream field tests. The formulation is poured directly into the stream, applied by dispenser or sprayed over the water. In all cases, application into a turbulent portion of the stream increases the likelihood that it will mix well and become effective at, or slightly below, the point of application. Emulsions are widely used because of the ease with which they can be dispersed through the water, wettable powders disperse well, but tend to settle quickly and so have little "carry"; solutions must be sprayed as fine droplets into streams for maximum effectiveness, either by hand or power sprayer from the ground or from

aircraft. The cross-section and rate of flow of the stream must be determined to calculate the concentration in parts per million per unit time. It should be noted that swiftly flowing turbulent streams may require higher concentrations than those required for equivalent control in slow streams with stretches of slack water. In many cases not enough larvicide will traverse a large pool to remain effective at the pool outlet even at high concentrations (WHO 1968,1973).

Ground application is typically used when there are one or a few accessible streams to be treated and aerial application when there are many and/or inaccessible streams which require treatment. With the ground application technique there is an initial overdose but the gradually diminishing concentration remains effective for many miles below the single point of application.

Effectiveness can be evaluated by comparing larval populations above and below the treatment point not more than one day before and one day after treatment. Longer periods allow larval drift, egg hatching and pupation of black fly larvae to obscure the data. Samples should be taken at various distances below the point of treatment to determine how far downstream below the point of application the toxicant becomes effective and beyond that point how far downstream it remains effective (Jamnback 1969). Effectiveness is evaluated by larval counts, either on a standardized number of sticks, stones, trailing grass, etc. or preferably on standardized attachment units, e.g., plastic tags, strips, cones or ceramic tiles that are placed in suitable black fly attachment sites in the stream several days prior to the pre-treatment evaluation (Lewis and Bennett 1974).

Additional experimental single stream tests should be carried out in a variety of stream sizes and types, e.g., rivers, creeks and even small temporary brooks, both slow and fast, turbulent and less turbulent, with and without pools, depending on the breeding habits of the black fly species under attack. In all cases, streams with relatively high and uniform populations should be used as this simplifies evaluation of effectiveness, especially "carry". If possible, a variety of formulations should be tested to determine those which are the most effective.

Adults

Laboratory Evaluation

Black fly adults typically have high mortality rates in captivity and have not been tested for insecticide susceptibility although a satisfactory method can undoubtedly be developed using a modified WHO susceptibility testing kit for mosquitoes. At present, systematic research on adult control of black flies is a much neglected field, however, there have been a number of unpublished observations that some nonpersistent insecticides applied by aircraft as sprays or from truck-mounted foggers may provide short term protection from black fly attack, but these have not been systematically evaluated.

Effectiveness of large scale adult control operations and area-wide larvae control operations can be evaluated by population estimates inside and outside of the control area before and after treatment. Landing rates can be made using an 18-inch square piece of dark blue flannel spread on the ground beside the observer (Davies 1951). Where black fly populations are high, a one-minute count beginning after a five-minute waiting period is satisfactory. Alternatively, 10 directed continuous standardized sweeps about the head of the observer after a five-minute wait will provide a useful index to the annoyance rate. Counts should be taken simultaneously by observers outside and inside margins of the treated plot. Records of air temperature, wind velocity and direction, and light intensity should be taken. Low temperatures below about 12°C and wind velocity above 10 kilometers greatly reduce black fly activity. After dusk black fly attack rates decrease quickly. They do not attack man at night. Light traps, sticky traps, CO_2 traps and silhouette traps may catch black flies, sometimes in large numbers, but require far more work and cost more than the landing rate or net methods and have the disadvantage that nonnuisance species are collected (sometimes in large numbers), as well as those that attack animals.

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CHIRONOMIDAE AND CHAOBORIDAE (Midges)

Aquatic midges include species of the family Chironomidae and Chaoboridae. The chironomids form a large group of insects, the immature stages are mostly aquatic and provide an ample source of food for aquatic vertebrates and invertebrates. They breed in lakes, reservoirs, ponds, streams, ditches and rivers. The chaoborids form a small group of insects, their immature stages are also aquatic, they feed on zooplankton and in turn are used as food by many aquatic organisms.

