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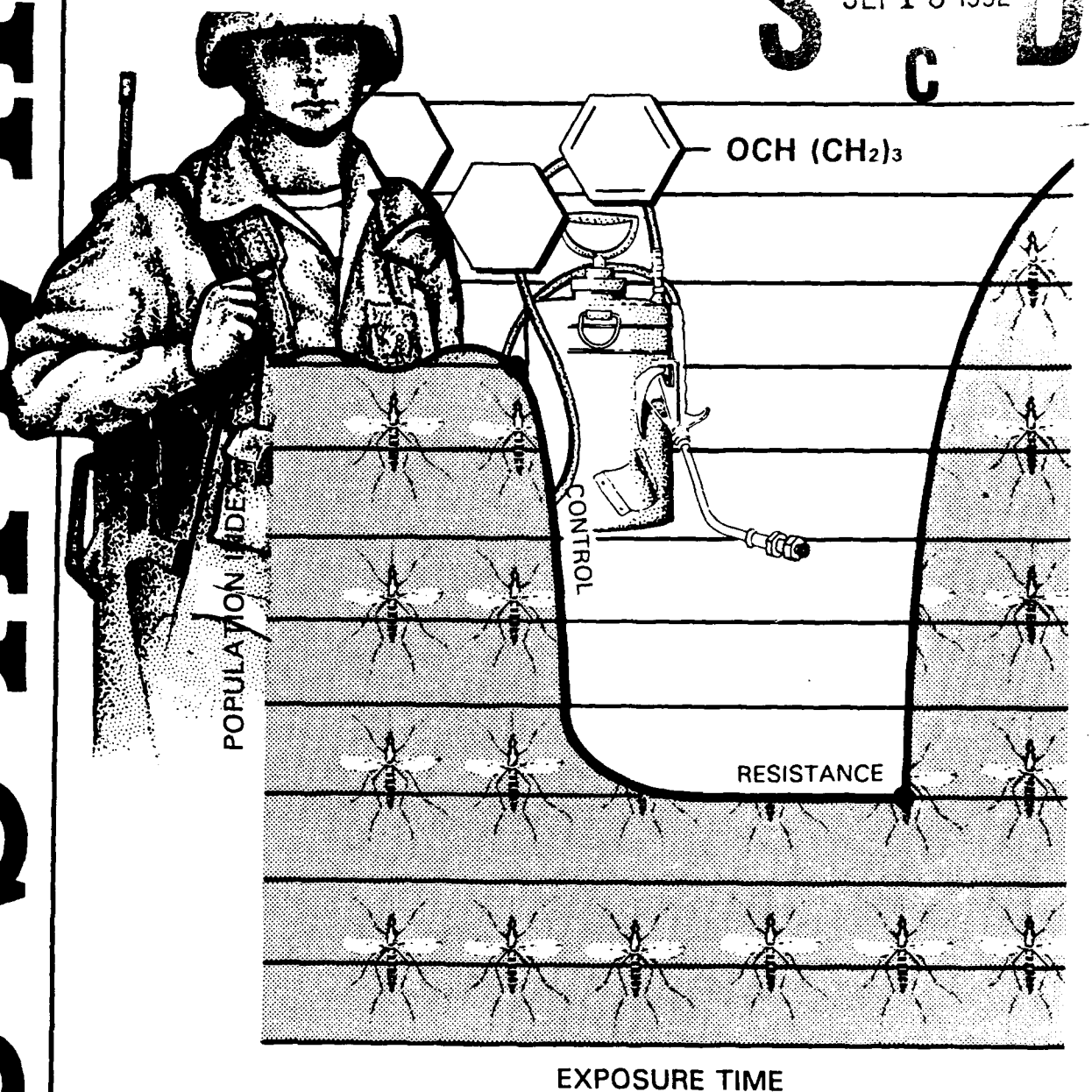
# USAEHA

## *Procedures for the Diagnostic Dose Resistance Test Kits for Mosquitoes, Body Lice, and Beetle Pests of Stored Products*








*U.S. Army Environmental Hygiene Agency*
*Aberdeen Proving Ground, Maryland 21010-5422*

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

| | |
|---|---|
| 1a. REPORT SECURITY CLASSIFICATION<br>UNCLASSIFIED | 1b. RESTRICTIVE MARKINGS |
| 2a. SECURITY CLASSIFICATION AUTHORITY | 3. DISTRIBUTION/AVAILABILITY OF REPORT<br>Approved for public release; distribution unlimited. |
| 2b. DECLASSIFICATION/DOWNGRADING SCHEDULE | |
| 4. PERFORMING ORGANIZATION REPORT NUMBER(S)<br>Technical Guide No. 189 | 5. MONITORING ORGANIZATION REPORT NUMBER(S) |
| 6a. NAME OF PERFORMING ORGANIZATION<br>U.S. Army Environmental Hygiene Agency<br>6b. OFFICE SYMBOL (If applicable)<br>HSHB-MR-E | 7a. NAME OF MONITORING ORGANIZATION |
| 6c. ADDRESS (City, State, and ZIP Code) | 7b. ADDRESS (City, State, and ZIP Code) |
| 8a. NAME OF FUNDING/SPONSORING ORGANIZATION<br>8b. OFFICE SYMBOL (If applicable) | 9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER |
| 8c. ADDRESS (City, State, and ZIP Code) | 10. SOURCE OF FUNDING NUMBERS<br>PROGRAM ELEMENT NO. / PROJECT NO / TASK NO / WORK UNIT ACCESSION NO |

11. TITLE (Include Security Classification)
Procedures for the Diagnostic Dose Resistance Test Kits for Mosquitoes, Body Lice, and Beetle Pests of Stored Products

12. PERSONAL AUTHOR(S)
Zeichner, Brian

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| Technical Guide | FROM _____ TO _____ | 92.08.01 | 39 |

16. SUPPLEMENTARY NOTATION

| 17. COSATI CODES | | | 18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) |
|---|---|---|---|
| FIELD | GROUP | SUB-GROUP | resistance testing, control mortality, medically important insect pests |
| | | | |
| | | | |

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In military operations, the control of medically important insect pests (such as mosquitoes, body lice, and beetle pests of stored products) is essential in preventing disease and the loss of food. The use of effective insecticides will maximize the control success.