The aquatic midges have become a serious nuisance problem in some urban, resort and industrial communities. They pose a serious economic and nuisance problem in resorts, newer sub-divisions encroaching on eutrophic bodies of water (Grodhause 1963, Mulla 1974). Their abatement in some communities is an essential part of environmental health programs. Control of larvae in the aquatic habitat is the most commonly used practice. Control of adults resting in or on vegetation and structures is also practiced in some communities.

CHIRONOMID MIDGES

Larvae

Larvicides include those compunds which act quickly on the larvae, producing mortality at practical concentrations or dosages in 24-48 hours. In contrast, IGR's, materials which induce delayed mortality or disrupt the developmental traits of the larvae, are slow acting materials. Their evaluation requires special and different techniques which will be discussed later.

Laboratory Evaluation

Conventional Larvicides:--Biologically active compounds can be evaluated against chironomid midges as technical or formulated materials. The technical material should be dissolved in acetone or ethyl alcohol as 1% solution and further serial dilutions should be made in the same solvent if necessary. Formulated materials such as WP (wettable power), EC (emulsifiable concentrate) or flowable concentrate (FC) formulation should be diluted with water or other appropriate diluents. Solutions of toxicants in organic solvents should be kept refrigerated when not in use. Preparation of formulated materials in water should be made fresh.

Larvae of uniform size (third or early fourth stage) should be obtained from field populations or laboratory colonies. Field larvae may be obtained by taking bottom mud samples using an Ekman dredge or another type of scoop. The sample is stirred in water, sieved through

a 50-mesh or another appropriate sieve to remove most of the mud and detritus. The larvae will remain on the top of the screen. The larvae are washed with water from the screens into cups, placed in cool ice chest and brought into the laboratory. Care should be taken to avoid direct contact between larval containers and the ice in the ice chest (Ali and Mulla 1976, Anderson et al. 1964, Mulla et al. 1973).

In the laboratory, the sample is transferred to white enamel pans or other appropriate containers. Larvae of the desired stage and species are picked up by a screen loop, tweezers, aspirator, eye dropper or pipet and placed in the test units. They may also be recovered by flotation using saturated aqueous solution of Epsom salt (see below).

Several types of containers may be employed as test units. For ease, disposable treated paper cups (ca. 100 ml) are normally employed, as these are discarded after each test. Custard cups, ointment jars, beakers and other glass containers may be used, but these have to be thoroughly washed and decontaminated after each use.

The test units are provided with 100-200 ml (depending on the size of the unit) of tap or field water. It is essential that 5-10 g of dry standard masonry sand or other fine sand from a stream, river or sand dune is added to each unit. Most chironomid larvae are tube builders and the sand will provide the necessary material for tube building. Provision of sand also reduces cannibalism in the tube-building species (Mulla and Khasawinah 1969). Twenty uniform size larvae are transferred to each unit. The units are then treated within 1-2 hours after addition of larvae with the desired concentrations of the toxicants. Several concentrations of each toxicant should be evaluated for establishing dosage response line. Each concentration should be replicated at least two times and each treatment should be run on at least three different occasions, yielding a total of six replicates per concentration. Check units receiving the highest amount of the solvent or diluent should be run along with each test. The test units should be kept at temperatures approximating those to which they are accustomed. Increased of 5-6°C over the normal temperature can be tolerated by many species.

Mortality should be assessed 24 hours after treatment. For this short period, it is not necessary to feed the larvae. The average mortality after correction (if needed) for each concentration is calculated as given under Data Analysis.

Care must be exercised in distinguishing dead and moribund larvae from living larvae (Mulla and Khasawinah 1969). Some of the criteria employed in distinguishing dead and moribund larvae are:

1. Lack of movement when touched with a dissecting needle.

2. Moribund larvae, although showing some indication of movement, are unable to return to their natural position. They usually are curved and unable to make undulating movements.

3. In pigmented red larvae, some areas become deeply colored, while others develop light colored areas, thus showing blotchy appearance.

Insect Growth Regulators and Antimetabolites:--Insect growth regulators and antimetabolites, unlike standard larvicides, act slowly and induce delayed response in the treated populations. In the case of some of these compounds, the effects may not be realized until the treated larval population has either pupated or is undergoing emergence. Therefore, the standard larval bioassay techniques will not provide the desired information. The assessment techniques discussed below will be applicable to all bioactive compounds inducing delayed response in the population. In most assessment procedures, the development of the treated or untreated population is scrutinized to the time of the emergence of the test individuals.

Technical or formulated materials may be evaluated. The technical material is dissolved in acetone or ethyl alcohol, making a 1% solution. Formulated materials are diluted with water. Serial dilutions are prepared as needed. The stock solutions should be stored in a refrigerator when not in use. In no case more than 1 ml of the solution should be added to the test units.

Since testing of IGR's and similar compounds takes longer (5-10 days) than that of standard larvicides, only glass containers should be used for testing. Half-pint wide-mouth Mason jars or glass custard cups are ideal units. The units are filled with 200 ml water (tap or field), provided with about 5-20 g of dry masonry quartz sand or fine sand obtained from a stream, river or sand dune. Twenty third to fourth instar larvae from a laboratory colony or field collected material are transferred to the units by means of screen loops, eye droppers or syringe. The larvae are allowed to burrow into the substrate. The units are aerated by forcing air (50-60 ml/min.) through a hypodermic needle in each unit. The units are provided with food prior to treatment and then once every other day. Tetramin® fish food has been found satisfactory. Many other types of high protein food may be substituted. The food is suspended in water and about 1 ml of the food suspension containing 50 mg of the dry food is added to each unit each time. Each treatment, including check, is replicated at least two times and the tests are repeated on three different occasions (Mulla et al. 1974) yielding six replicates.

The larval units are then treated with the dilute preparations of the test materials. The units are kept at temperatures approximating, or somewhat higher than that of normal populations and followed until most of the population has emerged in the check units. Emerging adults are collected in small plastic cups provided with a screen patch to allow for ventilation. They are counted and removed daily or every other day. Only adults under-going complete eclosion are counted as going through normal emergence. In tests where emergence is less than 50% in the check units, the test results should be discarded. In this manner, the inhibition of emergence in the treated and check units can be assessed.

The inhibition of emergence for a given treatment is calculated by the procedure presented under Data Analysis.

Field Evaluation

Chironomid midges causing nuisance problems in urban situations propagate in ponds, reservoirs, residential-recreational lakes, store drains, flood control channels, sewage disposal systems and other water-carrying structures. Some of the midges are benthic where the larvae reside in tubes constructed from detritus, sand and other particles, or they swim freely in the bottom ooze. Larvae of some species attach to plants and other submerged substrates. Therefore, different sampling techniques have to be employed for these larvae, depending on where they are found.

Conventional Larvicides:--For field evaluation, chemical control agents may be employed as liquid or solid formulations. The liquid and wettable powder formulations are diluted with water and sprayed onto the water surface with hand or power sprayers. Each treatment should be replicated at least two times (if possible) and at least three to four discrete larval samples taken from each treatment. At the same time, check plots should be run along with the treated plots. If it is not possible to run check plots, then the whole body of water should be treated as one plot.

Benthic larvae are sampled by means of an Ekman grab or dredge (15 x 15 cm) from the deeper portions of water or by a scoop that can be operated from the shoreline. The mud samples are stirred in water and sieved through a 50-mesh or other appropriate sieve. The residue on the screen is transferred with water to pint cups with lids and placed in a cool ice chest for transport to the lab. The cups containing larvae should not come in direct contact with ice in the chest (Mulla et al. 1971, 1973). The larvae are floated by adding 100-200 ml of saturated aqueous solution of Epsom salt (see below).

Free-swimming and drifting larvae (in flowing water) are sampled by dipping (Norland and Mulla 1975) or tow nets provided with a collection vial. The dipping samples are taken close to vegetation where larvae abound.

In flowing water, the sampling technique consists of placing tubes or cloth bags which are colonized by midge larvae, and these are retrieved when needed for larval assessment (Polls et al. 1976). After concentrating, these larvae are transferred to cups and transported to the laboratory in the manner described above. Surber samplers or modified Surber samplers have also been employed in sampling larvae in shallow (less than 50 cm deep) flowing water (Ali et al. 1976). The frame of the sampler is placed on the substrate which is scraped and stirred in the flowing water. The larvae and substrate are collected into a fine-mesh

net, from where it is transferred into cups, transported to the laboratory. The larvae are floated and counted in the manner described above.

The larval samples, after transport to the laboratory, are placed at room temperature for one hour. The larvae are then floated by adding 100-200 ml of saturated aqueous solution of Epsom salt ($MgSO_4$). The larvae are counted under a magnifying lamp. They should be identified to the genus or species, whichever identification is convenient.