Public health insect pests' resistance to insecticides has been documented from around the world. The possibility of resistance affecting control should be considered before any disease vector control operation.

This technical guide assists entomologists and preventive medicine specialists working under the supervision of an entomologist in using the diagnostic dose resistance test kits for several medically important insect pests.

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|---|---|---|
| 20. DISTRIBUTION/AVAILABILITY OF ABSTRACT<br>☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS | 21. ABSTRACT SECURITY CLASSIFICATION<br>Unclassified | |
| 22a. NAME OF RESPONSIBLE INDIVIDUAL<br>Brian Zeichner | 22b. TELEPHONE (Include Area Code)<br>(410) 671-3613 | 22c. OFFICE SYMBOL<br>HSHB-MR-E |

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

CONTENTS

DEPARTMENT OF THE ARMY
U. S. ARMY ENVIRONMENTAL HYGIENE AGENCY
ABERDEEN PROVING GROUND, MARYLAND 21010-5422

REPLY TO ATTENTION OF

HSHB-MR-E

August 1992

USAEHA TECHNICAL GUIDE No. 189

# PROCEDURES FOR THE DIAGNOSTIC DOSE RESISTANCE TEST KITS FOR MOSQUITOES, BODY LICE, AND BEETLE PESTS OF STORED PRODUCTS

## Chapter 1
## Introduction

### 1-1. Purpose

a. This technical guide (TG) provides guidance to entomologists and to preventive medicine specialists working under the supervision of an entomologist on using the diagnostic dose resistance test kits for several medically important insect pests. These test kits may be used for rapid resistance testing in the field for the following--

(1) Adult and larval mosquitoes.

(2) Body lice.

(3) Beetle pests of stored products.

b. This TG also provides guidance in--

(1) Determining the need for resistance testing of medically important insect pests.

(2) Using test results to make decisions regarding changing insecticides.

(3) Obtaining test kits from the U.S. Army Environmental Hygiene Agency (USAEHA).

(4) Using personal protective measures when conducting diagnostic dose resistance testing.

(5) Using insecticide-treated papers and test solution concentrates.

(6) Reducing control mortality.

(7) Recording diagnostic dose test results.

(8) Collecting test specimens.

## 1-2. Explanation of Abbreviations and Terms

Abbreviations and special terms are explained in the glossary.

## 1-3. References

References are listed in appendix A.

## 1-4. Background

a. In military operations, the control of medically important insect pests (such as mosquitoes, body lice, and beetle pests of stored products) is essential in preventing disease and the loss of food. The use of effective insecticides will maximize the control success for these insects and will minimize the cost, resources, and risks involved in the application of the insecticides. The ability of certain insects to develop resistance to insecticides has greatly reduced the effectiveness of many insect control programs worldwide.

b. The World Health Organization (WHO) has developed field resistance testing techniques using the diagnostic dose test method. This test method involves establishing a specific insecticide dosage that will kill all specimens of a susceptible population. When testing a field population against this specific dose, the presence of survivors indicates resistance.

c. This TG is a modification of the WHO's protocols on their diagnostic dose test kits for mosquitoes and body lice. The test kit for beetle pests of stored products uses a diagnostic dose test kit that was developed by the Food and Agriculture Organization of the United Nations. The protocols for beetle pests of stored products in this guide were provided by the U.S. Department of Agriculture, Stored-Product Insects Research and Development Laboratory, Savannah, Georgia.

d. All diagnostic dose resistance test kits and insecticide standards described in this TG are available to U.S. Department of Defense components. These can be obtained from Commander, USAEHA, ATTN: HSHB-MR-E, Aberdeen Proving Ground, Maryland 21010-5422. (See para 1-6.)

## 1-5. Determining the Need for Resistance Testing

Many instances of medically important insects' resistance to insecticides have been documented around the world. (See WHO/VBC/81.806.) Therefore, insecticide control programs should consider the possibility of resistance. To determine the need for resistance testing, consider the following--

a. Characteristics of past insecticide control programs.

(1) Frequency of insecticide application. More applications per insect pest generation will increase the selection pressure.

(2) Percentage of insect population treated. Treating a high percentage of the insect population will result in fewer susceptible individuals. The presence of fewer susceptible individuals will cause the resistant individuals to dominate the population more quickly.

(3) Types of insecticides. A more persistent insecticide produces a greater selection pressure. Consider the potential for the development of cross-resistance between previously used insecticides and those that have not yet been used.

b. Insect pest's binomics. The greater any of these are then the possibility of resistance development increases.

(1) Generations per year.

(2) Number of offspring.

(3) Exposed life stages.

(4) Proportion of the population exposed.

(5) Percentage of the insects' life that they are exposed.

c. Agricultural insecticides. Many insect field populations are exposed directly to agricultural insecticide sprays and indirectly to runoff. For example, riceland mosquitoes are exposed directly because they inhabit agricultural areas. In another example, body lice have developed resistance to malathion, simply because agricultural workers were contaminated with the malathion they were using to treat coffee trees.

## 1-6. Technical Assistance

Obtain program support from the following--

a. Commander, USAEHA, ATTN: HSHB-MR-E/Pest Resistance Coordinator, Aberdeen Proving Ground, Maryland 21010-5422, DSN 584-3613, commercial (410) 671-3613; 24-hour answering machine (410) 671-3773; or facsimile to DSN 584-2037 or commercial (410) 671-2037. USAEHA can provide the following--

(1) Test kits.

(2) Diagnostic dose test report forms.

(3) Insecticide standards.

(4) Resistance test data on arthropod disease vectors worldwide.

(5) Test result interpretations.

b. Armed Forces Pest Management Board, Defense Pest Management Information Analysis Center, Forest Glen Section, Walter Reed Army Medical Center, Washington, DC 20307-5001, DSN 291-5366, commercial (202) 472-5365. The Armed Forces Pest Management Board can provide resistance test data on arthropod disease vectors worldwide.

c. World Health Organization (WHO), Division of Vector Biology and Control, 1211 Geneva, 27-Switzerland, Country Code 22, Exchange 791-2111, Telex. 27821, or OMS Facsimile 910746. Resistance test kits can be purchased from the WHO.