Larvae attached to macrophytes and other collectable substrates can be sampled by removing a standard quantity (volume or weight) of the substrate. The substrate should be stirred vigorously in a bucket of water and then discarded. The dislodged larvae and detritus in the bucket are poured through a 50-mesh or other appropriate sieve. The residues on the sieve are transferred with clean water into a cup. The cups are transported in a cool ice chest to the laboratory where the larvae are floated and counted in the manner described above.

In semi-field evaluation utilizing small shallow ponds, larvae are sampled by isolating bottom mud in 15 X 15 cm baking pans placed in the natural habitat for 3 to 4 days. At desired intervals, 3 to 4 of these trays containing mud and larvae are retrieved. The sample is washed through the sieve and the larvae are transported to the laboratory in the manner described above (Mulla and Khasawinah 1969). In further refinement of this technique, sheet metal enclosures (.6-1 m in diameter) have been used for preliminary evaluation of larvicides against field population. In this regard, the enclosed water and bottom mud is considered as one plot, wherein several trays of bottom mud are placed.

For detailed and critical examination, the larvae and the residues may be transferred to enamel pans or lit counting trays, where they are counted and identified to genus or species.

In assessing the biological activity, larval samples should be taken prior to and 4, 7, 15 days or longer after treatment when the effectiveness is worn out. It is important that three to four days be allowed between treatment and the first assessment, as it takes this period of time for dead larvae to decompose or lose turgidity and form. Extent of control in the treatments, run along with checks or without checks, should be calculated in the manner described under Data Analysis.

Insect Growth Regulators and Antimetabolites:--Two approaches are used in semi-field and field evaluation of IGR's, antimetabolites and other delayed acting bioactive compounds against chironomid midges in aquatic habitats. Since some of these compounds induce delayed mortality in the larval stages, assessment of larval population, as described under Conventional Larvicides will provide the needed information on the action of these types of compounds on midge larvae.

For assessment of the efficacy of most of these compounds, however, it will be necessary to determine the inhibition of emergence of adults. Emergence is assessed readily by setting submerged, emerged or floating emergence straps. For shallow waters .3-6 m deep, cone type sheet metal traps provided with a collection jar or chamber are employed (Mulla et al. 1974, Mulla et al. 1975a, Norland and Mulla, 1975). These will also work in shallow, flowing water.

To evaluate the efficacy of a given IGR or similar acting compounds, field plots should be treated with EC, WP, FC or granular formulations of the compound. The first three formulations should be diluted with water and sprayed onto the surface of the water. Granular formulations are applied by a broadcaster mounted on a boat or on an airplane. If the body of water lends itself to be divided into plots, check plots should be run along with the treated plots. In some situations, it may not be possible to divide the habitat into plots. In these situations, the whole body of water is treated.

For assessment of efficacy, emergence of adult midges is obtained prior to treatment and then at intervals after treatment. Emergence traps should be placed in the treated as well as check plots if included in the design of the experiment. The emergence of midges as a function of time is plotted and the trend of midge production as influenced by the treatments can be ascertained until emergence level reaches pre-treatment level. If check plots are run along with treated plots and pre- and post-treatment samples are taken, the inhibition of emergence (EI) for a given period is calculated by Formula II given under Data Analysis. If no pre-treatment samples are taken, then, the EI is calculated by Formula III. However, if the whole body of water is treated as a single plot, then the extent of efficacy (EI) is determined by comparing emergence during post-treatment intervals with that during the pre-treatment period, using Formula I.

It is important that at least four traps be used per plot or 12 traps per whole body of water. In general, plotting the emergence of midges from check and treated plots yields the needed information. Similarly, emergence during pre-treatment and post-treatment intervals provides a basis for assessing effectiveness of IGR's and similar types of compounds. The emergence assessment should be continued until it reaches to 30-50% of the emergence prior to treatment or the emergence in the check.

Adults

As discussed earlier, adulticiding measures are employed on a limited scale for the control of midges. Nevertheless, many quick knockdown and effective insecticides have become available recently and these offer good tools for the control of adults.

Laboratory Evaluation

No techniques have been developed specifically for the evaluation of adulticides against chironomid adults. The techniques and protocol presented for the evaluation of mosquito adulticides will serve well for midge adulticides. It should be pointed out that due to the short life span of midges, adults not older than 24 hours be used in bioassays and that the mortality be read 4-8 hours after treatment. Otherwise, the procedures given for mosquitoes should apply equally to adult midges.

Field Evaluation

Aerial and ground application of aerosols and mist sprays provide temporary relief from nuisance midges. Normally, the adult midges rest in vegetation, on structures and other protective sites. Discharge of an adulticidal aerosol will yield high mortality of the resting adults. The swaths should be applied in such a way that the aerosol cloud is carried over the harborage sites by wind.

Assessment of adult populations should be made before and at intervals after treatment in the check area and the treated area. After population assessment, the level of reduction or control should be calculated by Formula I if no check plots are used, or by Formula II if check plots were established.

Adult populations may be sampled by one of the following techniques:

1. Insect sweep nets. Resting sites, especially vegetation, lend readily to this technique. At least five samples of five sweeps each should be taken at four to five locations at each sampling time.

2. Counting on substrates. Adult midges rest on leaves, stems of plants and on surfaces of buildings and other protected niches. Counts should be made at the same time of the day and converted to numbers/unit area (Patterson et al. 1966).

3. Shelters. Artificial standard shelters can be made and placed in the infested area. During the heat of the day, adult midges will enter the shelters where they can be counted easily.

4. Light traps. The New Jersey light trap and other similar traps will attract midges during the night. Trap collections give valuable information on the population activity and levels of adult midges. Several traps should be employed in the check and treated areas prior to and at intervals after treatment. The light traps should be located away from other artificial lights. The procedures outlined for mosquito adults will be applicable here too.

CHAOBORID MIDGES

These midges breed in eutrophic lakes, ponds and reservoirs and at times are co-existing with chironomid midges. The chaoborid larvae are relatively transparent and hard to see in water, and hence, they are also known as "phantom larvae."

Chemical control measures are primarily aimed at larvae by treating the aquatic habitat where the larvae are prevailing. In temperate zones, the midges become a nuisance problem in June-September and treatments, therefore, are necessary during this period. At most, three to four chemical treatments are needed during a given season.

Larviciding is the most effective measure for the control of *Chaoborus* species. Adult control has not been found practical and effective and, therefore, is not practiced except under certain circumstances. Equipment and methodologies employed for the control of adult mosquitoes and chironomid midges will be equally applicable here. Assessment of adult populations should be made in the manner described for those two groups.

Eggs of these midges float mostly on the water surface and are blown as drifts by wind. Treatment of egg drifts with petroleum oils (high aromatic content) has yielded good control of eggs (Dolphin and Peterson 1960). Notwithstanding this, ovicidal measures are not practical as a good portion of the eggs sink and hatch in the deeper portions.

Larvae

Laboratory Evaluation

Chaoborus species have not been colonized in the laboratory as yet; therefore, larvae for bioassays are collected from their breeding sources. Young instar larvae (first and second) are pelagic, occupying the upper portions of a lake, pond or reservoir, while the older larvae (third and fourth) are benthic, occupying the deeper portions, primarily residing at the mud and water interface during the daylight hours and in winter months. The younger larvae are collected by plankton-tow nets drawn vertically or horizontally through the water. The older larvae and pupae are sampled by means of an Ekman dredge or similar device. Larvae collected by tow nets are transferred to the laboratory in the lake water in a chilled ice chest. Mud samples secured by the dredge are washed through a 50-mesh screen, the residue containing larvae are transferred in water to jars or cups and transported in a cool ice chest to the laboratory (Brydon 1956, Cook 1965).

For bioassay, the larvae from the mud residue are floated with saturated solution of Epsom salt (magnesium sulfate). The larvae are transferred to filtered or screened lake water in beakers or fruit jars. Normally, 100-200 ml water is placed per unit, to which 20-25 larvae are transferred by pipet, screen loop or an aspirator. Both first and fourth instars are used in separate tests.

Technical insecticides are dissolved in acetone as 1% solution, serial dilutions when needed are made with water. Formulated fluid or wettable powder formulations are diluted with water and serial dilutions made in water. Aliquots of the solution to yield the desired concentrations are added to the test units containing the larvae. Each concentration should be replicated two times and each test repeated at least three times on different occasions. The units are held in a room with a temperature of 20-25°C. Checks are run along each test to which the solvent alone is added. Chaoborid larvae are susceptible to acetone and other solvents; therefore, the quantity of solvent added per unit should not be more than 0.1 ml.