## Chapter 2
## Diagnostic Dose Testing

### 2-1. Concept

a. The diagnostic dose for insecticides is determined by exposing susceptible insects to a series of insecticide concentrations. The lethal dose to kill 99.9 percent of the test population ($LD_{99.9}$) should be determined. The diagnostic dose should be twice the $LD_{99.9}$.

b. Diagnostic dose testing is an abbreviated and simplified resistance test. By comparison, tests to definitively define resistance would entail determining concurrently the $LD_{50}$ and $LD_{90}$ of the test population and of a known susceptible population.

c. These definitive test procedures are time-consuming and labor intensive. They require a well-equipped laboratory and a known susceptible strain of the insect pests. Obviously, these requirements are beyond the resources and capabilities of many small laboratories or mobile military units. Diagnostic dose testing is useful in providing resistance information where time and resources are limited.

d. Ideally, diagnostic doses should be determined before exposing the target insect pest population to insecticides. Then, through regular monitoring, the development of resistance can be readily traceable as a greater percentage of the insect pest population survives the diagnostic dose of the insecticide.

e. In reality, testing an insect pest population before it has been exposed to insecticides has rarely been done. Therefore, diagnostic doses of certain insecticides have been determined by using susceptible laboratory insect strains. The diagnostic doses of insecticides have been determined for relatively few insects and insecticides of interest to the U.S. Armed Forces. Tables 3-1 through 3-4 contain the determined diagnostic doses of insecticides for several insect pests.

f. The provided test kits are not limited only to the species and insecticide combinations for which there are determined diagnostic doses. USAEHA has access to WHO's database which contains the results of diagnostic dose testing completed since the 1960s. This database contains 16,199 test results on 159 species and 40 insecticides, for a total of approximately 640 unique species and insecticide combinations. This database, as

well as the published literature, can be used to interpret the test results for species and insecticide combinations that do not have a determined diagnostic dose.

g. Contact USAEHA for any information regarding a species and insecticide combination not specifically mentioned in this guide. (See para 1-6.)

## 2-2. Using Test Results for Decision Making

a. A high percentage of survivors indicates resistance. A small number of survivors may occasionally appear by chance. This is especially true in the adult mosquito test kit where adults that stay on the untreated ends of the test tube do not come in contact with the insecticide. However, if survivors consistently appear in the testing, then resistance is indicated. Deliberate and consistent avoidance of the insecticide-treated papers could indicate behavior resistance.

b. Hard and fast guidelines on when to change insecticides are not available. The best data available to answer this question was work done on DDT resistance in mosquitoes. (See Bulletin of the WHO, 49: 475-483.) This work found that if more than 20 percent of the population survived the diagnostic dose of insecticide, then resistance was present at a level that affected control.

c. Based on the available information, seriously consider changing insecticides when 20 percent or more of the insect pest population survive the diagnostic dose testing. Consider these factors when deciding to change insecticides--

(1) The epidemiology of the disease. Less than perfect control may effectively break or significantly reduce the disease transmission cycle. Therefore, a given insecticide may be effective in reducing disease even though more than 20 percent of the population survived exposure to the insecticide.

(2) Alternative insecticides. The characteristics and logistical considerations of alternative insecticides will influence the decision to change insecticides. Examples of these characteristics and considerations are as follows--

(a) Cost. Do you have funds available to procure and apply sufficient quantities of the replacement chemical if it is more expensive, which it probably will be? An insecticide with a low level of resistance may be more effective when applied

thoroughly at the highest rate allowed, than an insecticide with no indication of resistance that is applied at too low a rate and on too small an area. Alternatively, if a threat of a serious disease exists, then cost may be relatively unimportant, and it would be prudent to use a proven insecticide to which there was no indication of resistance, even if it was more expensive.

(b) Availability. Do you have the ability to procure and apply the material in a timely fashion?

(c) Compatibility. Can the existing application equipment apply the insecticide?

(d) Longevity. Do you have the ability to implement more frequent treatment regimes if the insecticide has a shorter residual life?

d. If resistance is being monitored over the years in an ongoing insecticide control program, seriously consider the appearance of regular survivors in the diagnostic dose test, even if less than 20 percent. This is a warning that resistance is developing to that insecticide. While the level of resistance may still be so low that control is not reduced, reexamine the insecticide control program and seek and implement ways to reduce the continued development of resistance.

# Chapter 3
# Diagnostic Dose Test Procedures to Determine Resistance to Insecticides

## Section I
## General Procedures

### 3-1. Equipment

a. Test kits may be borrowed from Commander, USAEHA, ATTN: HSHB-MR-E/Pest Resistance Coordinator, Aberdeen Proving Ground, Maryland 21010-5422, DSN 584-3613, commercial (410) 671-3613 or facsimile (410) 671-2037. When requesting kits, indicate what insect pest species will be tested and what insecticides will be used. When the diagnostic dose testing has been completed, return the test kits to USAEHA.

b. Test kits include all equipment necessary to perform the test. The only collecting equipment provided is a mouth aspirator and tweezers; however, other collecting equipment may be requested.

### 3-2. Personal Protective Measures

a. While the concentration of insecticide on the treated papers is low, always wear protective gloves when handling these papers. Do not store the insecticide-treated papers with foodstuffs or medical supplies.

b. Use care when collecting and handling insect pest specimens, since they may be carrying disease. The most important line of defense is to always wear protective clothing that covers as much of the body as possible.

c. Carefully weigh the use of any insect repellents. If collecting equipment becomes contaminated with insecticide from the hands or clothing, catching a sufficient number of insect pests may be impossible. If an insecticide, such as permethrin is used, mortality and invalidation of the test may be caused.

## 3-3. Insecticide-Treated Papers and Solutions

a. Treated papers are identified with the insecticide and the concentration of the insecticide solution. The concentration of the insecticide solution is identified by milligram of active ingredient per milliliter of solution (mg/mL). The insecticide and dose are found on the papers and the foil wrap. All papers are treated at the rate of 2.75 mL of the insecticide solution per 180 centimeters squared ($cm^2$). The correlation between WHO and USAEHA manufactured/labeled papers is as follows. A WHO 1 percent paper would be equivalent to a--

(1) 2.57 mg/mL USAEHA paper for organophosphates.