Larval mortality is assessed 24 hours after treatment under a microscope; larvae with no movement are considered dead. Mean larval mortality is plotted against concentration on a probit log paper and the points fitted with a straight line. LC_{50} and LC_{90} concentrations are read off this line or from a computer analysis of the data.

Larvae of *Chaoborus* can survive for 24-48 hours without food. Longer holding of larvae requires provision of food in the form of live zooplankton, as the larvae are predacious. For the evaluation of IGR's, antimetabolites and other slow-acting bioactive compounds, evaluation procedures in the laboratory are yet to be developed. These procedures, however, will be quite similar to those employed in mosquito and chironomid midge studies, except that live food items have to be offered to the larvae at regular intervals during the test period.

Field Evaluation

Field evaluation of larvicides, IGR's and other bioactive compounds against the larvae is quite similar to that presented for chironomid midges. For methods of application and dilutions, see those procedures.

Larval samples are taken in the manner as described under Laboratory Evaluation for obtaining live larvae for laboratory bioassays. For counting, the field larval samples are heated to 55-60°C for a few minutes. This temperature will kill the larvae recovered from the plankton tow-nets or bottom mud samples. The dead larvae float to the top of the water in the jar from where they can be decanted, aspirated or screened out and counted in a dark photographic tray (Cook 1967, Hazeltine 1962, Lindquist et al. 1951, Snell and Hazeltine 1962).

The larval counts prior to and after treatment are compared for level of control. The % reduction is calculated by Formula I, as reported in Data Analysis. If check plots are run along with the treated plots, then the level of control or reduction is calculated by Formula II.

Adult emergence can be assessed by a submerged emergence trap as used for chironomid midges or by a floating type trap. The level of inhibition of emergence is to be calculated in the manner described for chironomid midges.

Adults

Adulticiding measures are employed on a limited scale for the control of midges. Many quick knockdown and effective insecticides have become available and these offer good tools for the control of adults.

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CERATOPOGONIDAE (Biting Midges, Punkies, Sand Flies)

Although biting midges transmit a number of parasitic diseases (Kettle 1965), in the United States they are of particular importance as bloodsucking pests of man. Some species, adapted to brackish water, are vicious pests near salt marshes and beaches; others are severe pests in northern forests. The immature stages of biting midges breed primarily in moist to wet soil of salt marshes, beaches, swamps, margins of ponds, small puddles, hoofprints, treeholes, decaying leaves, etc. (Hair et al. 1966). These habitats are often shaded and protected by living and decaying plants on the ground surface.

Although it has not been rigorously demonstrated, the larvae are probably more susceptible to insecticides than the eggs or the pupae. Because the larvae live in a protected habitat, control usually is impractical unless persistent insecticides are used. There has been a general tendency to rely on environmental manipulation, e.g., filling, flooding or draining larval breeding areas (Linley and Davies 1971, Foulk 1966).

Larvae

Laboratory Evaluation

Biting midges, with due allowance for their size, are handled much like mosquitoes for laboratory larvicide screening tests. Colonies of a number of species have been established to facilitate laboratory testing (Linley 1968).

One ml of acetone solution of each of a number of insecticides in serial dilutions is pipetted into 190 ml of distilled water in 250 ml flasks with 1 ml of acetone serving as a check. After 30 minutes, 10 ml of distilled water containing 25 fourth instar larvae is added to each beaker. The flasks are held at about 25-30°C for 24 hours after which time mortality readings are made. A minimum of three tests should be reported at each of three to four concentrations with mortalities between 10% and 90%. Slopes, LC_{50}'s and LC_{90}'s are then calculated as given under Data Analysis. Larvae are considered dead if they are incapable of swimming normally or do not respond when probed with a needle. Larvae that pupate during the test are discarded. If more than 10% of the control larvae pupate, or the control mortality is more than 20%, the test should be discarded (Harris and Jones 1962).

Field Evaluation

Small plots experimentally treated with larvicides can vary in

size from 1 or 2 to 40 square m or more. The larger sizes reduce the likelihood of larval migration into treated plots from adjacent untreated areas effecting the results. Small plots can be treated with hand sprayers (or spreaders for granules) while larger plots usually require power sprayers, and still larger plots aircraft.