(2) 2.78 mg/mL USAEHA paper for organochlorines.

(3) 2.42 mg/mL USAEHA paper for pyrethroids.

b. The treated papers should be used prior to the expiration date. A treated paper may be used up to 20 times if it is used within 3 weeks upon removal from the package. After using the papers, return them to the package, and then place the package in a cool dry place, if possible. Do not refrigerate at temperatures below 9° centigrade (C) or 48° fahrenheit (F), because crystallization of propoxur may occur.

c. Insecticide solutions are provided with the diagnostic dose test kits for mosquito larva. Immediately after using insecticide solutions, tightly close the containers to prevent evaporation of the solvent. Evaporation would cause an increase in the concentration of the insecticide.

## 3-4. Reducing Control Mortality

a. Minimizing trauma to the insects during collection, handling, and holding of test specimens is important.

(1) Test insects as soon as possible after collection. Perform tests in areas that are--

(a) Free of insecticidal contamination.

(b) Sheltered from the wind and sun.

(2) Provide food and water when and where appropriate, such as placing an apple slice in the mosquito holding cage.

(3) Protect insects from direct sun or excessive temperatures. If the humidity is low, increase it by adding wet paper towels or sponges to the holding area. The temperature and relative humidity of the testing environment should be recorded at different intervals throughout the testing period. Extremes in these readings can affect test results.

(4) Transfer insects as gently as possible to reduce physical damage.

b. Ensuring that mortality is attributable to the insecticide exposure and not other causes can be one of the most difficult aspects of conducting resistance tests on specimens caught in the field. The mortality of the untreated control insect pests will indicate if death was due to other causes.

### 3-5. Recording Diagnostic Dose Test Results

a. The diagnostic dose resistance test kits are designed to be read and interpreted at a given endpoint, generally 6 or 24 hours. However, recording mortality at various convenient intervals throughout the test can be very informative and useful.

b. *Consider the following hypothetical scenario:* The treated insects all die in the first 2 hours. The control insects start dying at 12 hours and reach 70 percent mortality at 24 hours. If the test could not be repeated because of time and resources and the 24-hour mortality was the only time the test had been read, then conclusions about the population's susceptibility could not be drawn because of the high control mortality. However, the earlier mortality readings (treated and control insects) suggest that the population is susceptible; therefore, having the earlier readings provides some basis for action if a decision was required immediately.

c. For testing controls, AEHA Form 315-1-R (Diagnostic Dose Test Report Continuation Sheet) can be used to record the test results. For reproduction purposes, a blank copy of AEHA Form 315-1-R is located in the back of this TG. The top of the sheet should be identified as follows: Control Testing.

d. AEHA Form 315-R (Diagnostic Dose Test Report) can be used to record the results for all provided tests. For reproduction purposes, a blank copy of AEHA Form 315-R is located in the back of this TG. Each test kit will contain additional copies of the forms. A copy of the forms must be forwarded to USAEHA for incorporation into the resistance database. (See para 1-6.) The following instructions should be followed to report the test results on AEHA Form 315-R:

(1) Test Identification: Fill in the test identification with any designator specific to the test. This identification correlates the first page of the report to continuation sheets.

(2) Page _____ of ____ Pages: Fill in the page ____ of ____ pages to connect the first page with the continuation sheets.

(3) Insecticide and Dose: Record the name and dosage of the insecticide tested. For example, malathion (14.1 mg/mL).

(4) Date: Record the date the test began.

(5) Time insects placed in test container: Record the time of day the insects were placed in the testing container.

(6) Time insects were exposed to insecticide: Record the time of day that the insects were exposed to the tested insecticide. For some tests, such as the mosquito larval test, the time of day will be the same as when the insects were placed in the testing container.

(7) Time insects removed from insecticide: Record the time of day that the insects were removed from the testing container to the holding container. This is not applicable to all tests.

(8) Temperature and Humidity Readings: Record the temperature and humidity readings at four different times throughout the course of the test.

(9) Collection Site: Identify the geographic location where the insects were collected.

(10) Testing Site: Identify the facility and/or location where test was conducted.

(11) Investigators: Record name(s) of the individual(s) performing the test.

(12) Column 1: Record the replicate number. Horizontal lines are not drawn under this column so that the form can be adjusted for various tests depending on the number of species that may be present. Decide how many lines per replicate you want and draw a horizontal line across the page.

(13) Column 2: Identify the species of insect by using the scientific name.

(14) Column 3: Record the number of insects that were dead (probably due to handling) after the 1-hour holding period. Not applicable to all tests.

(15) Column 4: Estimate the number of individuals for each species that are in each replicate. This estimation is optional.

(16) Columns 5 and 6: Record the time of day for each intermediate reading and the number of alive or dead insects at each reading. Be sure to scratch out dead or alive as appropriate. The form is set up this way because when few insects are dead it is easier to count the dead individuals, and when many insects are dead it is easier to count the alive individuals.

(17) Columns 7 and 8: Record the time of day for the final reading and the number of dead and alive insects.

(18) Column 9: Calculate the corrected mortality for damaged insects by subtracting the number of dead insects in column 3 or (A) from the final number dead in column 7 or (B). Record this calculation.

(19) Column 10: Count the total number of insects, alive and dead, in a given replicate.

(20) Column 11: Calculate the correction for damaged insects by subtracting the number of dead insects after the 1-hour holding period in column 3 or (A) from the total number of insects in a given replicate in column 10 or (D). Record this value.

(21) Column 12: Calculate the percentage of dead insects by dividing the corrected mortality in column 9 or (C) by the correction for damaged insects in column 11 or (E). Record this value as a percentage. For those tests where the 1-hour mortality readings are not taken, the corrected mortality is the same as the number dead at the final reading, and the total corrected for damaged insects is the same as the exact total number of insects, alive and dead, in a given replicate.