Effectiveness is much more difficult to evaluate for biting midge larvae than for mosquito or black fly larvae since they live in soil or decaying organic matter and are small and slender. Species identification is necessary to determine which areas are to be treated bacause many species that are nonbiting live in habitats superfically similar to those inhabited by biting species. Populations of larvae can be sampled by collecting a standard area and volume of suspected breeding media. These are taken to the laboratory where they are washed individually with tap water through a 10-mesh sieve and a 20-mesh sieve into an 80-mesh sieve. Biting midge larvae are washed through the first two sieves but retained by the 80-mesh sieve. The material retained by the 80-mesh sieve is placed in a low broadmouthed glass container which is filled with a saturated solution of magnesium sulfate (Epsom salt) and stirred (Kettle et al. 1956). The container is then placed under a low-power dissecting microscope and the surface of the solution examined. Because of the high specific gravity of the solution, biting midge larvae, pupae and other small organisms float to the surface along with plant detritus. The larvae or pupae can then be transferred to clean water and counted or reared. Variations of these methods are described by Bidlingmayer (1957), Jamnback and Wall (1958), and Foulk (1966).

A second method of evaluating the efficiency of larviciding is emergence trapping (Davies 1966). Traps can be made of inexpensive cones of roofing felt joined with paper fasteners. The basal opening should be about 0.2 square m and the opening at the top about 10 cm. A glass jar with the inside walls coated with castor oil is placed over the top. The emerging insects are attracted to light, enter the jar and are trapped on the sides. These are taken to the laboratory at regular intervals and the biting midges removed, identified and counted. The traps should be removed after each count as the larvae tend to migrate into the shaded areas beneath the trap from adjacent areas.

Adults

Laboratory Evaluation

Biting midges are attracted to light and frequently enter houses. They are small enough to pass through window screens and so can be a serious nuisance at night. Painting or spraying window screens with a pesticide can prevent annoyance by killing the midges that alight on the screen mesh in the process of entering a room.

The toxicity of insecticides to adult biting midges can be measured in the laboratory using standard WHO susceptibility kits in which the standard screen through which the midges can pass is replaced with a fine mesh screen or nylon stockings (Service 1968).

Formulation can also be tested in small chambers with one end of the chamber covered by a transparent plastic and the other end opaque. A screen is inserted between the light and dark ends of the container. The adult midges are introduced into the container at the dark end. They are attracted to light and quickly alight on and pass through the treated screen at which time they are exposed to insecticide. The duration of the effectiveness of such treatment can be measured by preparing a large number of screens in the same way and exposing them to weathering. Evaluation of effectivess is measured by determining the time from passing through the screen (immediately after they are introduced) until mortality (Jamnback 1961, Linley and Davies 1971).

Field Evaluation

Adult populations estimates can be made using either landing rate counts or light trap collections. Whichever method is used, counts should be taken simultaneously both inside and outside of the treated area before and at regular intervals after treatment. In this way, normal hourly and seasonal changes in activity and populations levels can be taken into account when evaluting the effectiveness of control.

Landing rate counts preferably taken by two individuals at each site at the same time should not begin until five minutes after arrival at a given site as counts taken immediately on arrival are lower and less uniform. When biting midges are abundant, counts of the number landing on one bared arm exposed to ca. 10 cm above the elbow over a two minute period will provide a useful estimate (Jamnback and Watthews 1963).

Adult biting midges are attracted to light and can be collected in light traps in large numbers in the same way as mosquitoes to evaluate the effectiveness of larval or adult control programs. However, all species are not equally attracted to light and biased counts may result. Another drawback is that many species of insects other then nuisance *Culicoides* are attracted to light, and extensive sorting is required even when only aliquots of the collection are identified. If light traps are fitted with jars of alcohol rather than ethyl acetate, sorting, handling and identification are simpler. The larger moths, beetles, flies, etc. can be strained out with 10- and 20-mesh sieves. The larger insects are retained by these sieves and the *Culicoides* by a 60-mesh sieve.

Tests on insecticide aerosols have not been reported under field conditions although they are routinely used in many areas. The efficacy can be evaluated by taking landing rate counts inside and outside of the control area before and at intervals after treatment as described above.

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