### 3-6. Adjusting for Control Mortality

a. If the control mortality rate among the control insects is between 5 and 20 percent, then adjust the percent mortality of the treated insects by using Abbott's formula. (See fig 3-1.) Insert the percent test dose mortality and the percent control mortality as calculated on AEHA Form 315-R into the formula. The number that is generated is the adjusted percent mortality that should be recorded. This value is used to make decisions regarding changing insecticides.

$$\text{Adjusted percent mortality} = \frac{\text{Percent test dose mortality} - \text{Percent control mortality}}{100 - \text{Percent control mortality}} \times 100$$

Figure 3-1. Abbott's Formula for Adjusting Control Mortality

b. For example, if the treated replicates had an average test dose mortality of 78 percent and the control mortality in the control replicates averaged 14 percent, then calculate the adjusted percent mortality as follows:

$$\text{Adjusted percent mortality} = \frac{78 - 14}{100 - 14} \times 100 = 74.4 \text{ percent}$$

In this example, more than 20 percent (25.6 percent) of the population survived the diagnostic dose. Therefore, seriously consider using another insecticide. (See para 2-2.)

c. If the control mortality is below 5 percent, no adjustment is necessary.

d. Tests with control mortality rates above 20 percent are unsatisfactory but should be recorded. Examine the causes of the control mortality rates and take steps to reduce them. If time does not permit repeating the test, then the intermediate readings may provide a clue as to the status of the population. (See para 3-5.)

## Section II
## Adult Mosquitoes

### 3-7. Collection of Test Specimens

a. Sixty to one hundred test specimens are needed for each insecticide tested, plus an additional sixty to one hundred specimens are needed for the control tubes.

b. Female specimens can be aspirated from houses and bait or resting stations. Conventional light traps can also be used to collect the test specimens; however, conventional light traps may cause too much damage to the insects as they pass through the fan blades. Light traps can be modified to blow the insects into the collecting bag without passing through the fan blades. To modify the trap,--

(1) Turn the fan and motor upside down.

(2) Reverse the fan direction to blow downward by reversing positive and negative leads.

(3) Cut holes in the trap body below the fan. This will allow the insects to fall into the collecting bag as they enter the trap. (See Sandoski, C. A. et al., 1983.)

c. Modified light traps can be obtained from USAEHA. (See para 1-6.) While these traps do reduce stress on the insects, their drawback is that collection efficiency is not as great.

d. The diagnostic doses of insecticides for mosquitoes were determined by using females that had not had a blood meal. Table 3-1 presents the diagnostic doses of several insecticides used on adult mosquitoes. Field collections may contain blood-fed females; if these are used, remember that they may be more tolerant of the dose. However, the increase in tolerance would account for no more than a small percentage of survivors (less than 5 percent). An advantage to using blood-fed females is that there is less of a control mortality rate.

Table 3-1
Diagnostic Doses of Insecticide for Adult Mosquitoes

| Insecticide | Treated Paper Dosage mg/mL | Exposure time in hours for: Anophelines | Culex quinquefasciatus | Aedes aegypti |
|---|---|---|---|---|
| Malathion | 12.85 | 1 | 1 | _ |
| Propoxur | 0.257 | 1 | 2 | _ |
| Chlorpyrifos | 6.1[a] | _ | _ | 1 |
| Fenitrothion | 2.57 | 2 | 2 | _ |
| Permethrin | 0.605 | 1 | 3[b] | _ |
| Resmethrin | 5.45[a] | _ | _ | 1 |

[a] Tentative diagnostic dose.

[b] Lay exposure tube horizontally.

e. If collecting a sufficient number of adults is difficult, consider collecting larvae and rearing them to adulthood. If this is done, collect larvae from a variety of sources so that a representative gene pool is collected.

## 3-8. Test Kit

The adult mosquito diagnostic dose resistance test kit for one insecticide contains--

a. Four control tube sets and four treated tube sets. Request additional tube sets if planning to test more than one insecticide concurrently. (See para 1-6.) A tube set consists of--

(1) One green-dot tube for holding the insects and one red-dot tube for exposing the insects to the control or insecticide-treated papers.

(2) One slider.

(3) Two end caps.

b. Unlabeled and untreated paper.

c. Paper that is labeled control.

d. Paper that is labeled with the insecticide and dose.

e. Vinyl gloves.

f. Silver rings.

g. Copper rings.

h. A mouth aspirator.

### 3-9. Test Conditions

Test temperatures should not exceed 32° C (90° F) or fall below 16° C (61° F). High humidity will increase the survival of the control specimens; 80 to 90 percent relative humidity is optimum.

### 3-10. Test Procedures

a. Insert a piece of clean, unlabeled, and untreated paper into each of the plastic tubes. Roll this paper into a cylinder shape before inserting.

b. While wearing vinyl gloves, insert a paper labeled "control" into each of the red-dot tubes (control exposure tubes) of the control tube sets, and then insert a silver ring into the tube to press the paper against the tube. The control paper will be labeled control and will have been treated with carrier oil only.

c. Insert a paper labeled with the insecticide and dose into each of the red-dot tubes (treated insecticide exposure tubes) of the treated tube sets, and then insert a copper ring into the tube to press the paper against the tube.

d. Connect the green-dot tube (holding tube) to the slider.

e. Once the mosquitoes have been collected and the tubes assembled, begin the testing by performing these steps--

(1) Introduce, with a mouth aspirator, 15 to 25 mosquitoes to each green-dot tube (holding tube). The control mortality rate will increase because of overcrowding if more than 25 mosquitoes per tube are used.

(2) Set the green-dot tubes (holding tubes) in an upright position with the screen end up for 1 hour. Note and record any damaged or moribund mosquitoes on AEHA Form 315-R.

(3) Attach a red-dot tube (exposure tube) to the slider. Open the slide and gently blow the mosquitoes into the red-dot tube (exposure tube). This procedure is easier when the open end of the red-dot tube is held up to the light. Close the slide; detach the green-dot tube (holding tube) and set aside.

(4) Allow the red-dot tube (exposure tube) to stand upright, screen end up, for the entire exposure time specified in Table 3-1. When testing Culex quinquefasciatus against permethrin insecticide, lay the red-dot tube (exposure tube) on its side during the specified exposure time so that when the mosquitoes are knocked down they will remain in contact with the insecticide-treated paper.

(5) Transfer the mosquitoes back to the green-dot tube (holding tube) by reversing step (3) above. If any mosquitoes have been knocked down during the exposure period, hold the red-dot tube (exposure tube) horizontally and tap it softly to remove the insects from the slide before withdrawing the slider. Set the green-dot tube (holding tube) upright so that it stands on the slide. Place a pad of moist cotton or sliced apple on the screen.

(6) Maintain the green-dot tube (holding tube) for 24 hours after exposure. Since control mortality is often a problem in the adult mosquito test, AEHA Form 315-R includes two additional columns for recording mortality at various intervals prior to the 24-hour reading. (See para 3-5.) Record the temperature and relative humidity at different intervals throughout the 24-hour period.

(7) Make the final mortality counts 24 hours after the insects have been placed in the green-dot tube (holding tube). Count those specimens that are unable to stand or walk as dead. If all specimens are of the same species, then make the final mortality count in the green-dot tube (holding tube).

(8) If more than one species is involved, then separate the dead specimens from the live specimens for identification by--

(a) Holding the green-dot tube (holding tube) vertically with the screen end up.

(b) Gently moving the slide so that the small hole is under the green-dot tube (holding tube).

(c) Tapping the tube lightly so that the dead mosquitoes fall into a collecting container. Take care to prevent the escape of live mosquitoes.

(9) Kill the live specimens by exposing them to either heat or cold, and then remove them for identification.

(10) Record the test results and adjust for control mortality per paragraphs 3-5 and 3-6.

## Section III
## Larval Mosquitoes

### 3-11. Collection of Test Specimens

a. Between 80 and 100 third and early fourth instar mosquito larvae of the same species are needed for testing one insecticide. An additional 80 to 100 larvae are needed for controls. Collect the larvae from various sources to ensure genetic diversity.

b. To ensure pesticide ingestion, proper determination of the instars is important because pesticide exposure must coincide with larval feeding periods. Late fourth instars should not be used since they stop feeding prior to pupation.

c. Some generalizations regarding instar identification follow--

(1) The size of the head capsule is the best indicator of instar number. The body of the larva will enlarge within a given instar, but the size of the head capsule remains constant.

(2) The setae increase in number and size as the larvae mature and are more pronounced in the late fourth instar stage.

d. Carefully collect larvae and retain in the same water in which they were collected until selected for testing. The following factors can cause stress that may affect the test results--

(1) Crowding.

(2) Depletion of natural food supply.

(3) Change in water temperature.

The longer the larvae are held prior to testing, the greater these effects can become.

### 3-12. Test Kit

The larval mosquito diagnostic dose resistance test kit contains--

a. Four 237 mL cups for the control test and four 237 mL cups for each insecticide treated.

b. Four 120 mL cups for the control test and four 120 mL cups for each insecticide treated.

c. One small, flat screen dipper.

d. Fifteen mLs of the insecticide test solution concentrate.

e. One wooden applicator for each insecticide tested and for the control test.

f. Fifteen mLs of alcohol control solution.

g. One pipet for each insecticide tested.

### 3-13. Test Conditions

a. The water used for these tests should be as clean and free of sediment as possible. If chlorinated water is used, allow it to stand in an open container for at least 8 hours to allow the chlorine to evaporate. Maintain the water temperature between 20° and 30° C (69° and 86° F).

b. Certain salt marsh or tree hole species of mosquitoes may suffer high control mortality rates when they are removed from their breeding water and placed in fresh water. With these species, use water from their breeding source if it is free of insecticides and detritus.

### 3-14. Test Procedures

a. Once the larval mosquitoes have been collected, begin the testing by performing the following steps--

(1) Scoop up 20 to 25 larvae with the small, flat screen dipper.

(2) Place groups of 20 to 25 larvae in the four 120 mL cups for the control replicates and in the four 120 mL cups for each tested insecticide.

(3) All 120 mL cups should contain 25 mLs of water.

(4) Label the 237 mL cups with the date and treatment information.

(5) Fill each 237 mL cup with 74 mLs of water. An additional 25 mLs of water will be added with the larvae.

(6) Add 1 mL of the appropriate insecticide test solution concentrate to each 237 mL cup, and then stir with a wooden applicator. Use a new wooden applicator for each different insecticide and concentration. Table 3-2 presents the diagnostic doses of several insecticides used in the larval mosquito test.

Table 3-2
Diagnostic Doses of Insecticide for Mosquito Larvae

| Insecticide | Test Solution Concentrate mg/mL | Final ppms for: Anophelines | Culex quinquefasciatus | Aedes aegypti |
|---|---|---|---|---|
| Malathion | 0.312 | 3.12 | | |
| Malathion | 0.1 | | 1.0 | 1.0 |
| Temephos | 0.025 | 0.25 | | |
| Temephos | 0.002 | | 0.2 | 0.2 |
| Chlorpyrifos | 0.0025 | 0.025 | | |
| Chlorpyrifos | 0.001 | | 0.01 | 0.01 |
| Fenthion | 0.005 | 0.05 | 0.05 | 0.05 |
| Propoxur | 0.3 | | | 3.0 |

NOTE: To achieve the final ppm of the diagnostic dose, add 1 mL of test solution concentrate to 99 mLs water.

(7) Add 1 mL of the alcohol control solution to each of the 237 mL cups used in the control test, and then stir.

(8) Within 15 and 30 minutes of preparing the test concentrations, expose the mosquito larvae to the insecticide by pouring the larvae and 25 mLs of water into the 237 mL cups. If the larvae and water are swirled and poured quickly, then the larvae should not stick to the sides of the 120 mL cup. If any do stick to the cup, use a clean wooden applicator to gently slide them into the 237 mL cup.

b. Make the final mortality counts and species verification 24 hours after the larvae are exposed to the insecticide. Consider those larvae that do not exhibit a normal escape response when lightly prodded with a wooden applicator as dead.

c. Repeat the test if more than 10 percent of the control larvae pupate. Since feeding stops prior to pupation, larvae that have pupated may not have ingested enough insecticide. Therefore, survival may not be attributed to resistance.

d. Record test results and adjust for control mortality per paragraphs 3-5 and 3-6.

## Section IV
## Body Lice

### 3-15. Collection of Test Specimens

a. Collect between 60 and 100 recently fed, healthy, adult body lice (either sex) to test each insecticide. Collect an equal number for controls.

b. Collect body lice from the clothing of several individuals; this will ensure a representative sample of genetic diversity. Place specimens in pill vials (or similar containers) in lots of 15 to 20. Use each pill vial as a replicate. Discard any specimens damaged during the collection process.

### 3-16. Test Conditions

Lice do not survive well away from the host; therefore, carefully collect and handle test specimens. From the time of collection and during the test, keep the specimens in a dark environment. Maintain a temperature of between 25° and 29°C (77° and 84° F) and a relative humidity of at least 50 percent. The higher the humidity, the higher the survival rate.

### 3-17. Test Kit

The body lice diagnostic dose resistance test kit contains the following--

a. Five control papers.

b. Five insecticide-treated papers.

c. Five petri dishes for the control test, and five petri dishes for each insecticide tested.

d. Rubber bands.

e. Five pill vials for the control test, and five pill vials for each insecticide tested.

### 3-18. Test Procedures

a. Once the body lice have been collected, begin the testing by performing the following steps--

(1) Place the control and insecticide-treated papers inside the larger half of each petri dish. Use four replicates and four controls for each tested insecticide. Table 3-3 presents the diagnostic doses of several insecticides used for body lice.

Table 3-3
Diagnostic Doses of Insecticide for Body Lice

| Insecticide | Paper Dose mg/mL | Exposure time* in hours |
|---|---|---|
| Lindane | 0.449 | 6 |
| Malathion | 6.137 | 6 |
| d-Phenothrin | 3.035 | 6 |

*Time starts when insects are first placed in contact with the insecticide.

(2) Place the body lice from each replicate onto the smaller half of the petri dish.

(3) Pour the body lice onto the center of the test paper.

(4) Place the two halves of the petri dishes together and secure with a rubber band. Maintain the petri dishes in a horizontal position throughout the test so that the body lice will remain in constant contact with the insecticide.

b. Take the final mortality reading at the end of the indicated exposure time. (See table 3-3.) To determine knock-down or mortality--

(1) Turn the petri dish on its side so that it is perpendicular to a horizontal surface.

(2) Tap lightly against the horizontal surface.

c. Those body lice that are knocked-down will tumble to the bottom of the dish because they cannot cling to the vertical filter paper. This process requires close observation so that the following misleading situations can be corrected.

(1) Mating. Occasionally two lice will be mating and both will fall because the female can not support both of them on the vertical filter paper. In falling, the two may also knock down other specimens. If this occurs,--

(a) Return the petri dish to the horizontal plane.

(b) Observe the mating pair to see if the female can walk. If so, count both as alive.

(c) Additionally, observe any dislodged specimens for their ability to cling.

(2) Gluing. Sometimes a dead louse will be glued to the test paper by its excrement. Upon close observation and a firmer tap, this can be determined. Count these specimens as knocked-down.

d. Record the test results and adjust for control mortality per paragraphs 3-5 and 3-6.

## Section V
## Beetle Pests of Stored Products

### 3-19. Collection of Test Specimens

a. Preferably, collect adult beetles from each different commodity or habitat where infestations are detected. Collect between 80 and 100 adults for each tested insecticide and an equal number for the controls.

b. Use the following methods to collect beetles--

(1) Sieving the commodity with an appropriate sized sieve.

(2) Brushing the floor, walls, or the surface of bags.

(3) Aspiration.

### 3-20. Test Conditions

The beetles should be substantially separated from the commodity. If the test can begin quickly, no nourishment is needed; otherwise use a small amount of the commodity as food.

### 3-21. Test Kit

The beetle diagnostic dose resistance test kit contains the following--

a. Five insecticide-treated papers for each insecticide tested.

b. Five untreated papers for the control tests.

c. Five petri dishes for the control tests, and five petri dishes for each insecticide treated.

d. Two pairs of vinyl gloves.

e. Rubber bands.

f. Forceps.

### 3-22. Test Procedures

a. Once the beetles have been collected, begin the testing by performing the following steps--

(1) Place the appropriate insecticide-treated papers on the inside of the larger half of a petri dish. Table 3-4 presents the diagnostic doses of malathion used for the beetle pests of stored products test.

Table 3-4
Malathion Diagnostic Doses for Beetle Pests of Stored Products

| Species | Paper Dosage mg/mL | Exposure Period in Hours |
|---|---|---|
| Sitophilus oryzae | 1.55 | 6 |
| S. zeamais | 1.55 | 6 |
| S. granarius | 1.55 | 6 |
| Rhyzopertha dominica | 2.58 | 24 |
| Tribolium castaneum | 0.52 | 5 |
| T. confusum | 0.52 | 6 |
| Oryzaephilus surinamensis | 1.03 | 5 |
| O. mercator | 1.03 | 5 |

(2) While wearing vinyl gloves, place the control papers and then the insecticide-treated papers in the petri dishes. Use four replicates and four controls for each insecticide.

(3) Place 15 to 20 beetles on each of the control and insecticide-treated papers.

(4) Place the two halves of the petri dish together and secure with a rubber band.

b. Record the final mortality count at the end of the required exposure time. (See table 3-4.) Mortality for beetles is their inability to stand and walk. A gentle push with forceps is usually sufficient to determine whether the insect is alive or dead.

c. Record the test results and adjust for control mortality per paragraphs 3-5 and 3-6.

## Appendix A
## References

1. Davidson, G. & A. R. Zahar. "The practical implications of resistance of malaria vectors to insecticides." Bulletin of the World Health Organization, 49: 475-483, 1973.

2. Method No. 15, Tentative methods for adults of some major beetle pests of stored cereals with malathion or lindane. Food Agriculture Organization, In Recommended methods for detection and measurements of resistance of Agricultural Pest to Pesticides, Plant Protection Bulletin No. 22, pp. 127-137.

3. Sandoski, C. A. et. al. "Effects of collection and handling techniques on riceland mosquitoes used in laboratory and field insecticide susceptibility tests." Mosquito News, 1983: 43 #4: 445-448.

4. World Health Organization. Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorine, organophosphate and carbamate insecticides-diagnostic test. WHO/VBC/81.806, 1981.

5. World Health Organization. Instructions for determining the susceptibility or resistance of mosquitoe larvae to insecticides. WHO/VBC/81.807, 1981.

6. World Health Organization. Instructions for determining the susceptibility or resistance of body lice and head lice to insecticides. WHO/VBC/ 81.808, 1981.

7. World Health Organization. Resistance of vectors and reservoirs of disease to pesticides. Tenth report of the WHO expert committee on vector biology and control. Technical Report Series 737, WHO/VBC/81.806, 1986.

## Glossary

Section I
Abbreviations

**C**
centigrade

**$cm^2$**
centimeters squared

**DDT**
Dichloradiphenyltrichloroethane

**F**
fahrenheit

**$LD_{50}$**
Lethal dose to kill 50 percent of the test population

**$LD_{90}$**
Lethal dose to kill 90 percent of the test population

**$LD_{99.9}$**
Lethal dose to kill 99.9 percent of the test population

**mg**
milligram

**mL**
milliliter

**USAEHA**
U.S. Army Environmental Hygiene Agency

**WHO**
World Health Organization

Section II
Special Terms

**Behavior Resistance**

The ability of insects to avoid insecticides.

**Control Mortality**

The mortality of the insects in the control replicates, such as those not exposed to the insecticide.

**Selection Pressure**

Forces that favor the propagation of a particular genotype within a species.

TEST ID: _____ PAGE _____ OF _____ PAGES

# DIAGNOSTIC DOSE TEST REPORT

(Instructions on Reverse and in TG 189)

Insecticide and Dose: _____

Date: _____

Time insects placed in test container: _____

Time insects were exposed to insecticide: _____

Time insects removed from insecticide: _____

| Readings: | (1) | (2) | (3) | (4) |
|---|---|---|---|---|
| Time: | | | | |
| Dry Bulb: (Temp) | | | | |
| Wet Bulb: | | | | |
| Humidity: | | | | |

Collection Site: _____

Testing Site: _____

Investigator: _____

| | | | | INTERMEDIATE READINGS | | FINAL READING | | CALCULATIONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rep # | Species | (A) No. Dead After 1 Hr Holding Period | Estimate of Total in Container | No. Dead Alive Time _____ | No. Dead Alive Time _____ | (B) No. Dead Time _____ | No. Alive Time _____ | (C) Mortality Corrected for Damaged Insects (B-A) | (D) Exact Total in Container | (E) Total Corrected for Damaged Insects (D-A) | (F) % Dead (C/E) |
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Remarks:

*Instructions for Completing the Diagnostic Dose Test Report*

**Test ID:** Fill in the test identification with any designator specific to the test. This identification correlates the first page of the report to continuation sheets.

**Page _____ of _____ Pages:** Fill in the page _____ of _____ pages to connect the first page with the continuation sheets.

**Insecticide and Dose:** Record the name and dosage of the insecticide tested. For example, malathion (14.1 mg/mL).

**Date:** Record the date the test was begun.

**Time insects placed in test container:** Record the time of day the insects were placed in the testing container.

**Time insects were exposed to insecticide:** Record the time of day that the insects were exposed to the tested insecticide. For some tests, such as the mosquito larval test, this time will be the same as when the insects were placed in the testing container.

**Time insects removed from insecticide:** Record the time of day that the insects were removed from the testing container to the holding container. This is not applicable to all tests.

**Temperature and Humidity Readings:** Record the temperature and humidity readings at four different times throughout the course of the test.

**Collection Site:** Identify the geographic location.

**Testing Site:** Identify the facility and/or location where test was conducted.

**Investigator:** Record name(s) of the individual(s) performing the test.

**Column 1:** Record the replicate number. Horizontal lines are not predrawn under this column so the form can be adjusted for various tests depending on the number of species that may be present. Decide how many lines per replicate you want and draw a horizontal line across the page.

**Column 2:** Identify the species of insect by using the scientific name.

**Column 3:** Record the number of insects which were dead (probably due to handling) after 1 hour holding period. Not applicable to all tests.

**Column 4:** Estimate the number of individuals for each species that are in each replicate. This estimation is optional.

**Columns 5 and 6:** Record the time of day for each intermediate reading and the number of alive or dead insects. Be sure to scratch out dead or alive as appropriate. The form is set up this way because when few insects are dead it is most accurate to count the dead individuals, and when many are dead it is most accurate to count the alive individuals.

**Columns 7 and 8:** Record the time of day for the final readings and the number of dead and alive insects.

**Column 9:** Calculate the corrected mortality for damaged insects by subtracting the number of dead insects in column 3 (A) from the final number dead in column 7 (B). Record this value.

**Column 10:** Count the total number of insects, alive and dead, in a given replicate.

**Column 11:** Calculate the correction for damaged insects by subtracting the number of dead insects after the 1 hour holding period in column 3 (A) from the total number of insects in a given replicate in column 10 (D). Record this value.

**Column 12:** Calculate the percentage of dead insects by dividing the corrected mortality in column 9 (C) by the correction for damaged insects in column 11 (E). Record this value as a percentage. For those tests where the 1 hour mortality readings are not taken, the corrected mortality is the same as the number dead at the final reading, and the to[illegible] corrected for damaged insects is the same as the exact total number of insects, alive and dead, in a given replicate.

TEST ID: _____ PAGE _____ OF _____ PAGES

# DIAGNOSTIC DOSE TEST REPORT

(Instructions on Reverse of AEHA Form 315-R and in TG 189)
(Continuation Sheet)

| | | | | INTERMEDIATE READINGS | | FINAL READING | | CALCULATIONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rep # | Species | (A) No. Dead After 1 Hr Holding Period | Estimate of Total in Container | No. Dead Alive Time _____ | No. Dead Alive Time _____ | (B) No. Dead Time _____ | No. Alive Time _____ | (C) Mortality Corrected for Damaged Insects (B-A) | (D) Exact Total in Container | (E) Total Corrected for Damaged Insects (D-A) | (F) % Dead (C/E) |
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Remarks: